Optimization of Camelina Protein Extraction and Screening of Diverse Lines for Differences in Protein Profile, Structure, and Functionality

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Dedication

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Abstract

Current consumer trends such as vegan and flexitarian diets and interest in sustainably sourced ingredients are driving the demand for plant proteins. Camelina sativa, a novel oilseed crop with several environmental benefits, is high in oil (30-49%) and protein content (20-30%). Although the protein-rich camelina meal, a byproduct of oil extraction, has been explored for animal feed applications, the utilization of camelina protein for food applications has been less researched.

Producers are seeking alternative sources of plant proteins to replace soy protein, which has limitations from being sourced from a GMO crop as well as being one of the 'Big Eight' allergens. With its non-GMO and non-allergenic status as of yet, camelina shows potential to be developed as a source of novel plant protein to replace traditional protein ingredients in food products to provide optimal nutrition and functionality. To develop functional protein ingredients from camelina, effective protein extraction methods need to be investigated. Moreover, protein functionality for various food applications should be explored through evaluation of diverse camelina lines with differences in protein composition and profile.

The specific objectives of this study were: 1) Optimize protein extraction methodology to produce camelina protein isolates (CPI) with optimal yield, purity, structural characteristics, and functional properties; 2) Screen camelina lines for protein profile, structure, functionality, and nutritional quality to develop optimal lines for food use.

The protein profile of 40 diverse camelina lines with differences in their genotypes and origins was evaluated. Differences in cruciferin and napin subunits as well as the abundance of other protein polypeptides among camelina lines were observed. Based on their differences in protein profile, two camelina lines were selected for the further comprehensive characterization of camelina protein for its potential utilization in food applications. For optimization of camelina protein extraction, defatted camelina meal was subjected to two protein extraction procedures, pH extraction and salt extraction coupled with membrane filtration. In pH extraction, addition of sodium sulfite to improve the color of the camelina protein extracted under high alkalinity (pH-CPI) was tested. The concentration of sodium sulfite was optimized based on protein purity, yield, profile, and

color of the isolates. In salt extraction coupled with membrane filtration, the concentration of NaCl needed for protein solubilization was determined for optimal protein purity and yield of salt extracted CPI (salt-CPI). The optimized extraction conditions from each method were used to produce CPI from winter (WL) and spring (SL) camelina lines. The impact of the extraction method and of camelina genotype on structural characteristics of camelina protein was evaluated by determining the protein profile using SDS-PAGE, protein denaturation by DSC, surface hydrophobicity using a spectrophotometric method, surface charge by measuring zeta potential, and secondary structure by Fourier transform infrared spectroscopy (FTIR). The functional properties including protein solubility, gelation, water holding capacity, and emulsification properties of the CPIs were evaluated as impacted by the structural differences. Additionally, the structural and functional characteristics of CPIs were compared to those of commercial soy (cSPI) and pea protein isolates (cPPI).

Presence of sodium sulfite at 0.1% (w/v) during the pH extraction showed optimal protein purity and yield, as well as significant improvement in color, with no protein polymerization. The optimized NaCl concentration needed for protein solubilization during salt extraction was determined to be 0.5 M. The optimized extraction conditions for both methods produced CPI with protein purity of 83-84%. However, pH extraction resulted in a significantly higher yield (63%) than salt extraction (55%).

Protein profiling showed pronounced differences in the relative abundance of different protein fractions between pH-CPI and salt-CPI, and between CPI extracted from winter line (WL-CPI) and from spring line (SL-CPI). Overall, salt-CPI showed lower denaturation, and lower or comparable surface hydrophobicity and charge as compared to pH-CPI. The SL-CPI showed higher denaturation of cruciferin than WL-CPI and higher or comparable surface hydrophobicity and charge.

Due to its lower denaturation state and surface hydrophobicity, salt-CPI demonstrated better solubility than pH-CPI at both neutral and acidic conditions, and comparable or higher solubility than cSPI under acidic conditions. The WL-pH-CPI had comparable or inferior solubility but superior gelation and emulsifying properties compared to SL-pH-CPI. On the other hand, SL-salt-CPI had better or comparable gelation and emulsifying properties as compared to WL-salt-CPI. cSPI demonstrated higher gel

strength than CPIs, while CPIs showed comparable water holding capacity to cSPI and cPPI. However, CPIs had superior emulsification properties than both cSPI and cPPI, owing to their better balance of surface charge and hydrophobicity. These functional properties were linked to the denaturation state, extent of protein aggregation, and to the surface properties of CPI.

Overall, this study showed that CPI with optimal purity, yield, color, and functionality can be produced using industry feasible extraction methods, thus advancing its commercialization as an alternative plant protein. The structural and functional characterization of the different camelina lines provided useful information to the camelina breeding programs that aim at developing camelina protein for food use. Further investigation on targeted protein modification and crop diversity/breeding of camelina is needed to enhance its protein functionality for widespread food applications.

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Chapter 1: Review of Literature

1.1 Introduction

There has been a steep increase in demand for high protein products over the past decade. Specifically, plant-based proteins have shown a huge rise in popularity among consumers. Changes in consumer trends including increase in vegetarian, vegan, and flexitarian diets coupled with heightened awareness of health benefits associated with consuming plant-based food have majorly fueled these market demands. Concerns about the environment and food insecurity to feed the increasing population have further evoked interest in plant-based, sustainably sourced ingredients.

To address this market need, novel crops with lower input and cost are being explored to replace the expensive traditional protein sources. *Camelina sativa* is one such novel oilseed crop that has been grown over thousands of years in European countries (Zubr, 2010). Due to their high oil content, camelina seeds have been primarily used as a source of oil for fuel and cooking purposes (Zubr, 2010; Larsson, 2013). Oil can be extracted from camelina seeds by means of mechanical pressing, solvent extraction, and supercritical carbondioxide extraction (Shukla, Dutta and Artz, 2002; Moslavac *et al.*, 2014; Stroescu *et al.*, 2015; Zhao *et al.*, 2015; Raczyk *et al.*, 2016; Belayneh *et al.*, 2017). The residual meal after oil extraction has been mainly used for enriching protein and/or omega-3 content in animal feed or regarded as waste (Rokka *et al.*, 2002; Ryhänen *et al.*, 2007). The camelina meal has high protein and fiber content, thus gaining attraction as a potential source for food ingredients (Pekel *et al.*, 2009; Almeida *et al.*, 2013; Colombini *et al.*, 2014; Kahindi *et al.*, 2014; Sarv, Trass and Diosady, 2017; Boyle, 2018).

Being a short season crop, camelina can be readily integrated in crop rotations while contributing to several environmental benefits including preventing soil erosion, enhancing nutrient sequestration, and reducing nitrate leaching (Gesch and Archer, 2013; Berti *et al.*, 2016). Thus, utilizing camelina meal as a source of high-value ingredients, namely protein, will not only add to the economic sustainability of camelina crop but it will also result in multifaceted prosperity to the farmers.

In order to create a unique and competitive place for novel protein crops like camelina in the market, it is important to explore their protein production and utilization.

Furthermore, demonstration of equivalent or superior/new functions of novel plant proteins is needed. Traditional protein sources, such as dairy, soy, and egg, are currently facing challenges in terms of cost of production, allergenicity, vegetarian and vegan trends, and genetically modified organism (GMO) status. Thus, food producers are seeking more technical information to incorporate alternative plant proteins in food.

Soy protein, the most utilized plant protein ingredient in the market, contributes key functional properties in various food applications. However, the allergenic and non-GMO status of soy, coupled with consumer's desire to add variety in their plant-protein sources, have been paving the way for novel plant-based proteins (Mintel, 2020). However, the functional properties of alternative plant proteins must match or outperform those of soy protein to be successfully utilized in various food applications (Singh *et al.*, 2008). While pea protein ingredients are being increasingly utilized in various food products replacing soy protein, they lag behind soy protein in their sensory, functional, and nutritional qualities (Srivastava, 2020).

Boyle *et al.* (2018) found that camelina protein exhibits comparable and, in some cases, superior functionality to soy protein. Thus, camelina protein shows potential to be commercialized for food applications. To be able to enhance camelina's marketability, there is a need to investigate cost effective and industry feasible protein extraction processes. Moreover, it is important to enhance the sensory perception and functional integrity of camelina protein for improved success in food applications.

Functional properties, namely protein solubility, gelation and water holding capacity, emulsification and foaming properties, are influenced by the structural characteristics of the protein (Foegeding and Davis, 2011). Plant proteins generally have inferior functionality as compared to other traditional protein sources due to their globular structure and low molecular flexibility (Martínez *et al.*, 2007; Wanasundara, 2011). Therefore, it is important to investigate optimization of protein extraction processes to preserve the integrity of the protein structure, and to study the potential enhancement in protein functional properties through breeding programs.

Depending on targeted end-product applications, germplasms accessions with desirable protein profile (type and proportion of proteins) and composition can be developed through breeding programs. As protein composition and structural

characteristics have a direct impact on its functionality, targeted breeding strategies could be utilized to enhance the functionality of camelina protein. This targeted breeding approach can be achieved through investigation of various existing camelina lines, which would further guide the identification of genetic markers responsible for superior protein functionality traits in camelina. The protein quality and functionality of different camelina accessions or varieties for food applications is a key area of research that has not been explored yet.

Furthermore, a deep investigation into cost effective, industry feasible protein production methods is needed to advance the commercialization of camelina protein ingredients. Evaluation of the protein profile, structural characteristics and functionality in different camelina lines will contribute to the breeding programs that aim at successfully adapting camelina as edible protein crop for food applications.

1.2 Hypothesis and Objectives

We hypothesize that various protein extraction conditions and processing techniques will influence the camelina protein profile and structure, consequently leading to differences in the functionality of the camelina protein isolates. Furthermore, we anticipate that variation in the protein composition and profile among camelina lines will result in the production of protein ingredients with unique structure and functionality.

The overall objective of the project was to evaluate various protein extraction methods and the impact of varietal differences on the structural and functional characteristics of the isolated protein. The specific objectives of this study were:

1. Optimize protein extraction methodology to produce camelina protein isolates (CPI) with optimal yield, purity, structural characteristics, and functional properties.
2. Screen camelina lines for protein profile, structure, functionality, and nutritional quality to develop optimal lines for food use.

1.3 Need for Novel, Sustainably Sourced Protein

In 2020, the global protein ingredients market was valued at USD 38.5 billion and is anticipated to grow at a rate of 10.5% from 2021 to 2028 (Grand View Research, 2021). Recognition of protein as a part of a balanced diet and healthy aging has been driving its increasing consumption among health-conscious and elderly consumers. Furthermore, the

COVID-19 pandemic contributed to the increased sales of plant protein-based products such as meat alternatives (Mintel, 2020). The increased sales for this segment are attributed to the consumer's refocus on wellbeing as a result of the pandemic.

Increased demand for plant protein-based products have been associated with the growing popularity of vegan and flexitarian diets coupled with overall increased traction to healthy diets. Plant protein ingredients have been known to be associated with several physiological benefits, including weight management, diabetes management, cancer prevention, and reduced risk of metabolic syndrome (Ahnen, Jonnalagadda and Slavin, 2019). Additionally, rising occurrences of health disorders, such as diabetes, obesity, and cardiovascular diseases, are driving the adoption of health promoting ingredients (Tuso *et al.*, 2013).

Considering the projected global population growth to 9.7 billion by 2050 (United Nations, 2019), there will be increased pressure on the world's limited resources to feed the growing population (Henchion *et al.*, 2017). Animal-based foods are generally associated with negative environmental impacts, higher greenhouse gas emissions, and higher need for water and land as compared to plant-based foods (Tilman and Clark, 2014). Such pressing issues are demanding the investigation of sustainable production of existing and alternative protein sources using the available resources. With the limited access to arable land, different approaches such as enhancing crop yield and crop intensity can be used to increase productivity (Alexandratos and Bruinsma, 2012). The crop intensity can be enhanced by implementing multiple cropping systems and/or reducing the fallow periods between plantings. Moreover, consumers are seeking sustainability and transparency in their food supply (Nielsen Global Survey, 2014). They are consciously adopting purchasing habits that positively impact the environment and promote animal welfare. Accordingly, food producers are seeking environment-friendly, sustainable ingredients.

Increasing incidences of protein allergenicity are further fueling the search for novel plant proteins. Eggs, dairy, and soy are included in the 'big eight' major allergens recognized by the Food and Drug Administration (FDA). Although soy has been the dominant plant-based protein source for various food applications, health and GMO concerns limit the consumption of soy-based foods. A study on plant-based meat

alternatives found that over 50% consumers are seeking more variety in the plant-based protein sources (Mintel, 2020). Demand for novel plant proteins from pulses (pea, lentils, chickpeas, and beans), oilseeds (canola, sunflower), cereals (oats, rice, corn), and ancient grains is increasing (Grand View Research, 2021). However, there is limited consumer and producer knowledge of plant proteins other than soy. A better understanding of plant proteins can result in partial or whole replacement of traditional protein ingredients to deliver optimal nutrition, flavor, and functionality. Additionally, considering the multiple functional roles of proteins, including structure building, stabilizing properties, and flavor enhancement, they have a potential to replace synthetic ingredients (e.g. mono and di glycerides, sucrose polyester, polyvinyl alcohol, polylactic acid) in various food applications (Ismail *et al.*, 2020). Therefore, producers are seeking functional, non-allergenic ingredients with an aim to develop cleaner labels.

Another opportunistic reason for producers' interest in plant proteins is their relatively lower cost of production as compared to traditional protein ingredients. The value and revenue from plant-based ingredients can be increased through byproduct valorization. Oilseed meals are a byproduct of oil extraction. With the increasing demand for edible oil and biodiesel, the production of oilseed meals is on the rise. These meals are majorly composed of protein and fiber, making them an attractive choice for production of food ingredients.

Camelina sativa, an oilseed crop from Brassicaceae family, has been gaining attention as a source of oil for food as well as biodiesel applications. Cultivation of camelina has agronomic benefits, making it a favorable choice for farmers. The short growing season of winter camelina allows its integration into cropping systems of other major crops such as soybean, maize, and sunflower (Gesch and Archer, 2013; Gesch, Archer and Berti, 2014; Berti *et al.*, 2015). Other benefits of growing camelina include reduced agricultural inputs, soil erosion, and nitrate leaching, as well as enhanced nutrient sequestration (Gesch and Archer, 2013; Berti *et al.*, 2016). However, farmers would be interested in planting it only when it has been developed and evaluated for food use.

The camelina meal, produced as a byproduct of oil extraction, contains approximately 30-45% protein, 51% dietary fiber, and some residual oil (Pekel *et al.*, 2009; Almeida *et al.*, 2013; Colombini *et al.*, 2014; Kahindi *et al.*, 2014; Sarv, Trass and Diosady,

2017; Boyle, 2018). Being rich in protein, camelina meal has the potential to be used for the production of novel protein ingredients. Investigation of camelina as a novel plant protein source will not only lead to economic gains to the farmers and the food industry, but also promote the production of this sustainable crop that performs varied ecosystem services.

1.4 Camelina Sativa Production

1.4.1 Camelina Production History

Archaeological studies have traced the early cultivation of *Camelina sativa* in the Bronze Age (1500-400 B.C.) in Europe (Zubr, 1997; Karg, 2012; Larsson, 2013). Though its cultivation was found to be closely associated with flax, an archaeobotanical study by Larsson (2013) concluded that camelina was grown independently for its oil-rich seeds. Furthermore, archaeological investigations of Iron Age sites have found camelina seeds in the form of solid brown pressed cake and as an ingredient in bread and porridge, indicating that camelina was being cultivated for oil and food purposes (Zubr, 1997; Hansson, 2009; Karg, 2012). In recent years, camelina has received renewed interest due to its environmental adaptability and benefits, and to its potential to be introduced as a winter crop into the conventional cropping systems (Zanetti *et al.*, 2017).

1.4.2 Agronomic Potential and Ecosystem Benefits of Camelina Production

Unlike spring camelina cultivars, winter camelina cultivars require an additional vernalization (exposure to prolonged cold) treatment to induce flowering (Berti *et al.*, 2016; Anderson *et al.*, 2018). The exceptional survivability of winter camelina in low temperatures makes it a favorable option as an annual winter cover crop (Gesch and Cermak, 2011; Gesch and Archer, 2013). Moreover, winter camelina has a short growing season, thus, it can be harvested early in the summer allowing the full development of second crops such as soybean, corn, oilseed sunflower, sorghum, and forage foxtail millet (Gesch and Archer, 2013; Gesch, Archer and Berti, 2014; Berti *et al.*, 2015).

The integration of winter camelina as a rotational crop in double-cropping and relay-cropping system has been shown to be economically beneficial (Gesch and Archer, 2013; Gesch, Archer and Berti, 2014). A study evaluating the camelina-soybean dual

cropping system noted a higher yield in seed oil with the relay system in comparison to the conventional monocropped full-season soybean. Relay-cropping with winter camelina keeps the agricultural land covered in the period between the cultivation of summer annual crops. Camelina crops can help retain nutrients, prevent soil erosion, suppress weeds, and scavenge the excess nitrogen before the next summer crop is planted (Staver and Brinsfield, 1998; Gesch, Archer and Berti, 2014; Berti *et al.*, 2016). Moreover, camelina requires comparatively low agricultural inputs such as fertilizer and herbicides (Putnam *et al.*, 1993).

Along with the environmental and agronomic benefits, both spring and winter camelina provide nectar and pollen for pollinators, thus contributing to a healthy ecosystem. Especially winter camelina that is cultivated in the Upper Midwest region begins to flower in late May, which is relatively early as compared to other common oilseeds such as soybean, canola, and flax in the region (Eberle *et al.*, 2015; Thom *et al.*, 2016). The nectar and pollen offered by these early camelina flowers provide a good food source for honeybees and other pollinators in the area. The amount of nectar sugar produced during their flowering period was reported to be higher for camelina (100 kg ha^{-1}) as compared to pennycress (12 kg ha^{-1}) and canola (82 kg ha^{-1}) (Eberle *et al.*, 2015). While the pollinator activity may contribute to the development of wild camelina populations with genetic diversity, growing interest in camelina has further encouraged the investigation of targeted camelina breeding systems.

1.5 Camelina Breeding

In the 1980s, the genetic variation of camelina germplasm accessions was investigated by Seehuber (1984). The first camelina breeding program focused on developing the base populations for further yield improvement (Seehuber, Vollmann and Dambroth, 1987). Later, breeding activities were initiated in the United States, in Canada, and in several European countries (Eynck and Falk, 2013). The development of genetic and genomic tools in *Camelina sativa* are greatly facilitated due to its close similarity with the genetic model plant Arabidopsis, whose genome has been widely studied (Hutcheon *et al.*, 2010; Nguyen *et al.*, 2013).

One of the key activities in plant breeding is the creation of genetic variation, which may be introduced naturally or artificially. In a study investigating the pollination system

of *Camelina sativa*, the flowers were found to be visited by honeybees, wild bees, and other flies. However, no significant difference in yield was noted between open pollination and self-pollination (Groeneveld and Klein, 2014). Thus, camelina is predominately considered an autogamous (self-pollinated) species (Vollmann and Eynck, 2015a).

Séguin-Swartz *et al.* (2011, 2013) explored the hybridization technique to induce variability for improvement of camelina. The researchers concluded that it is possible to cross *Camelina sativa* with other camelina species such as *Camelina alyssum*, *Camelina microcarpa*, and *Camelina rumelica*, while gene flow between *Camelina sativa* and its distant relatives such as *Brassica juncea* (mustard), *Brassica napus* (oilseed rape), and *Brassica rapa* (turnip rape*)* is unlikely. Other breeding approaches that have been investigated to develop camelina populations aligning with various demands of camelina producers, food manufacturers, and consumers to advance its marketability will be discussed in the following sections.

1.5.1 Objectives and Achievements of Camelina Breeding

1.5.1.1 Yield and Physiological Traits

Camelina producers are interested in traits such as high yield, early maturity, drought resistance, and herbicide resistance, that enable profitable and efficient crop production. The growing need to feed the increasing population in an efficient and sustainable manner on the available land necessitates the development of high yield cultivars. To date, several studies on different breeding lines aimed at increasing the camelina seed yield (Vollmann and Eynck, 2015a). Guy *et al.* (2014) reported yields over 3000 $\mathrm{kg\,ha^{-1}}$ for camelina genotypes grown in favorable locations with higher precipitation and lower temperature. Gesch *et al.* (2018) explored different winter cultivars for early maturity trait, which allows the early establishment of the second crop in double-cropping systems. Another important breeding objective from producer's perspective includes increasing the seed size of camelina to facilitate the seeding and harvest with large farm equipment (Vollmann *et al.*, 2007; Gesch *et al.*, 2018). The larger seeded forms have also demonstrated higher oil yields than small seeded camelina (Vollmann *et al.*, 1996). The increase in yield and seed size could increase the economic value of camelina as cover crops in double cropping systems (Berti *et al.*, 2016).

The advent of recombinant DNA technology has led to the development of *Agrobacterium*-mediated transformation method that involves precise gene transfer from bacteria into plant cells. Using this method, new genes have been successfully expressed in *Camelina* sativa (Lu and Kang, 2008; Liu *et al.*, 2012). Transgenic camelina seeds with improved agronomic characteristics including higher yield and drought resistance, have been produced using this technique (Lee *et al.*, 2014; Roy Choudhury, Riesselman and Pandey, 2014).

Crop adaptation, which refers to the crop's growth response to environmental factors such as plant pathogens and herbicide residues, can also be enhanced through breeding. Disease resistant camelina cultivars that produce phytoalexins, camalexin and methoxy-camalexin, which act against pathogens, have been investigated. Studies have concluded that *Camelina sativa* is resistant to diverse insect pests, which may further assist in developing disease and pest resistance in other Brassicaceae crops, too (Vollmann and Eynck, 2015a). Exposure of camelina seeds to ethyl methane sulfonate (EMS), a chemical mutagen employed to induce genetic variation, has developed mutants with increased resistance to acetolactate synthase (ALS) inhibitor herbicides (Walsh *et al.*, 2012). Camelina mutants with herbicide-resistant traits can reduce the risks associated with growing camelina in soils containing herbicide residues.

1.5.1.2 Compositional Traits

Food manufacturers are mainly interested in the enhancement of quality traits including appearance, storage quality, processing quality, and nutritional value of camelina. Depending on the intended use of the product and processing requirements, camelina cultivars with enhanced expression of quality traits are desired. Food and non-food applications of camelina oil have encouraged breeding efforts that focus on increasing the oil yield. A considerable variation in the oil content (30-49%) either through modification in the genetic composition, or due to changes in the growing environment has been reported (Vollmann and Eynck, 2015a).

In response to the growing interest in the nutritional quality of food products, breeding to enhance nutritional traits, and to reduce or remove antinutritional traits is being explored. Hutcheon *et al.* (2010) characterized a *fatty acid desaturase* (*FAD2*) and a *fatty acid elongase* (*FAE1*) gene in different camelina species. The manipulation of these genes

has been successful in modifying the fatty acid profile of camelina oil for targeted production or reduction of individual fatty acids.

Due to adverse effects of erucic acid on heart muscle, low-erucic breeds in the *Brassicaceae* family are being investigated (Vetter, Darwisch and Lehnert, 2020). Camelina accessions and mutants with low levels of erucic acid (within the permitted limit of 2%) have been reported (Budin, Breene and Putnam, 1995; Vollmann *et al.*, 1997). The genes responsible for erucic acid biosynthesis in camelina have also been investigated (Gehringer *et al.*, 2006). These studies indicate promising breeding efforts to develop camelina lines with permitted levels of erucic acid.

Higher linoleic acid content is desirable for increasing the omega-3 fatty acid content of the oil. Consumption of omega-3 fatty acids has health benefits, particularly prevention of cardiovascular and inflammatory diseases (Surette, 2013). Both seed irradiation with gamma rays and seed treatment with EMS have been demonstrated to enhance the linolenic acid content in mutant camelina lines (Vollmann *et al.*, 1997; Büchsenschütz-Nothdurft, Schuster and Friedt, 1998). Ruiz-Lopez (2014) demonstrated the generation of omega-3 polyunsaturated fatty acids (PUFA) in transgenic camelina seeds, equivalent to those in fish oils, thus providing a sustainable, plant source of omega-3 PUFAs.

Considering the increasing contribution of plant-based protein sources to the global protein supply (Henchion *et al.*, 2017), breeding for improved protein content in crops is crucial to address both nutritional needs and food security. Numerous studies have successfully enhanced the nutritional quality and bioavailability of various seed proteins by enriching specific amino acids and/or by altering the ratio of storage proteins. An example of this is the development of Quality Protein Maize with higher lysine content, higher levels of digestible glutelins, and a desirable ratio of leucine to isoleucine for enhanced niacin production (Acquaah, 2012).

To advance the breeding efforts towards improvement of camelina seed quality, Nguyen *et al.* (2013) built a transcriptome profile of *Camelina sativa*. The transcriptome analysis provided fundamental information on genes coding for seed storage proteins in *Camelina sativa*, facilitating the enhancement or modification of camelina protein. Further, the researchers used the sequence information from the seed transcriptome to reduce the

expression of 2S napins, thus demonstrating the development of camelina lines with modified protein composition through targeted suppression of specific genes. Lyzenga *et al.* (2019) utilized CRISPR/Cas9 gene editing technology to alter the expression of cruciferin isoforms and other seed storage proteins without impacting the total protein content in *Camelina sativa*. The mutant line also demonstrated modified amino acid composition with high levels of alanine, cysteine and proline and low levels of isoleucine, tyrosine, and valine. Redistribution in the abundance of specific storage proteins was also reported. Such manipulation of seed storage proteins could be utilized to enhance the nutritional content of camelina seeds. It is important to note that changes in amino acid profile and altered ratio of specific proteins may also lead to the accumulation of high-value proteins as well as influence the physiochemical/functional properties of camelina protein. Thus, it would be beneficial to study how these changes in protein profile impact the utilization of camelina protein for different food applications.

Considering the current advances in sequencing and bioinformatics, genetic resources available for camelina, such as molecular markers, transcriptome profile, and genomics, show a great potential in advancing camelina breeding. However, further understanding of synthesis and regulation of storage proteins in *Camelina sativa* is needed for its targeted enhancement. Expression of mutant genes during breeding may also result in non-targeted changes. For example, high lysine products developed through breeding have undesirable attributes such as lower yield, susceptibility to breakage and rot pathogens, and chalky appearance (Frost and Robinson, 1971). These attributes may lead to economic losses to farmers and manufacturers. Therefore, it is essential to investigate and optimize the influence of breeding on yield, physiological traits as well as compositional traits (e.g. oil, fiber, protein) of camelina, thus facilitating its successful commercialization, similar to other well-established oilseeds in the market.

1.6 Camelina Nutrients

Camelina seeds contain around 30-49% oil, 30% dietary fiber, and 20-30% protein (Budin, Breene and Putnam, 1995; Zubr, 2003; Vollmann *et al.*, 2007; Moser and Vaughn, 2010; Sintim *et al.*, 2015; Sarv, Trass and Diosady, 2017; Zanetti *et al.*, 2017; Boyle *et al.*, 2018). Camelina seeds are a good source of vitamin B1 (thiamin), vitamin B3 (niacin), vitamin B5 (pantothenic acid), and iron (Zubr, 2010). Camelina seeds also contain phenolic

compounds such as tocopherols, sinapine, sinapic acid, flavanols, and flavonols (Zubr and Matthaus, 2002; Salminen *et al.*, 2006; Abramovič, Butinar and Nikolič, 2007; Salminen and Heinonen, 2008; Terpinc *et al.*, 2012). The major constituents of camelina, namely oil, dietary fiber, and protein, and their potential utilization as food ingredients will be discussed in the following sections.

1.6.1 Camelina Oil

Camelina oil can be extracted from the seeds using mechanical (hot or cold) pressing (Shukla, Dutta and Artz, 2002; Zhao *et al.*, 2015; Raczyk *et al.*, 2016), solvent extraction (Stroescu *et al.*, 2015), and/or supercritical carbon dioxide extraction (Moslavac *et al.*, 2014; Belayneh *et al.*, 2017). Application of heat during hot pressing may increase the susceptibility of oil to oxidation, degrade bioactive compounds, and consequently lower the oil quality (Geow *et al.*, 2021). Thus, cold pressing is the preferred method to extract camelina oil as it is known to ensure maximum retention of the bioactive compounds such as essential fatty acids and tocopherols in the extracted oil (Hrastar *et al.*, 2011; Teh and Birch, 2013).

The fatty acid profile of camelina oil depends on cultivar, location, growing conditions, and oil extraction method. Camelina oil generally is comprised of oleic acid (14-16%), linoleic acid (15-23%), α-linolenic acid (31-40%), and eicosenoic acid (12-15%). The proportion of α-linolenic acid and linoleic acid in camelina oil contributes to a ratio of omega-6 fatty acid to omega-3 fatty acid between 1:1 and 4:1, which is desirable for a balanced intake of essential fats (Budin, Breene and Putnam, 1995; Zubr, 1997; Simopoulos, 2002; Eidhin, Burke and O'Beirne, 2003; Abramovič and Abram, 2005). The camelina oil also contains other fatty acids including palmitic, stearic and erucic acid (Putnam *et al.*, 1993; Budin, Breene and Putnam, 1995; Zubr, 1997; Singh, Bala and Rai, 2014). Several studies investigating the composition of camelina oil have reported the content of erucic acid to be around 2-3%, which is relatively low as compared to other members in the Brassicaceae species (Zubr, 1997, 2003; Eidhin, Burke and O'Beirne, 2003; Abramovič and Abram, 2005; Vollmann and Eynck, 2015b). Considering the detrimental effects of erucic acid on the heart muscle, the maximum permitted level of erucic acid in vegetable oils is 2% in the USA (21 C.F.R. § 184, 2020). A recent study showed that the average erucic acid content in oil from winter genotypes (0.1%) is lower

than that from spring genotypes (3.4%), thus showing potential of utilizing winter varieties for production of oil (Kurasiak-Popowska, Graczyk and Stuper-Szablewska, 2020). Breeding of camelina to control the level of erucic acid can ensure successful utilization of camelina oil for food applications.

Oxidation of oil may cause formation of undesirable flavors affecting its sensory acceptability. Oxidation may also degrade bioactive compounds such as essential fatty acids, tocopherols, phytosterols, and carotenoids in the camelina oil, leading to loss of nutritional quality (Ratusz *et al.*, 2018). Studies have documented considerable oxidative stability of the oil despite the unsaturated fatty acid content being higher than 85% (Eidhin, Burke and O'Beirne, 2003; Zubr, 2003; Abramovič and Abram, 2005; Eidhin and O'Beirne, 2010). It has been reported that camelina oil contains 700-800 $\mathrm{mg\,kg^{-1}}$ of tocopherols, predominantly γ-tocopherol, and 123 $\mathrm{mg\,kg^{-1}}$ (expressed as chlorogenic acids) of phenolic compounds. Researchers have linked the stability of the oil to the antioxidant properties of the tocopherols and phenolic compounds (Zubr, 1997; Zubr and Matthaus, 2002; Abramovič, Butinar and Nikolič, 2007). Fish oils are popular for their high levels of omega-3 fatty acids, however their high susceptibility to lipid oxidation hinders their applicability. Eidhin *et al.* (2003) observed higher stability of camelina oil against oxidation in comparison to fish oil and linseed oil. The authors also reported reasonable quality of the camelina-based spread providing approximately 1 g of omega-3 fatty acids per day during a shelf-life of 16 weeks. Accordingly, camelina oil is a potential ingredient in functional foods as an alternative, stable source of omega-3 fatty acids. Studies have established the acceptability and stability of camelina oil in salad dressings, mayonnaise, and spread, however, it was not found to be stable enough for deep frying (Eidhin, Burke and O'Beirne, 2003; Eidhin and O'Beirne, 2010).

1.6.2 Camelina Dietary Fiber

The dietary fiber comprises about 30% of the camelina seeds and more than 50% of the defatted camelina meal by weight (Boyle, 2018). Small amount (<0.5%) of monosaccharides, glucose and fructose, are present in the defatted camelina meal (Zubr, 2010; Boyle, 2018). Zubr (2010) reported that camelina seeds contain 5.5% sucrose, while Boyle (2018) reported that defatted camelina meal contains 3.4% sucrose. Differences in the reported values could be attributed to the growing location, growing conditions, seed

genotype, maturity of the seeds, and specificity of the analytical method (Trugo, Almeida and Gross, 1988; Macquet *et al.*, 2007; Boyle, 2018). Raffinose and stachyose together constitute 1.1-1.2% of the camelina meal (Boyle, 2018). Raffinose and stachyose are known to encourage the growth of bifidobacteria and exert positive effects on intestinal cells and the immune system, suggesting its potential use as a prebiotic ingredient in functional foods (Martínez-Villaluenga, Frías and Vidal-Valverde, 2005). The amount of starch in camelina is around 1.21% as reported by Zubr (2010), which could partially get hydrolyzed to maltose during processing.

The presence of mucilage, a soluble fiber found in the outermost layer of the seed, contributes to the formation of gel when camelina seeds are soaked in water. Mucilage consist of a mixture of polysaccharides and its composition varies significantly depending on its source (Ziolkovska, 2012; Elboutachfaiti *et al.*, 2017). A study characterizing the gum isolated from camelina seed reported that the major monosaccharides found in the camelina mucilage were galactose (58%), glucose (25%), rhamnose (12%), and xylose (5%) (Li *et al.*, 2016). Boyle (2018) noted measurable quantities (>3%) of pectin, a known gelling substance, in the defatted camelina meal. The study also reported that the insoluble dietary fiber fraction contained pectin in small quantities possibly due to being bound to insoluble fiber such as cellulose (Macquet *et al.*, 2007). The camelina pectin investigated by Boyle (2018) showed low degree of methylation, classifying it as a low methoxy (LM) pectin. As LM pectin is able to form strong gels without the presence of sugar, it can be utilized to impart thickness to low-calorie products and mimic desirable attributes of fat in sauces (McCleary and Prosky, 2001).

Boyle (2018) also reported the presence of rhamnose, arabinose, xylose, mannose, galactose, and glucose in the dietary fiber fractions extracted from the defatted camelina meal. The monosaccharide composition of camelina fiber indicated the presence of galactomannans, arabinoxylans, xyloglucans, and cellulose in the insoluble dietary fiber fraction, and pectin and galactomannans in the water-soluble fraction that precipitated in 78% aqueous ethanol. The diverse composition of the insoluble dietary fiber fraction suggested the presence of hemicellulose, which is comprised of cellulose molecules substituted with other monosaccharides. Rhamnose is typically present as a constituent of pectin in the form of the polysaccharide rhamnogalacturonan type I (RGI) (McCleary and

Prosky, 2001). The identification of RGI in the mucilage on the seed coat of *Arabidopsis thaliana* by Macquet (2007) and the genetic relationship between *Camelina* and *Arabidopsis* confirms that the rhamnose detected in the soluble fraction is possibly a portion of the RGI structure on the seed coat that contributes to its water binding properties (Boyle, 2018). The high content and unique composition of dietary fiber in camelina indicate its potential to be incorporated in food for its nutritional benefits and functional properties.

1.6.3 Camelina Protein

The protein content in camelina seeds ranges from 20% to 30% depending on the variety, soil composition, and growing environment (Budin, Breene and Putnam, 1995; Moser and Vaughn, 2010; Sintim *et al.*, 2015; Sarv, Trass and Diosady, 2017; Zanetti *et al.*, 2017). Camelina meal that is produced after pressing and/or defatting of camelina seeds contain 30-45% protein (Pekel *et al.*, 2009; Almeida *et al.*, 2013; Colombini *et al.*, 2014; Kahindi *et al.*, 2014; Sarv, Trass and Diosady, 2017). The level of protein in the camelina meal is comparable to canola (33-41%) and flaxseed meal (33-37%) and slightly lower than soybean meal (50-53%) (National Research Council, 2001; Singh *et al.*, 2008; Colombini *et al.*, 2014; Wanasundara *et al.*, 2017; Hao *et al.*, 2020). As camelina meal is rich in protein as well as essential PUFA, current applications are mainly focused on its utilization as an animal feed additive. Recent studies have explored the potential applications of camelina protein as adhesives, biodegradable polymers, and food ingredients (Reddy *et al.*, 2012; Zhao *et al.*, 2014; Li *et al.*, 2015; Zhu, Wang and Sun, 2016; Boyle *et al.*, 2018). In a recent study investigating the potential of camelina protein for food applications, Boyle *et al.* (2018) found that the functional properties of camelina protein such as solubility in acidic environment, emulsification capacity, and foaming capacity are comparable to or better than soy protein. Moreover, the non-allergenic and non-GMO status of camelina, as of yet, also suggests that it has potential to compete with soy protein. Development of functional and competitive protein ingredients will allow camelina to gain a position in the market as a novel source of plant protein.

1.7 Protein Ingredients from Oilseeds

The plant protein ingredients based on their protein contents can be majorly classified as: defatted flour (30-60%), protein concentrate (60-80%), and protein isolate (>80%) (Ismail *et al.*, 2020). The processing of each ingredient influences the protein content in the final ingredient, and subsequently its classification (Arntfield and Maskus, 2011). For oilseeds, dehulling (removal of outer coat of the seed) and oil extraction are typical initial concentration steps before protein extraction and purification. Although dehulling is often used as a pre-treatment for oilseeds (e.g. soybean), in case of small oilseeds such as canola (genetically modified rapeseed containing <2% erucic acid in oil and <30 μmol of glucosinolates in meal) and camelina, the hull tends to adhere to the endosperm making it difficult to separate (Thakor *et al.*, 1995). Further, due to their high oil content, oilseeds are often subjected to oil extraction before protein extraction and purification. Mechanical pressing or flaking, which is used to expel the oil from the seeds, results in the production of pressed cake or flakes. The oil content is generally reduced further using solvent extraction, followed by milling to produce a defatted flour (Deak *et al.*, 2008; Thrane *et al.*, 2017). The production and utilization of soy protein ingredients are widely studied due to its current usage in the industry. The undesirable beany/grassy flavors in the soy flour limits its inclusion levels in food.

Protein concentrates from oilseeds are generally produced from the defatted flour through isoelectric precipitation, aqueous ethanol extraction, or heating to precipitate the proteins and separate soluble carbohydrates (Kinsella, 1979a; Thrane *et al.*, 2017). Soy protein concentrates with least off-flavors are produced using ethanol extraction (Deak *et al.*, 2008). However, alcohol extraction being highly denaturing produce protein concentrates with limited functionality. In general, soy protein concentrates can be utilized to produce texturized soy products (Singh *et al.*, 2008).

Protein isolates are produced by extracting the proteins from the defatted flour and removing both soluble and insoluble carbohydrates (Deak *et al.*, 2008; Thrane *et al.*, 2017). Proteins can be extracted and purified using pH solubilization/precipitation, salt extraction, membrane filtration, and/or chromatography. The commercially available protein isolates from soy are commonly produced using pH solubilization/precipitation. The defatted flour is solubilized at an alkaline pH, which solubilizes the proteins and low molecular weight

carbohydrates while starch and/or fiber is separated later by centrifugation. The soluble sugars and oligosaccharides are then separated by precipitating the protein at its isoelectric point. The precipitated proteins are washed, neutralized, and spray dried to obtain protein isolates. Production of protein isolates from canola using this method have also been reported in literature (Arntfield, 2011). The techniques and extraction conditions employed during protein isolation impact the structure of the protein and resulting functionality of the protein ingredients. The functional properties of the protein ingredients determine its utilization in various food applications.

Proteins from different sources have different structural characteristics, resulting in differences in their functionality and applicability in food. Furthermore, the protein's nutritional quality also plays a role in enhancing its adoption in the food industry. In efforts to develop viable protein ingredients from the novel crop, camelina, it is important to understand the nutritional quality, structure and profile, and functional properties of camelina protein.

1.8 Camelina Protein Composition and Nutritional Quality

The protein requirement for most of the healthy adults is 0.83 g/kg per day (WHO/FAO/UNU, 2007). However, the daily protein requirement is influenced by its nutritional value, which is mainly determined by its essential amino acid content and digestibility. The protein quality in human diets is often assessed using the protein digestibility-corrected amino acid score (PDCAAS). PDCAAS value is calculated by correcting the first limiting amino acid score for the true fecal digestibility of the test protein. The first limiting amino acid score is expressed by comparing the amount of the first limiting amino acid in the protein (mg/g test protein) to its amount in the recommended requirement pattern (WHO/FAO/UNU, 2007). High quality proteins have all essential amino acids in sufficient quantities and are highly digestible, resulting in a PDCAAS value of 1.0. The amino acid composition, with an exception of methionine, and the digestibility of soy protein have been shown to be comparable to high quality animal proteins (Wolf, 1970; Istfan *et al.*, 1983; Singh *et al.*, 2008). Considering its adequate amino acid score and high digestibility, soy protein has a PDCAAS value of 0.91 (Schaafsma, 2000; Friedman and Brandon, 2001). In comparison, canola protein has a PDCAAS value of 0.86 (Fleddermann *et al.*, 2013).

Table 1 shows the amino acid profile of camelina seed and other oilseeds. Camelina protein contains approximately 40% essential amino acids, slightly lower than canola protein (42%) (Li *et al.*, 2012), and soy protein (49%) (Khorasani *et al.*, 1990). Nevertheless, the amino acid profile of camelina meal is similar to that of canola meal (Li *et al.*, 2014). Camelina meal is deficient in lysine, which is one of the essential amino acids (Zubr, 2003). Among the remaining essential amino acids, methionine is present in low concentrations in camelina meal. Low methionine could be due to relatively low levels of sulphur containing amino acids in globulins, the predominant storage proteins in seeds of Brassicaceae family (Aider and Barbana, 2011). Similar to other oilseeds, glutamic acid is the most abundant amino acid in camelina.

The protein quality is also influenced by its digestibility, which is the degree of bioaccessibility of amino acids. The protein digestibility depends on the protein conformation, presence of antinutritional factors (e.g. trypsin and chymotrypsin inhibitors, lectins, tannins, and phytate) and protein's interaction with other components such as polysaccharides and dietary fiber (Damodaran, 2017). Moreover, processing treatments including heat and high pH can also limit the digestibility of proteins and bioavailability of amino acids. Protein extraction using sodium hydroxide may lead to degradation of amino acids (Tzeng, Diosady and Rubin, 1988). Excessive thermal treatment leads to denaturation and polymerization of the protein, causing reduced digestibility (Zhang *et al.*, 2020). Although there have been many studies on protein digestibility of camelina meal for animal feed, further research is needed to understand the digestibility of camelina protein for human consumption and to determine its PDCAAS value.

The different groups of amino acids classified based on their polarity, reactivity, and structure influence the physicochemical properties of the protein. The hydrophilic residues consist of amino acids with ionizable groups such as asparagine, glutamine, lysine, arginine, and histidine, which carry a net charge at neutral pH (Damodaran, 2017). The hydrophobic amino acids with aliphatic (alanine, isoleucine, leucine, methionine, proline, and valine) and aromatic (phenylalanine, tryptophan, and tyrosine) groups prefer to be in organic solvent rather than an aqueous phase. These residues tend to locate themselves in the interior of the protein.

Table 1. Amino acid composition (g/100g protein) of camelina and other oilseeds

Amino acid	Camelina	Rapeseed	Soy	Flax
Alanine	4.61	4.0	4.8	5.5
Arginine	8.15	6.7	7.5	11.1
Aspartic Acid	8.71	6.6	12.7	12.4
Cysteine	2.12	3.0	1.3	4.3
Glutamic Acid	16.4	18.1	19.0	26.4
Glycine	5.44	4.7	4.5	7.1
Histidine*	2.60	3.1	3.2	3.1
Isoleucine*	3.96	4.1	3.1	5.0
Leucine*	6.63	6.3	7.3	7.1
Lysine*	4.95	6.5	6.1	4.3
Methionine*	1.72	1.7	1.3	2.5
Phenylalanine*	4.19	3.5	5.0	5.3
Proline	5.09	6.0	6.0	5.5
Serine	5.04	4.0	5.6	5.9
Threonine*	4.25	4.5	4.2	5.1
Tryptophan*	1.15	-	1.3	1.7
Tyrosine	3.04	2.4	3.9	3.1
Valine*	5.42	6.0	32	5.6

*Denotes essential amino acid; ^Value not reported; Source: Zubr (2003).

The composition and reactivity of amino acids determine the overall hydrophobicity and net charge of the protein molecules. These characteristics influence the structural conformation and molecular interactions of the protein, which in turn contributes

to its functional properties. Thus, the amino acid composition that governs the formation of higher levels of protein structure of camelina protein can provide useful insights about its physicochemical, biological as well as functional properties.

1.9 Camelina Protein Profile and Structure

Plant protein components, specifically in oilseeds and pulses, are commonly classified based on their sedimentation coefficient, which is a factor of the molecular weight of the protein. A high sedimentation coefficient corresponds to a large protein. The predominant seed storage proteins in Brassicaceae crops are cruciferin (11S or 12S globulin) and napin (2S albumin) (Wanasundara, 2011). Cruciferin and napin constitute about 32-53% and 25-45%, respectively, of the total seed protein in rapeseed, depending on variety (Malabat *et al.*, 2003). Structural proteins such as oleosins that are associated with oil bodies, and metabolic proteins such as lipid transfer proteins, protease inhibitors, Ca^{2+} dependent-calmodulin binding proteins, and dehydrins have also been reported to be present in smaller amounts in Brassicaceae oilseeds (Wanasundara, 2011).

Cruciferin from *Brassica napus* (rapeseed) has lower proportion (10%) of α-helix and higher proportion (50%) of β-sheet secondary structures (Zirwer *et al.*, 1985). On the other hand, higher proportion of α-helical structure (40-46%) than β-sheet conformation (12%) has been reported in napins from canola (genetically modified rapeseed) (Tan *et al.*, 2011).

Protein chains with ordered secondary structures (α-helix and β-sheet) typically fold into a compact three-dimensional tertiary structure. The conformation of a protein molecule is dictated by its amino acid composition and sequence. The formation of tertiary structure involves rearrangement of most of the hydrophilic residues to the protein-water interface, and rearrangement of most of the hydrophobic residues away from the aqueous environment and towards the interior of the protein molecule. The protein molecules with large fraction of hydrophobic residues in its amino acid sequence acquires a globular (sphere-like) shape. The globular conformation has least surface area to volume ratio, allowing burial of most of the hydrophobic residues in the interior of the protein.

The majority of plant proteins, including cruciferin and napin from camelina and other Brassicaceae crops, have a globular tertiary structure (Wanasundara, 2011). When proteins contain greater than 30% of hydrophobic amino acids, as is the case for camelina

and other oilseed proteins and most plant proteins in general, relocation of all of the nonpolar residues in the interior becomes impractical. Such proteins tend to form quaternary complexes (oligomers) due to the presence of large hydrophobic patches on the surface that interact with hydrophobic patches of other proteins. These oligomers (dimers, trimers, tetramers, etc.) are composed of monomers of either homologous polypeptides or heterogeneous polypeptides.

Cruciferin protein exists in the form of a hexamer that is formed by stacking of two trimers (Wanasundara, 2011) **(Figure 1)**. Non-covalent interactions, namely electrostatic interactions, hydrogen bonding, hydrophobic interactions, and van der Walls, hold together the trigonal antiprism conformation of the hexamer (Wanasundara, 2011; Fahs and Louarn, 2013). Each trimer consists of three monomers, each with a heavy (~30 kDa), acidic α-subunit and a light (~20 kDa), basic β-subunit that are linked by one disulfide bond (Dalgalarrondo, Robin and Azanza, 1986; Fahs and Louarn, 2013) **(Figure 2)**. Protein profiling using 1D and 2D gel electrophoresis of camelina protein concentrates confirmed the presence of α- and β-subunits of cruciferin protein in camelina (Boyle *et al.*, 2018). The number and type of amino acids in the acidic and basic polypeptide chains are different. However, certain regions in their sequence have conserved homology. The molecular weight of each monomer is approximately 51 to 56 kDa, resulting in a total of about 300 kDa for the cruciferin hexamer (Wanasundara, 2011). A number of genetic variants of cruciferin subunits exists among different species (e.g. canola), which vary slightly in amino acid composition and length (Wanasundara, 2011).

The isoelectric point (pI) of cruciferin is ~7.2, which also varies depending on the genetic variation of its subunits (Schwenke *et al.*, 1980). Glycinin, the 11S globulin protein of soy, is the most structurally related protein to cruciferin outside of the Brassicaceae oilseed family (Wanasundara, 2011). However, glycinin has a larger molecular weight of around 350 kDa and a much lower pI (4.5) (Qi *et al.*, 2011). The difference pI is related to the relative abundance of acidic vs basic amino acids (Patrickios, 1995). The pI of acidic subunits of glycinin is around pH 5 whereas basic subunits show a wider pI between pH 4.5-8.0 (Mo *et al.*, 2006). On the other hand, the pI of acidic subunits of cruciferin have been reported to range between 6.7-8.8, and that of its basic subunits to range between 5.9-9.5 (Nietzel *et al.*, 2013).

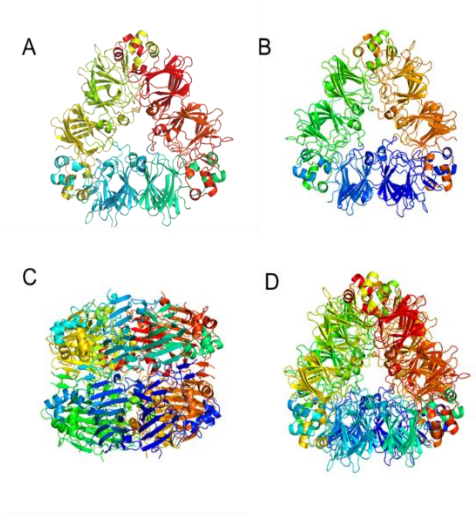

Figure 1. Illustration of the crystal structure of the cruciferin hexamer. (A) the cruciferin homotrimers at the top of the cruciferin hexamer. (B) the cruciferin homotrimers at the bottom of the cruciferin hexamer. (C) the side view of cruciferin hexamer. (D) the top view of cruciferin hexamer. (Picture credits: Fan Bu)

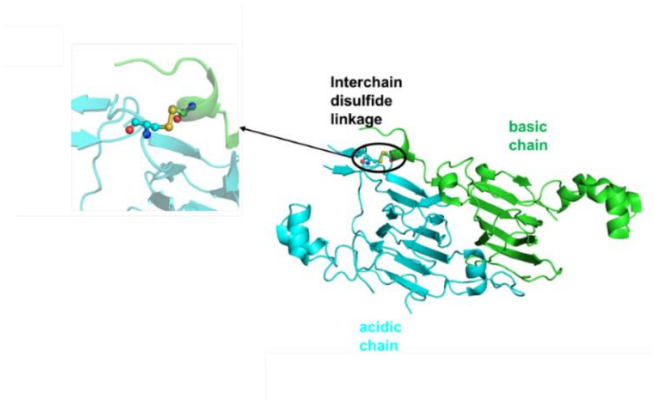

Figure 2. Illustration of the disulfide linkage between the acidic chain and basic chain in the cruciferin monomer. (Picture credits: Fan Bu)

Napin is a 2S albumin of 14-16 kDa consisting of a small (~4.5 kDa) subunit and a larger (~10 kDa) subunit that are linked by two disulfide bonds (Gehrig *et al.*, 1996) **(Figure 3)**. The napin subunits are hydrophilic in nature (Jyothi, Singh and Appu Rao, 2007). Both subunits are basic with the isoelectric pH of the small chain being 10.89 to 12.16 and that of the large chain being 10.25 to 11.30 (Wanasundara, 2011). Boyle *et al.*

(2018) confirmed the basic isoelectric pH (higher than 10) of napin subunits in camelina protein using isoelectric focusing electrophoresis.

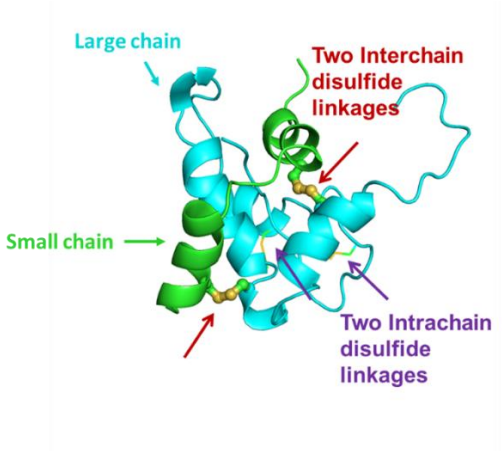

Figure 3. The interchain and intrachain disulphide linkages in the two subunits of 2S napin. (Picture credits: Fan Bu)

Along with the predominant cruciferin and napin protein fractions, previous studies have also shown the presence of glutelins in camelina (Li *et al.*, 2014; Boyle *et al.*, 2018). Boyle (2018) observed a glutelin-type polypeptide of about 15 kDa to 20 kDa. Considering the structural differences between the protein fractions, the ratio of individual protein fractions in the isolated camelina protein may impact its overall physicochemical and functional properties.

Changes in environmental factors such as pH, ionic strength, temperature, and extracting solvent composition may cause conformational changes in proteins. Protein denaturation refers to the major conformational changes in the secondary, tertiary, and quaternary structure of the protein. The hexameric assembly of rapeseed cruciferin, which exists at an ionic strength above 0.5 μ, undergoes reversible dissociation into 7S trimers upon reduction in ionic strength (Schwenke and Linow, 1982; Schwenke *et al.*, 1983). Moreover, extreme pH conditions can further cause irreversible dissociation of the trimers (Schwenke *et al.*, 1983). The denaturation temperature of camelina cruciferin extracted using a salt solution was reported to be ~95°C. However, complete denaturation of cruciferin was observed during its extraction using alkaline solution (pH ~ 12) (Boyle *et al.*, 2018). Cruciferin from canola also demonstrated temperature-induced unfolding with

a denaturation temperature of 83°C (Perera, McIntosh and Wanasundara, 2016). On the other hand, napin from canola exhibits thermally stable structural characteristics. Depending on the degree of protein denaturation, the resultant structural changes may have a positive or negative effect on the functional properties of the protein.

The protein structure is the outcome of various intramolecular interactions between the inherent amino acid residues as well as intermolecular interactions with other components in its environment. The different protein fractions in camelina exhibit varied structural properties, which may further undergo conformational changes during protein extraction and processing. Thus, a basic understanding of camelina protein structure under various environmental factors is important to develop appropriate processing strategies that maintain its functional integrity for food applications.

1.10 Camelina Protein Functionality

Proteins play a major role in achieving desirable attributes such as texture, color, appearance, and flavor of foods. Protein functionality refers to the physicochemical properties that influence the behavior of proteins during processing and storage. The physicochemical properties of proteins such as size, structural conformation, net charge, hydrophobicity to hydrophilicity ratio, surface properties, and molecular flexibility govern the functionality of the protein. The functional properties of the proteins have been broadly classified as: (i) properties involving water-protein interactions, (ii) properties involving protein-protein interactions, and (iii) properties involving water-oil and water-air interactions. These properties influence the processing characteristics, quality attributes, and acceptance of the final product. Therefore, to be able to utilize novel plant protein ingredients in food products, it is important to investigate their functional properties. Various research groups have investigated functional properties of proteins using different seed varieties, protein extraction conditions, analytical methods, and different assay conditions, making it difficult to compare functional properties of various plant proteins among different published studies. Nevertheless, a basic understanding of the physicochemical properties associated with each of the functional properties of plant protein ingredients can help enhance their utilization in food products.

1.10.1 Properties Involving Water-Protein Interactions

Water-protein interaction is important for most functional properties of proteins including solubility, viscosity, water-holding capacity, gelation, emulsification, and foaming. The water binding capacity of proteins is influenced by its proportion of hydrophilic amino acids (Damodaran, 2017). The charged groups in proteins bind more water molecules as compared to uncharged polar residues, which in turn bind more water molecules than nonpolar groups. Protein solubility is a critical factor for other functional properties, especially thickening, foaming, emulsifying, and gelling (Kinsella, Damodaran and German, 1985). Net charge and surface hydrophilicity are the intrinsic physicochemical properties that influence the solubility of the protein (Damodaran, 2017). Higher net charge on the surface of the protein causes repulsive electrostatic forces, which favors solvation. Proteins with low hydrophobic groups on the surface have high solubility.

The intrinsic profile and physicochemical characteristics of the proteins play an important role in determining their solubility. Protein isolates of *Brassica carinata* (Ethiopian mustard) showed lower solubility at acidic pH as compared to the original meal due to loss of certain proteins that are soluble at low pH during protein isolation (Pedroche *et al.*, 2004). Napin-rich protein ingredients show considerably higher solubility values under a wide pH range than cruciferin-rich protein ingredients due to the hydrophilic nature of napin subunits (Wanasundara, 2011).

Environmental conditions including ionic strength, type of salt, pH conditions, and heat influence the solubility of proteins. The solubility of soy protein isolates, for instance, increase up to ionic strength of 0.1 and is reduced at ionic strengths over 0.5 (Jiang *et al.*, 2015). At low salt concentrations, salt ions partially shield the charges on protein molecules, reducing the electrostatic interactions with other protein molecules and enhancing protein-water interactions (Duong-Ly and Gabelli, 2014). With an increase in salt ions, charges on the protein's surface are mostly shielded, leading to reduced repulsion and increased hydrophobic interactions among protein molecules and subsequent aggregation and precipitation (Zhou, 2005; Novák and Havlíček, 2013).

At or near the isoelectric point (pI), proteins have minimal or zero net charge, which causes protein-protein interactions to dominate, thus leading to the formation of protein aggregates and reduced solubility. Accordingly, higher solubility of camelina protein

concentrates was observed at pH 3.4 as compared to pH 7 (Boyle *et al.*, 2018), owing to the increase in net charge on the protein surface at pH values away from its basic pI (Boyle *et al.*, 2018). Similar trends were also reported for pea, soy, lentil, and canola protein isolates, showing high net charge and consequently high solubility at pH values away from their corresponding isoelectric points (Chang *et al.*, 2015).

Boyle *et al.* (2018) demonstrated that protein extraction methodology impacts camelina protein denaturation and thus its solubility. Alkaline conditions during protein extraction caused severe protein denaturation and polymerization, which resulted in low solubility of camelina protein. On the other hand, salt extraction compared to alkali extraction had minimal effect on camelina protein denaturation, thus resulting in lower surface hydrophobicity and better solubility.

During heat treatments, proteins may undergo denaturation. The unfolding of polypeptide chains upon denaturation of globular proteins exposes the hydrophobic groups located in their interior moiety. The increased hydrophobic interactions among denatured protein molecules lead to polymerization and aggregation, thus causing decreased solubility. Boyle *et al.* (2018) reported a decrease in the solubility of salt-extracted camelina protein upon heating at 80°C for 30 minutes.

Along with solubility, water holding capacity (WHC) of proteins is also determined by water-protein interactions (Damodaran, 2017). The mouthfeel of gel-based food products such as meat or meat analogues, yogurt, and baked goods, are influenced by the protein's WHC. WHC is the ability of the protein to retain water in its network against different external forces. Interactions between proteins are needed for the formation of a protein network. The formation of a protein gel network allows physical entrapment and retention of water against gravitational forces within the protein matrix. Water may be held in the protein gels through hydrogen bonding, electrostatic interactions, or capillary forces.

WHC is influenced by the amino acid composition of the protein, particularly the type and number of polar and charged hydrophilic groups (Damodaran, 2017). These hydrophilic residues are required for maintaining the protein-water interactions, which are important for water retention. Camelina protein gels showed water holding capacity higher than 98%, which was comparable to that of soy protein gels (Boyle *et al.*, 2018), probably due to the exposure of appreciable levels of hydrophilic residues. Thus, camelina protein

shows potential to replace soy protein in food applications where higher water retention is desirable.

1.10.2 Properties Involving Protein-Protein Interactions

The crosslinking of protein polymers through heat, pH shifts, divalent cations, and/or action of enzymes leads to the formation of a gel, which is a three-dimensional protein network that can entrap water molecules. The gel network is a balance of hydrophilic and hydrophobic interactions. Some level of repulsion among protein molecules and protein-water interactions are also required to form a balanced gel, entrapping water, otherwise the proteins would come together to form an aggregate.

In the case of heat-induced gels, the most common form of gels, thermal treatment causes partial unfolding of protein molecules and the exposure of hydrophobic residues and free sulfhydryl groups. Protein-protein interactions among the unfolded proteins via hydrophobic interactions and disulfide linkages enable the formation of a protein network (Léger and Arntfield, 1993; Damodaran, 2017). Intermolecular disulfide bonding are enhanced upon heating leading to stable protein interactions, thus enhancing the mechanical strength of the formed protein gels (Hoffmann and Van Mil, 1997).

On the other hand, pH-induced gels are formed by gradual change in pH towards the pI of the proteins (Alting *et al.*, 2002). The consequent low charges on the protein's surface near the pI lead to reduced electrostatic repulsions and enhanced protein-protein interactions, thus forming the gel protein network. Protein gels can also be formed by the addition of divalent cations such as Ca^{2+} and Mg^{2+}, which facilitate the formation of cross-links between negatively charged groups of protein molecules (Mleko and Foegeding, 2000; Damodaran, 2017).

In enzyme-induced gelation, transglutaminase enzyme is used to induce crosslinking, facilitating the formation of protein gel network. Transglutaminases catalyze the formation of intramolecular and intermolecular crosslinking between the glutamine and lysyl groups of the protein molecules (Gaspar and De Góes-Favoni, 2015; Damodaran, 2017). Moreover, a controlled treatment with proteases such as trypsin, ficin, and bromelin prior to cross-linking with transglutaminase showed improvement in the gelation properties of canola protein (Pinterits and Arntfield, 2007). The limited hydrolysis during the pretreatment causes partial unfolding of the protein structure, which exposes the

hydrophobic groups and increases the accessibility of lysine and glutamine groups that are needed for transglutaminase-induced crosslinking. The increased hydrophobic interactions and formation of larger polymers through the action of transglutaminase results in the formation of strong gel networks (Léger and Arntfield, 1993). However, excessive hydrolysis leads to release of small peptides, which has the opposite effect on gel formation (Huang, Catignani and Swaisgood, 1999).

Protein concentration is another critical factor that influences gel formation. A minimum protein concentration, known as the least gelation concentration (LGC), is required for the formation of coherent gels. The LGC is influenced by environmental factors (heat, pH, ionic strength) as well as the overall surface hydrophobicity of proteins. A relatively high surface hydrophobicity facilitates better protein/protein interactions that can enable gel formation even at low protein concentration. This was demonstrated in a study by Boyle *et al.* (2018), where the pH extracted camelina protein, which was more denatured and had higher surface hydrophobicity, formed a gel at lower protein concentration as compared to salt extracted camelina protein that had lower surface hydrophobicity.

Genotypic variation, on the other hand, impacts amino acid composition, which influences its functionality. A study investigating soy protein isolated from different varieties correlated the variation in its amino acid composition with differences in its gelation property. Soy proteins from Macon genotype compared to Enrei, FG1, and IL2 genotypes formed stronger and more elastic gel (Riblett *et al.*, 2001). The superior gelation characteristics of Macon genotype were attributed to the higher percentage of hydrophobic amino acid residues as compared to the other genotypes.

Additionally, the gelation property varies among different oilseed species. Camelina protein gels had lower gel strength as compared to soy protein gels (Boyle *et al.*, 2018). The lower gel strength of camelina protein was attributed to the relatively lower molecular weight of its cruciferin subunits (~20-30 kDa) as compared to that of globulin subunits (~20-82 kDa) in soy (Badley *et al.*, 1975; Kinsella, 1979b; Utsumi and Kinsella, 1985; Sathe *et al.*, 1987; O'Kane *et al.*, 2004; Boye, Zare and Pletch, 2010; Fukushima, 2011; Wanasundara, 2011; Fahs and Louarn, 2013). Larger molecular weight proteins have higher tendency to form larger polymers leading to a cohesive and stronger gel network

than lower molecular weight proteins (Tan *et al.*, 2011; Wanasundara, 2011). Differences among species are also attributed to differences in amino acid composition and sequence, which lead to differences in their surface charge and hydrophobicity, in turn influencing the interactions among protein molecules needed for the formation of gel network.

Extrinsic factors such as pH, temperature, and presence of other components in the environment also influence the gelation characteristics. Soy protein isolate did not form a gel at pH 9, and the strength of soy protein gel at pH 7 was lower than that at pH 5 (Kim, Varankovich and Nickerson, 2016). These observations were attributed to higher electrostatic repulsive forces between proteins due to the increased charges at pH values that are farther away from its isoelectric point (pH 4.2). At pH 5, which is closer to its isoelectric point, the charges on protein's surface are low, resulting in reduced electrostatic repulsions and increased protein-protein interactions, thus facilitating the gel network formation.

Heating conditions influence the formation and strength of protein gels. Heating temperature needs to be above the denaturation temperature of the protein to cause unfolding of the protein and the exposure of the buried sulfhydryl and hydrophobic groups, thus promoting protein-protein interactions (Renkema and Van Vliet, 2002). Generally, increase in the temperature is associated with more denaturation of the protein, enhanced hydrophobic interactions between protein molecules, and thus higher gel hardness (Woodward and Cotterill, 1986; Damodaran, 2017). An optimal heating rate is needed for rearrangement and alignment of the proteins into an ordered gel network (Barbut and Mittal, 1990; Sun and Arntfield, 2011a). However, very high temperature or heating rate may result in protein aggregation and precipitation instead of a balanced protein network.

Depending on its type and concentration, salt may influence the gelation characteristics. In the presence of NaCl above 0.1 M, soy protein gels with lower stiffness were produced as compared to those produced without NaCl (Schuldt *et al.*, 2014). Monovalent salts such as NaCl may shield the charges on the protein's surface, which reduces the electrostatic interactions between protein molecules and thus hinders the protein-protein interactions that are needed for the formation of a gel network (Bryant and Julian McClements, 1998). On the other hand, as discussed above, while divalent ions such as Ca^{2+} can be used to induce gelation, they may also be used to enhance the gel strength

of other type of gels. Addition of $CaCl_2$ (0.05 g/100mL) to soymilk resulted in an improved hardness of tofu (pH-induced soy protein gel) (Zhao *et al.*, 2020). These divalent ions have the ability to form salt bridges between negatively charged groups on the protein molecules and consequently promote protein-protein interactions and consequently enhance the gel strength (Hongsprabhas and Barbut, 1996, 1997; Bryant and McClements, 2000; Marangoni *et al.*, 2000).

The presence of other components such as phenolic acids may also influence the formation and characteristics of protein gels. The strength of canola protein gels was reduced in the presence of sinapic acid and thomasidoic acid (Rubino *et al.*, 1996a). Phenolic compounds interact with the proteins to form phenolic-protein complexes, which may prevent protein-protein interactions and thus hinder the formation of a protein gel network (Wanasundara, 2011; Campbell, Rempel and Wanasundara, 2016).

1.10.3 Properties Involving Protein-Oil and Protein-Air Interactions

The surface-active properties of proteins are useful for stabilizing the oil-water and air-water interfaces of emulsion and foam-type food products, respectively. Molecular flexibility is a crucial property that influences the surface activity of proteins. Native conformations of globular proteins, such as cruciferin and napin, usually have limited molecular flexibility, resulting in only partial unfolding at the interface and restricted surface activity (Wanasundara, 2011). Moreover, a balance of hydrophobic and hydrophilic groups on the protein surface is needed to maintain an equilibrium between hydrophobic interactions and electrostatic repulsion, ensuring the formation of a stable viscoelastic protein film at the interface (Damodaran, 2017). Partial denaturation of such globular proteins, either deliberately through protein modification strategies or accidently during processing, can expose its buried hydrophobic residues and enhance its molecular flexibility (Panyam and Kilara, 1996; Foegeding and Davis, 2011). Relocation of buried hydrophobic residues to the surface results in a good hydrophilic/lipophilic balance (HLB), which enables the proteins to interact with both water and oil or air phases.

The emulsifying properties of proteins are usually evaluated in terms of their emulsifying activity index (EAI), emulsion capacity (EC), and emulsion stability (ES). EC determines the amount of oil that can be emulsified per unit of protein. For high EC, proteins should have a relatively flexible structure and a good HLB. EAI represents the

ease of protein migration to the water:oil interface and the unfolding to orient their hydrophobic residues to the oil phase and hydrophilic residues to the aqueous phase. Proteins with flexible structures and good solubility demonstrate high EAI (Nakai, 1983; Barac *et al.*, 2010; Feyzi, Milani and Golimovahhed, 2018). ES represnts the period of time a protein can stabilize the emulsion before the oil and water phases begin to separate. Due to the positive free energy at the water:oil interface, emulsions are thermodynamically unstable (Kinsella, 1979a). Proteins can create a thick, continuous film around the oil droplets to decrease tension at the interface. The protein film acts as a physical barrier among oil droplets and prevents coalescence, providing long-term stability to the emulsions. Additionally, proteins mostly carry a negative charge at neutral pH that can lead to repulsion among proteins adsorbed on the surface of the oil droplets and thus delay phase separation (Kinsella, 1979a; Dagorn-Scaviner, Gueguen and Lefebvre, 1986). Homogenization coupled with the protein characterization impact the droplet size. The smaller the droplet size is the higher is the ES.

Comparable values for EC and EAI have been reported for camelina and soy protein. However, soy protein showed higher ES than camelina protein, owing to the higher charges and thus the better HLB on the surface of soy protein (Boyle *et al.*, 2018). Moreover, the relatively low protein solubility of camelina protein at neutral pH leads to less protein/water interactions and more protein/protein interactions, resulting in quicker coalescence and lower ES.

Environmental conditions during processing, such as temperature and pH, influence the protein structure and thus impact the emulsification properties of proteins. A reduction in the emulsifying properties of rapeseed protein was noted when oil pressing and protein extraction were carried out at higher temperatures (Östbring *et al.*, 2020). Similarly, a decrease in the emulsification properties was also observed with an increase in protein extraction pH used to isolate the protein from *Brassica carinata* (Ethiopian mustard) (Pedroche *et al.*, 2004). Extreme pH or prolonged heat treatment may cause complete protein denaturation and possible aggregation, thus impacting the HLB and protein flexibility, resulting in poor emulsification properties.

The environmental conditions during extraction may also influence the relative proportion of the protein fractions, thus impacting the emulsification properties. Depending

on relative surface activities, protein fractions in a mixture compete with each other for adsorption at the interface (Damodaran, 2017). The ratio of glycinin (11S) to β-conglycinin (7S) plays an important role in determining the emulsification properties of soy protein. Specifically, β-conglycinin has higher emulsifying abilities than glycinin due to its better HLB (Fukushima, 2011). Moreover, β-conglycinin has a smaller size and is deficient in disulfide linkages, allowing it to easily orient at the oil:water interface (Rickert, Johnson and Murphy, 2004; Fukushima, 2011). Similarly, ratio of cruciferin to napin may influence the emulsification properties of *Brassicaceae* proteins. Napin is abundant in basic amino acids, which favor electrostatic interactions. Thus, higher content of napin may lead to enhanced protein-water interactions and thus poor emulsification properties (Wu and Muir, 2008).

In addition to oil:water interface in an emulsion system, proteins can also be utilized to stabilize air:water interface in a foam system. The dispersion of gas bubbles in foams is important for the desirable mouthfeel of foam-based food products such as ice cream, cake, and whipped cream. The foaming property of proteins allows the incorporation and stabilization of gas bubbles. The foaming properties are evaluated by measuring the foaming capacity (FC) and foaming stability (FS) of protein solutions. The molecular characteristics that influence foamability include adsorption rate at the interface, molecular flexibility, and the HLB of proteins. FS is determined by the ability of the interfacial proteins to hold water in the film surrounding the air droplets (Aluko and McIntosh, 2001). FS is influenced by the rheological properties of the protein film, which depend on its hydration, thickness, and favorable intermolecular interactions (Damodaran, 2017).

Similar to ES, FS of canola and camelina proteins have also been shown to be positively correlated to their corresponding solubility (Aluko and McIntosh, 2001; Boyle *et al.*, 2018). Proteins with high solubility interact better with the aqueous environment and hold more water molecules at the air-water interface, contributing to higher FS.

Both cruciferin and napin from rapeseed are good foaming agents with high FC and FS (Nitecka and Schwenke, 1986; Nitecka, Raab and Schwenke, 1986). Camelina protein showed higher FC than soy protein, while both proteins had similar FS (Boyle *et al.*, 2018). Thus, there is a potential to explore camelina protein for plant-based food applications involving foam systems.

In conclusion, previous research has shown that some functional properties of camelina protein ingredients, such as solubility in acidic conditions, WHC, and emulsification and foaming properties, are comparable or better than soy protein. These findings indicate that camelina protein has the potential to be explored as a viable, alternative plant protein ingredient in different food applications. However, it is important to understand how the profile, structure, and functionality of camelina protein would be impacted under various extraction conditions involved in the production of camelina protein ingredients.

1.11 Extraction and Isolation of Camelina Protein

Considerable processing is needed to produce protein ingredients with high purity, quality and commercial viability. Plant protein processing involves isolation from other plant components and concentration. Oil is one of the major constituents of oilseeds such as camelina. Mechanical pressing, solvent extraction, supercritical carbon-dioxide extraction, or a combination of these methods have been explored to expel and separate oil from camelina seeds (Moslavac *et al.*, 2014).

Mechanical pressing of oil can be categorized into cold or hot pressing and can be achieved using either screw press or hydraulic press. In cold pressing, the seeds are pressed to obtain the cold-pressed camelina cake, while maintaining the temperature below 40°C. Cold pressing helps to prevent any potential heat damage to the protein during the oil extraction process (Campbell, Rempel and Wanasundara, 2016). On the other hand, hot pressing employs higher temperatures to facilitate the ruptures of cell wall material, leading to higher oil yield and lower oil content in the pressed cake (Östbring *et al.*, 2020). An oil expeller or expresser with an auger system uses both pressure and heat to break pre-heated seeds (50° to 130°C), compact them into pellets, and press out the oil (Pradhan *et al.*, 2011). Hot pressing using a screw press at 50°C, which is below the measured onset of denaturation of cruciferin and napin, has been found to result in partial denaturation of both proteins in camelina (Boyle *et al.*, 2018). However, there was no major impact of oil pressing conditions on the functionality of camelina protein.

Further reduction of oil content after pressing is needed to facilitate protein extraction without interference and formation of an emulsion that prohibits efficient protein extraction. Reduction of oil content is achieved by subjecting the pressed cake to solvent

extraction using organic solvents to obtain a defatted meal. Hexane is commonly employed in the industry to ensure maximum oil recovery and a higher oil yield (Wanasundara, 2011). Organic solvents, however, are known to cause protein denaturation (Khmelnitsky *et al.*, 1991). Extreme protein denaturation increases surface hydrophobicity and protein-protein interactions, leading to formation of large protein aggregates. Protein aggregation affects adversely the protein functionality. Therefore, optimization of oil extraction process is advantageous in recovering most of the oil, while preserving the protein quality.

Dehulling of *Brassicaceae* seeds for reduction in its fiber content is problematic due to their smaller size compared to soybean (Wanasundara, 2011). Therefore, seed coat of camelina seeds forms a considerable fraction of the defatted meal and necessitates considerable processing to produce camelina protein ingredients with high protein purity. Different wet processing methods for the extraction of camelina protein from the defatted meal have been explored. Unlike other members of the *Brassicaceae* family, *Camelina sativa* contains large amount of mucilage with high water binding capacity (Grady and Nleya, 2010). Thus, defatted camelina meal in water forms a thick slurry that complicates aqueous processing and leads to inefficient solubilization of protein, resulting in lower protein extraction yield. The consequent impact on the purity of the camelina protein extracts can be minimized by subjecting the defatted camelina meal to degumming prior to protein isolation. Degumming is carried out by dispersing the defatted camelina meal in water to solubilize the gums and soluble fiber, which are then separated (Li *et al.*, 2014). Water-soluble proteins may also get extracted in the water during the degumming step. Li *et al.* (2014) found that the water soluble proteins show lowest solubility at pH 2.5-3, thus allowing their precipitation and consequent separation from the soluble gums. Studies that investigated wet processing methods of camelina proteins investigated different solvents and pH conditions to solubilize the proteins and isolate them from the meal (Reddy *et al.*, 2012; Li *et al.*, 2014; Zhao *et al.*, 2014; Sarv, Trass and Diosady, 2017). Different protein concentration methods are then employed to recover the solubilized protein (Wanasundara, 2011).

1.11.1 Alkaline Extraction

Alkaline protein extraction approach is the most common solubilization method. Proteins are solubilized at an alkaline pH followed by separation of the insoluble

components mostly by centrifugation. The solubilized protein is then isolated from soluble sugars, oligosaccharides, and dietary fiber by acid precipitation at the protein's isoelectric pH. The precipitated protein is sometimes subjected to diafiltration to reduce the salt content and enhance purity.

Proteins from different sources have different solubilization and precipitation pH conditions, depending on its protein fractions and their physicochemical properties. Thus, it is necessary to determine pH conditions that result in maximum protein yield and purity for any given source. Typically, higher solubilization pH is required for higher protein yield (Li *et al.*, 2014). Ionization of carboxyl, hydroxyls, and sulfydryl groups in the protein at higher pH leads to enhanced interactions between charged groups and water, thus increasing the solubilization of proteins and resulting in higher yield (Green and Hughes, 1955).

The pH employed during protein extraction may also impact protein functionality and digestibility, as well as color. The ionization of amino acids at extremely high pH values increases intramolecular electrostatic repulsion within the protein molecules, resulting in unfolding (Damodaran, 2017). Protein unfolding causes exposure of hydrophobic and sulfydryl groups, which may cause protein crosslinking and aggregation, and subsequent loss of protein functionality (Alizadeh-Pasdar and Li-Chan, 2000). Moreover, protein aggregation also decreases protein digestibility and bioavailability of amino acids (Damodaran, 2017). Alkaline pH can cause racemization of amino acids from L- to D-, which are less absorbed in the body. Additionally, under alkaline conditions, cysteine and serine amino acids can be converted into nephrotoxic lysinoalanine compounds, resulting in loss of essential amino acids (Lam *et al.*, 2018).

Phenolic compounds such as tocopherols, sinapine, sinapic acid, flavanols, and flavonols, are present in camelina (Zubr and Matthaus, 2002; Salminen *et al.*, 2006; Abramovič, Butinar and Nikolič, 2007; Salminen and Heinonen, 2008; Terpinc *et al.*, 2012). Under alkaline conditions during protein extraction, the phenolic compounds are oxidized to quinones, and binding of quinones to proteins is enhanced. These large complexes usually result in the formation of brown pigments, which are co-extracted with the proteins and negatively impact the color and flavor of the final protein products (Matheis and Whitaker, 1984; Pourcel *et al.*, 2007). Reducing agents can be added to

prevent the oxidation and consequent darkening of protein isolates during alkaline extraction (Sarv, Trass and Diosady, 2017). Further investigation into the alkaline extraction of camelina protein is necessary to optimize the protein purity and yield, while preserving the functional, nutritional, and sensory quality of camelina protein isolates for food applications.

1.11.2 Salt Extraction

Salt extraction is another protein extraction method, yet not as commonly employed in industry. Salt extraction method involves increasing the ionic strength of the environment using salt to solubilize (salting-in) the protein and separate it from insoluble fiber. Typically, salting-in phenomenon is followed by salting-out, in which ionic strength of the environment is further increased beyond a specific threshold to precipitate the solubilized proteins and separate them from soluble sugars and fiber. The isolated protein is then subjected to diafiltration to get rid of the excess salt, which may impact the functionality of the protein. A similar salt extraction method has been investigated to produce camelina protein concentrates with acceptable functionality (Boyle *et al.*, 2018). However, the use of high amount of salt and the need for diafiltration makes this process not industrially feasible due to high water use and high waste stream. Due to the same reason, commercially available plant protein ingredients (mostly soy and pea protein isolates) are commonly extracted using pH solubilization/precipitation method even though a less functional ingredient is produced.

Ultrafiltration is an industry feasible method that has been explored to separate low molecular weight components from the solubilized protein. Apart from salt, the low molecular weight components solubilized with the protein may include small sugars and oligosaccharides, phenolics, phytates, glucosinolates and their breakdown products (Wanasundara, 2011; Sarv, Trass and Diosady, 2017). Prior to ultrafiltration, the interactions between phenolics and the protein molecules can be disrupted by solubilizing the protein extract in sodium chloride solution at 55-60°C for 30 minutes (Xu and Diosady, 2000a). Sarv *et al.* (2017) used the sodium chloride pretreatment followed by ultrafiltration to reduce the phenolics content by approximately 60% and produce camelina protein extracts with a purity of 40-70% and yield of approximately 11%. Further work is needed

to develop beneficial and industry feasible techniques such as ultrafiltration to maximize the yield and purity of camelina protein isolates.

As camelina contains a heterogenous mixture of various types of proteins, different extraction methods can result in protein isolates with unique protein profile, structure, and functionality. Exposure to extreme conditions such as high pH during the extraction of camelina protein may lead to amino acid destruction, undesirable interactions between protein and other components, protein denaturation and polymerization, resulting in deterioration of nutritional and functional quality of the protein. Boyle *et al.* (2018) demonstrated that salt extraction results in a less denatured camelina protein as compared to pH extraction. Due to lower denaturation and subsequently lower surface hydrophobicity, salt extracted camelina protein showed higher solubility, better emulsification properties, and higher foaming capacity than pH extracted camelina protein. However, considering the lower feasibility of salt extraction methods, further innovation in feasible extraction methods may enable the development of camelina protein ingredients with superior functional properties.

1.12 Limitations Associated with Camelina Use

1.12.1 Antinutritional Components in Camelina

Antinutritional components include phytochemicals produced by the plants that act as protective or defensive agents against various environmental stresses (Buntrock, 2012). Brassicaceae seeds, including camelina, contain antinutritional compounds such as glucosinolates, phenolics, phytates, and trypsin inhibitor. The genotype and environmental growing conditions influence the accumulation of such phytochemicals (Berhow *et al.*, 2014). The presence of these compounds hinders the bioavailability of proteins and minerals, thus restricting the utilization of camelina for human consumption.

Although glucosinolates in their original form are not harmful, the hydrolyzed products that are formed when acted upon by the myrosinase enzyme have shown toxic effects at higher doses, especially impacting the function of the thyroid gland (Wanasundara *et al.*, 2017). On the other hand, beneficial bioactivities of glucosinolates such as biocidal activity, bioherbicidal potential, antioxidant activity, antimutagenic and antiproliferative activity have also been documented (Vig *et al.*, 2009). Further research is

needed to assess the effect of specific glucosinolates found in camelina on human health to advance the utilization of camelina-based ingredients in food.

Glucosinolates are sulfur-containing compounds that are derived from amino acids (Czerniawski and Bednarek, 2018). The biosynthesis of glucosinolates in camelina is influenced by its sulphur amino acid content, which in turn is related to the sulphur concentration in the soil (Schuster and Friedt, 1998). The three types of glucosinolates present in camelina include glucoarabin (9-methylsulfinylnonyl-glucosinolate), glucocamelinin (10-methylsulfinyldecyl-glucosinolate), and 11-methylsulfinylundecyl-glucosinolate (Schuster and Friedt, 1998; Berhow *et al.*, 2013). The allowable levels of glucosinolates that have been defined for canola are 30 μmol/g (USDA Standards, 1992). Glucosinolates are soluble in water and are accumulated in the pressed cake after oil extraction (Tzeng, Diosady and Rubin, 1988). The amounts of glucosinolates in expeller pressed camelina meals range from 27 to 42 μmol/g (Almeida *et al.*, 2013). However, as these compounds are water soluble and have smaller size than proteins, glucosinolates are generally removed during protein extraction and isolation. Glucosinolate-free protein isolates were produced from canola meal using alkaline extraction, isoelectric precipitation, and membrane processing despite the presence of glucosinolates in the starting canola meal (Tzeng, Diosady and Rubin, 1990). Thus, production of camelina protein isolates with low levels of glucosinolates is warranted through the optimization of extraction conditions.

Phenolic compounds including lignans, tannins, and sinapine are reported to be present in camelina seeds (Matthäus and Zubr, 2000; Smeds, Eklund and Willför, 2012; Terpinc *et al.*, 2012). Phenolic compounds have a bitter flavor and dark greyish color (Wanasundara *et al.*, 2017). Camelina seeds have been reported to contain sinapine at high levels (Matthäus, 2002). The presence of sinapine in camelina lowers the palatability of animal feed due to its bitter taste (Berhow *et al.*, 2014).

The presence of phenolic compounds in camelina may impact the organoleptic and nutritional quality of its protein ingredients. The phenolic compounds interact with the protein to form protein-phenol complexes, especially during the alkaline extraction of proteins, resulting in darker color of the protein isolates. The high tannin contents in camelina have been reported to cause depression in the protein digestibility due to the

formation of such complexes and modification of protein structure that impacts the activity of digestive enzymes (Kahindi *et al.*, 2014).

The formation of protein-phenol complexes also influences the functional properties of the protein (Spencer *et al.*, 1988; Schwenke and Dąbrowski, 1990). Binding of free sinapic acid to canola cruciferin is enhanced at pH 4.5 and in the absence of NaCl (Rubino *et al.*, 1996b). Under alkaline protein extraction conditions, free sinapic acid is converted to thomasidioic acid, which can also bind to the protein. A negative effect on the formation of heat-induced gel and gel characteristics has been observed due to the binding of both sinapic acid and thomasidioic acid to the protein (Rubino *et al.*, 1996b).

Phytic acid may bind to essential dietary minerals and proteins, resulting in lower bioavailability and functionality, respectively. Due to its strong cation binding ability, phytic acid can bind metal ions, such as zinc, calcium, and iron, consequently causing a mineral imbalance in the diet (Tzeng, Diosady and Rubin, 1988; Wanasundara, 2011). Phytic acid also interacts with napins and cruciferins at pHs below their isoelectric point where the proteins carry a net positive charge. These electrostatic interactions lead to the formation of insoluble complexes that may further hinder protein functionality and digestibility. A decrease in solubility of the rapeseed cruciferin was observed due to the formation of phytic acid-protein complexes in the pH range of 3 to 7 (Gillberg and Tornell, 1976; Schwenke *et al.*, 1987; Kroll, 1991). The formation of stable electrostatic complexes between phytic acid and proteins may also diminish the surface activity of proteins (Krause and Schwenke, 2001). Different approaches including controlled extraction conditions, chemical modification, and the use of phytate degrading enzymes have been investigated to reduce the formation of phytic acid complexes in canola meals and canola protein concentrates (Wanasundara, 2011). Ultrafiltration of rapeseed protein, which was extracted using high salt concentrations (0.7-0.9M NaCl) to weaken the electrostatic interactions between phytates and proteins, has been shown to be effective in lowering the phytic acid levels in the protein concentrates (Serraino and Thompson, 1984).

Trypsin inhibitor activity in camelina depends on variety, seed origin, and growing conditions (Zubr, 2003). Trypsin inhibitor affects the activity of serine proteases and consequently lowers protein digestibility (Friedman and Brandon, 2001). Thus, reduction in trypsin inhibitor activity is needed to maintain the nutritional quality of camelina protein.

Heat treatment could be used for the reduction of trypsin inhibitor activity (Herkelman *et al.*, 1993). Considering the existing variation of trypsin inhibitor activity in camelina cultivars, selective breeding approach has also been proposed to reduce its levels (Berhow *et al.*, 2014).

Reduction in the level of antinutritional components of camelina protein isolates is important to preserve their functional, nutritional, and sensory qualities, as well as make them suitable for human consumption. The levels of antinutritional compounds in camelina cultivars can be minimized through innovation in selective breeding of camelina. Furthermore, appropriate processing methods such as ultrafiltration have successfully demonstrated reduction or elimination of antinutritional components in rapeseed protein products (Tzeng, Diosady and Rubin, 1988). Filters with high capacity and low cost are industrially available (Gésan-Guiziou, 2017), thus promoting the potential scope of utilizing ultrafiltration to produce camelina protein isolates.

1.12.2 Knowledge Gaps: Processing and Selective Breeding of Camelina

Although there has been research on protein isolation and characterization of camelina protein, more investigation is needed before camelina protein ingredients can be successfully marketed for food applications. Boyle *et al.* (2018) explored two different extraction methods, namely pH solubilization/precipitation and salt extraction, to isolate camelina protein. Characterization of the isolated proteins showed that salt extraction produced a less denatured and more functional camelina protein compared to pH extraction. However, salt solubilization/precipitation requires the use of a lot of water, resulting in an unsustainable and nonviable process. While other protein purification techniques such as membrane filtration and chromatography show potential to produce a functional protein ingredient more efficiently, limited research has been done on their utilization for purifying plant proteins. Additionally, to develop cost effective and industry feasible protein isolation processes, there is a need to further explore protein extraction methods for enhancing the yield, purity, and quality of the camelina protein ingredients. Moreover, the extraction conditions might impact not only the functionality, but also nutritional quality as well as the color and flavor of the resulting isolate. Preservation of functional, nutritional, and organoleptic quality of the protein during the extraction process

needs to be investigated to enhance the acceptability and commercialization potential of novel plant proteins such as camelina protein.

While breeding of soy protein to enhance protein quality has been studied extensively, little research exists on camelina protein. Unique functional properties of camelina protein can be developed through selective breeding. Although there have been ongoing breeding efforts to enhance the yield, adaptation, and cultivation of camelina, breeding to enhance the functional and nutritional quality of camelina protein has not been explored. Markers and tools for camelina need to be developed to initiate breeding strategies for direct enhancement in its protein functionality and nutritional quality. Evaluation of natural variations in existing camelina lines can provide useful information regarding protein quantity and quality. The inherent differences in protein content and profile among different camelina lines could arise as a result of genetic variation or differences in environmental growing conditions. On the other hand, advanced breeding techniques employed to enhance the agronomic traits in camelina may inadvertently impact the protein profile and structure. Considering the growing demand and utilization of novel plant proteins in the food industry, a deeper investigation into camelina accessions or varieties with superior traits for protein quality and functionality is needed. Such investigation will guide the development of genetic markers, thus enabling breeding of camelina lines for traits that are desirable for food applications. Therefore, an evaluation of protein profile and functionality among different camelina lines coupled with breeding programs for efficient introgression of desirable traits will advance the development of camelina as a novel plant protein source.

1.13 Conclusions

The rising popularity of plant-based protein ingredients are paving the way for utilization of camelina protein for food applications. Moreover, development of high-value applications for protein ingredients from camelina will benefit the farmers as well as food producers. The agronomic benefits associated with the cultivation of camelina include low agricultural inputs, short growing season, enhanced nutrient sequestration, reduced soil erosion, and decreased nitrate leaching. These agronomic benefits along with potential end-use market are incentives for farmers to cultivate camelina. Camelina protein also has shown potential to replace soy protein for some food applications, without the concerns

around GMO status and allergenicity. However, further work is needed to develop industry feasible production methods and to understand the applicability of camelina protein ingredients.

The extraction and processing conditions can be highly variable and could alter the protein profile and structure, either intentionally or consequentially. The variability in the extraction and processing conditions could lead to differences in the functional and nutritional properties of the resulting protein ingredients. In order to develop camelina protein isolates with good functional properties for various food applications, the native protein structure needs to be preserved. Thus, extraction and processing conditions should be optimized to maximize purity and yield of camelina protein isolates, while maintaining the structural and functional integrity as well as nutritional quality of the protein.

Alkaline extraction followed by isoelectric precipitation is the most common extraction technique in the industry to produce soy and pea protein ingredients. The protein ingredients produced using this method are generally dark in color and have a high degree of protein aggregation. The color of the isolates could be improved and aggregation via disulfide linkages could be reduced using reducing agents during the protein extraction. The usage levels of the reducing agents need be optimized to produce isolates with an acceptable color and protein characteristics.

Camelina protein produced using salt extraction has shown superior functionality as compared to pH-based extraction. However, salt extractions methods are less commonly used in industry due to the protein precipitation step that creates a waste-water stream with high salt concentration, making the process less viable. It may be possible to produce protein isolates using dilute salt concentrations for protein solubilization, followed by membrane filtration for salt removal. Development of such industry feasible methods may advance the production of camelina protein ingredients with superior functionality.

Finally, improvement in the germplasm for camelina is an on-going process for enhancement of desirable agronomic as well as food-related traits. The existing camelina lines need to be investigated for natural variations in the protein profile and structure. Variation in protein profile and structural characteristics among different camelina lines can lead to differences in protein functionality. The identification of superior genetic variants will provide baseline data to enhance the superior traits beyond their current usage,

and result in the development of new camelina lines with improved functional and nutritional quality. Breeding for quality traits would allow the utilization of camelina for widespread food applications. Therefore, there is a need to optimize protein extraction methods for enhanced protein functional and nutritional quality, and to investigate diverse camelina lines for the successful adoption of camelina protein in various food applications. Development of functional and nutritious camelina protein will address the growing demand for alternative plant proteins.

Chapter 2: Materials and Methods

2.1 Materials

Camelina seeds of the Joelle variety, a winter line, were grown at Rosemount Research Station (Minnesota, US) and harvested in the summer of 2019. All other camelina accessions and mutant lines were obtained through the breeding program at the University of Minnesota (identification numbers, varieties, growth habits, origins, growing locations, ad harvest years for all lines are summarized in **Table 10**, Appendix A). The spring camelina line originating from Canada was harvested in 2017, and later grown in the greenhouse at the Plant Growth Facilities (Minnesota Agricultural Experiment Station, St. Paul, MN) in 2020 for seed increase. Sunshine® Mix #8 was obtained from Sun Gro Horticulture (Agawam, MA, USA). Soy protein isolate (cSPI, 90% protein) and pea protein isolate (cPPI, 79% protein) were kindly provided by ADM Specialty Food Ingredients (Decatur, IL). All samples were stored at -20°C when not in use.

Bullet Blender Storm 24 and RINO® screw-cap tubes were purchased from Next Advance, Inc. (Troy, NY, USA). Criterion™ TGX™ 4-20% precast gels, Laemmli sample buffer, 10x Tris/Glycine/SDS running buffer, Imperial™ Protein stain, and Precision Plus™ molecular weight marker were purchased from Bio-Rad Laboratories, Inc. (Hercules, CA, USA). SnakeSkin™ dialysis tubing (3.5K molecular weight cutoff (MWCO)) and Sudan Red 7B were purchased from Thermo Fisher Scientific™ (Waltham, MA, USA). Electrophoresis grade sodium dodecyl sulfate (SDS), 2-mercaptoethanol (βME), Costar® solid opaque black 96-well plates, and 8-anilino-1-napthalenesulfonic acid ammonium salt (ANS) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Aluminum crucibles (40 µL, with pin) for DSC were purchased from Mettler-Toledo (Columbus, OH, USA). Folded capillary cuvettes for zeta potential were purchased from Malvern (Malvern, UK). Vivaflow® membrane ultrafiltration cross-flow cassettes (3kDa MWCO) were purchased from Sartorius™ (Gottingen, Germany). Protein Digestibility Assay Kit (K-PDCAAS) was purchased from Megazyme (Bray, Ireland). All other chemical grade reagents were purchased from either Thermo Fisher Scientific or Sigma-Aldrich. Pure corn oil of Mazola brand was purchased from grocery stores.

2.2 Screening and Cultivation of Camelina Lines

The protein profiles of 40 different camelina lines were screened following the method developed by Dr. Krishan Mohan Rai (Post-Doctoral Research Associate in the Department of Plant and Microbial Genetics at the University of Minnesota), with some modifications. In screw-cap tubes, 10 mg of camelina seeds were ground using Bullet Blender Storm 24. The meal was defatted by homogenizing with 1 mL of hexane, incubating for 5 minutes at room temperature, and then centrifuging at 6,400 x g for 2 minutes using Centrifuge 5415 D (Eppendorf, Hamburg, Germany). The hexane layer was decanted, and the residual hexane was left for evaporation under the hood for 10 minutes. For extracting the protein, the defatted meal was homogenized with 1 mL of extraction buffer (50 mM Tris-Cl, 100 mM EDTA, 100 mM NaCl), followed by incubation at room temperature for 10 minutes. After centrifugation at 9,016 x g for 2 minutes, the supernatant containing the solubilized proteins was transferred to a new tube to perform protein profiling using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), as described by Boyle et al. (2018). The supernatants were mixed 1:1 (v/v) with Laemmli buffer under reducing (using βME) and nonreducing conditions. Each sample (5 µL; containing ~2-4 µg protein) and Precision Plus^{TM} MW standard (10 µL) were loaded onto a Criterion™ TGX™ 4-20% precast Tris-HCl gradient gel. The gel was electrophoresed, stained, destained, and then scanned using Molecular Imager Gel Doc XR system (Bio-Rad Laboratories).

Based on the preliminary screening of 40 different camelina lines using the above method, four lines (one spring line, and 3 winter lines) with variation in protein profile were selected for cultivation to obtain larger seed quantity sufficient for production of protein isolates. These four lines were planted in the greenhouse at the Plant Growth Facilities (Minnesota Agricultural Experiment Station, St. Paul, MN). The seeds for the spring line were directly planted into the soil at the greenhouse in October 2020. Winter lines seeds were subjected to vernalization treatment (4°C for 21 days) before planting the resulting seedlings in the greenhouse in November 2020. All lines were grown in Sunshine® Mix #8. The crops were grown under 16-hour photoperiod and moderately watered once every three days until they were ready for harvest. Almost all plants from the spring line produced seeds, which were harvested in December 2020. Only ~20% of plants

from M5 220-1 (winter line) produced seeds, which were harvested in April 2021. Plants from both Ascn 413 and Ascn 259 (winter lines) did not produce any seeds. Out of the four lines, only spring line produced enough seeds sufficient for protein extraction and analysis. The harvested camelina seeds were evaluated for their protein content based on the Dumas method (AOAC 990.03) using LECO® FP828 nitrogen analyzer (LECO, St. Joseph, MI, USA) with a nitrogen-to-protein conversion factor of 6.25.

2.3 Production of Defatted Camelina Meal (DCM)

Joelle camelina seeds (winter line) and spring line were pressed for 36 hours at ambient temperature using a hydraulic press (Carver Model C 8-ton manual bench top laboratory press with 2094 cage equipment, Carver, Inc, Wabash, IN, USA) with a starting maximum pressure of 6,500 psi. The pressed seeds were ground using a KitchenAid® coffee grinder. The fat in the pressed, ground seeds was further reduced using 2 cycles of hexane (3:1 ratio) of 30 mins each, followed by overnight evaporation of residual hexane under the hood. The defatted meal was then milled to 50 mesh using a cyclone sample mill (Udy Corp, Fort Collins, CO, USA). The milled camelina flour was then subjected to additional 2 cycles of hexane (3:1 ratio) of 30 mins each. The optimization data for number of cycles and duration of hexane needed for extracting fat from Joelle winter line can be found in Appendix B (**Table 11**). The spring line needed 2 cycles of hexane (30 mins each) before milling and 3 cycles of hexane (30 mins each) after milling to reach a fat content less than 3% for efficient protein extraction. To prevent clogging of the mill, the fat content had to be reduced to less than 8% before milling. The defatted camelina meals from both winter line (WL-DCM) and spring line (SL-DCM) had ~3% fat content, as verified by the Mojonnier AOAC method 922.06 (AOAC International 2016).

2.4 pH Extraction of Camelina Protein

The optimization for pH extraction by alkaline solubilization coupled with isoelectric precipitation was performed using Joelle WL-DCM based on the extraction method described by Li et al. (2014) and Boyle et al. (2018), with modifications. To separate the soluble gums and polysaccharides, the WL-DCM was dispersed in distilled deionized water (DDW) at 2.5% w/v, stirred at room temperature for 1 hour, then centrifuged at 13,000 x g for 20 minutes. The water-soluble camelina proteins (WCP)

present in the supernatant were precipitated at pH 3 by centrifugation at 13,000 x g for 15 min, redispersed in DDW, and neutralized.

The pellet obtained after degumming was redispersed (2% w/v) in DDW or sodium sulfite solution (0.05% or 0.1%), followed by adjustment to pH 11.0 using NaOH. Solubilization pH was selected based on preliminary screening, which showed optimum solubilization yield (64.6%) at pH 11.0 (Appendix C, **Table 12**). The alkaline dispersion was stirred for 1 hour at room temperature, then centrifuged at 13,000 x g for 15 minutes. The pellet was redispersed (2% w/v) in DDW or sodium sulfite solution, adjusted pH to 11.0 using NaOH, stirred for 1 hour at room temperature, and centrifuged at 13,000 x g for 15 minutes. The supernatants from both solubilization cycles were combined and subjected to vacuum filtration, followed by pH adjustment to pH 5.0, and centrifugation at 13,000 x g for 10 minutes to precipitate the alkali-soluble camelina proteins (ACP). The precipitated protein pellet was redispersed in DDW and neutralized using NaOH. The two protein fractions, WCP and ACP, were dialyzed individually to remove salts, lyophilized and then combined to form pH extracted camelina protein isolates (pH-CPI). All extractions were carried out in triplicate. Samples were stored at -20°C when not in use.

The protein content of all fractions was determined based on the Dumas method. Mass balance was tracked to determine protein yield for the following fractions: ACP, WCP, pH-CPI, residual gums after precipitation of WCP, residual pellet after solubilization of proteins, and residual supernatant after precipitation of ACP. The protein yield/lost/residue for each fraction was calculated as shown in Appendix D and illustrated in **Table 13**. Ash contents of each pH-CPI sample was measured following the official AOAC method (AOAC 942.05).

The color of pH-CPI samples was evaluated using a Chroma Meter CR-221 (Minolta Camera Co., Osaka, Japan). The color for each sample was expressed using the CIE (International Commission on Illumination) 1976 L* a * b * color space system, where L* indicates lightness, ranging from 0 (black) to 100 (white); positive a* values represent level of redness; negative a* values represent level of greenness; positive b* values represent level of yellowness, while negative b* values represent level of blueness. The total color difference (ΔE) between the sample extracted with DDW (0% sodium sulfite) and the samples extracted with varying levels of sodium sulfite was determined. The

protein profile of each sample was visualized using SDS-PAGE. Optimum sodium sulfite concentration was determined based on protein purity, protein yield, ash, color, and protein profile. Using the optimized extraction conditions, scaled-up pH extraction was carried out to produce camelina protein isolates from winter line (WL-pH-CPI, 83.1% protein content, 3.9% ash) and from spring line (SL-pH-CPI, 80.6% protein content, 3.7% ash) for protein characterization.

2.5 Salt Extraction of Camelina Protein

The optimization trials for salt extraction were performed using the Joelle winter line. Camelina protein was extracted from WL-DCM by salt solubilization based on the salt extraction method reported by Boyle et al. (2018). However, membrane filtration technique was implemented to replace the salt precipitation step for isolating and purifying the solubilized proteins. As described previously, a degumming step was employed to separate the soluble gums and polysaccharides and the WCP were retrieved by precipitation at pH 3. The residual gums fraction was lyophilized, and the redispersed and neutralized WCP fraction was later combined with the salt solubilized protein supernatant.

During preliminary screening, the protein yield of solubilization in a phosphate buffer with NaCl used in previous studies (Wu and Muir, 2008; Boyle *et al.*, 2018) was compared to solubilization in NaCl solutions at different concentrations without phosphate buffer. A comparable protein solubilization yield was observed between 0.5 M NaCl solution with and without phosphate buffer (Appendix E, **Table 14**). Consequently, further investigation was carried out using 0.5 M and 0.75 M NaCl solutions. The degummed pellet was dispersed (2% w/v) in NaCl solution, stirred for 1 hour in a water bath at 50°C, and centrifuged at 13,000 x g for 20 mins. The pellet was re-dispersed in NaCl solution (2% w/v), stirred for another hour in the water bath at 50°C, and centrifuged at 13,000 x g for 20 mins. The residual pellet was lyophilized. Supernatants from the two solubilization cycles and WCP fraction retrieved from the degumming step were combined and subjected to cross-flow (tangential) ultrafiltration (UF) and dialysis to concentrate the protein and reduce the salt content.

For ultrafiltration, a benchtop Sartorius Vivaflow® 200 system was used with two Vivaflow® membrane cassettes running in parallel to increase the speed of filtration. According to manufacturer's instructions, the system was set up with the protein

supernatant in a feed reservoir and the feed tube connected to a peristaltic pump (Masterflex Easy Load Pump Head- Size 15, Masterflex Economy Drive Peristaltic Pump 230V, Sartorius) to pump the feed protein solution under pressure (2.5 bars) across the membranes. Membranes with a pore size of 3 kDa were used, so the components in the protein solution that were smaller than 3 kDa passed through filter pores as the permeate, which was collected in a waste bottle. Components that were larger than the pores were retained and recirculated to the feed reservoir. Initially, the volume of the protein solution was concentrated to 50 mL to minimize the amount of water needed for diafiltration. The samples were then diafiltered based on manufacturer's instructions, using 6 volumes of DDW (300 mL total) to continually reduce the concentration of salt and other low molecular weight compounds and further concentrate the protein. Finally, the protein solution was concentrated to 25 mL. To end UF, the feed tube was removed from the feed reservoir and some amount of air was pumped through the system in order to remove the residual protein solution from the tubing. The feed tube was then placed in DDW, and approximately 25 mL of DDW was pumped through the system in order to flush out any remaining protein solution and help increase protein recovery. The resulting protein extract was then subjected to dialysis using 3.5 kDa MWCO dialysis tubing to further reduce the salt and enhance the protein purity. The purified protein extract was lyophilized to obtain CPI produced by salt extraction (salt-CPI). All extractions were carried out in triplicate. Samples were stored at -20°C when not in use.

Protein purity of both salt-CPI was assessed following Dumas method. Mass balance was tracked by calculating the protein yield/lost/residue as shown in Appendix F and illustrated in **Table 15**. The residual salt in each isolate was evaluated by measuring the ash content. Optimum salt concentration for protein extraction was determined by comparing protein purity, protein yield, and ash of the salt-CPIs.

Using the optimized extraction conditions, scaled-up salt extraction was carried out to produce CPI from winter line (WL-salt-CPI, 82.26% protein content, 5.7% ash) and from spring line (SL-salt-CPI, 81.93% protein content, 7.6% ash) for protein characterization. For the scaled-up salt extraction, the solubilized proteins were ultrafiltered/diafiltered in the pilot plant instead of using the bench-top Vivaflow® membrane system, to allow for higher throughput. The ultrafiltration/diafiltration was

performed using a plate-and-frame unit (DD20, Osmonics) with a crossflow across a 3 kDa MWCO membrane (inlet pressure: 10-15 psi, outlet pressure: 25-30 psi) until the permeate solids was reduced to ~0%. The retentate after filtration was then dialyzed and lyophilized to obtain the CPI. The color of the isolates was evaluated using a Chroma Meter CR-221 as described earlier.

2.6 Protein Structural Characterization

2.6.1 Protein Profiling by Gel Electrophoresis

The protein profile of commercial protein isolates, defatted camelina meals, and camelina protein isolates (WL-pH-CPI, WL-salt-CPI, SL-pH-CPI, and SL-salt-CPI) were determined using SDS-PAGE, as outlined by Boyle et al. (2018). Protein samples were dispersed in DDW (20 mg protein/mL) and solubilized for 2 hours. The samples were then mixed 1:1 (v/v) with Laemmli buffer under reducing (using βME) and nonreducing conditions. Each sample (5 µL; containing ~0.01 mg protein) and Precision Plus™ MW standard (10 µL) were loaded onto a Criterion™ TGX™ 4-20% precast Tris-HCl gradient gel and electrophoresed at 200V. The gel was then stained using Imperial™ Protein Stain, followed by destaining with DDW. The gel was finally scanned using Molecular Imager Gel Doc XR system (Bio-Rad Laboratories).

2.6.2 Thermal Denaturation by Differential Scanning Colorimetry (DSC)

The protein denaturation temperature and enthalpy of denaturation for protein isolates were measured with a DSC instrument DSC 1 STARe System (Mettler Toledo Columbus, OH, USA), following the method adapted from Boyle et al. (2018). 20% protein (w/v) solutions, in triplicate, were solubilized in DDW overnight at room temperature. Aliquots of each sample (20 µL, delivering approximately 4 µg protein), in triplicate, were weighed in aluminum pans, which were then hermetically sealed. The pans were held at 25°C for 5 minutes, followed by ramping up to 115°C for camelina isolates and up to 110°C for commercial samples at 5°C/minute. An empty, sealed pan was run simultaneously as a reference. Thermograms were manually integrated to determine the peak denaturation temperature and enthalpy of denaturation using the Mettler Toledo's STARe Software version 11.00.

2.6.3 Protein Surface Properties

The surface hydrophobicity of protein isolates was analyzed spectrophometrically using an 8-anilino-1-napthalenesulfonic acid ammonium salt (ANS) probe. The method was adapted from Boyle et al. (2018) with modifications in fluorescence gain (40) and type of plate (black 96-well plate). Protein solutions (0.05% protein w/v) were solubilized, in triplicate, in 0.017M: 0.165M citric acid:sodium phosphate buffer (pH 7), in triplicate, and diluted to concentrations ranging from 0.05% and 0.005% protein (w/v). Aliquots (200µL) of each concentration for all samples and dilution buffer (as blank) were dispensed into the plate. The initial relative fluorescence intensity (RFI) was recorded at room temperature with excitation and emission wavelengths of 400/30 and 460/40 nm (wavelength/bandwidth), respectively, using a microplate reader (BioTek Synergy HT, BioTek Instruments, Winooski, VT, USA). ANS probe solution (20µL) was then added to the wells followed by gentle mixing with pipette tips. After holding the plate in dark for 15 minutes, the RFI was measured again under the same conditions. The net RFI was calculated as described by Alizadeh-Pasdar and Li-Chan (2000). The net RFI was plotted against percent protein concentration and the slope (S_0) was used as an index of protein surface hydrophobicity (sample calculations for RFI and surface hydrophobicity can be found in Appendix G and are illustrated in **Figure 7**).

Zeta potential of protein isolates was determined, in triplicate, using a dynamic light scattering instrument (Malvern Nano Z-S Zetasizer). Solutions of 0.1% protein (w/v) were solubilized in DDW, in triplicate, and stirred for 2 hours with pH adjusted to 7. An aliquot of each protein solution was then dispensed in the folded capillary cell and inserted into the Zetasizer. After a 30 second equilibration period, electrophoretic mobility was recorded by taking three sub-rep readings every 10 seconds. Zeta potential was determined using the Malvern's Zetasizer software (version 7.13) based on the Smoluchowski model.

2.6.4 Protein Secondary Structures by Attenuated Total Reflectance Fourier Transform Infrared Spectroscopy (ATR-FTIR)

ATR-FTIR spectra of protein isolates were recorded, in triplicate, using Fourier transform infrared spectrometer (Thermofisher Nicolett iS50 FTIR). Protein samples were placed on diamond ATR and scanned from 400-4000 cm^{-1} by DLaTGS detector. ATR spectra were converted to transmission spectra using OMNIC® software. Alpha-helix, beta-

sheet, beta-turn, and random coil were identified by obtaining the second derivative of Amide I band (1600cm^{-1} to 1700 cm^{-1}) using PeakFit v. 4.12 (Appendix H, **Figure 8**).

2.7 Protein Functional Characterization

2.7.1 Protein Solubility

Protein solubility was determined following the method outlined by Boyle et al. (2018), with modifications in protein concentration and duration of solubilization. Protein solutions were prepared, in triplicate, at either 1% or 5% protein w/v, adjusted to either pH 3.4 or pH 7.0 using 4 M NaOH or HCl solutions, and stirred for 2 hours. Aliquots (1 mL) were assessed at room temperature and after heating at 80°C for 30 minutes. Both non-heated and heated samples were centrifuged at 15,682 x g for 10 minutes. The protein contents of the initial solutions and of the supernatants were analyzed based on the Dumas method. The percent protein solubility was calculated using the following equation (sample calculation can be found in Appendix I):

$$\%\ protein\ solubility = \frac{\%\ supernatant\ protein}{\%\ initial\ protein}$$

2.7.2 Gel Strength

Strength of heat-induced gels was determined using the procedure described by Boyle et al. (2018), with modifications. Protein solutions (at either 15% or 20% protein w/v in DDW) were prepared, in triplicate, adjusted to pH 7 with 4 M NaOH, and stirred for 2 hours. Aliquots (1 mL) were dispensed into lightly oiled microcentrifuge tubes using a positive displacement pipette. Samples were heated in a water bath at 95°C (± 2°C) for 10 minutes or 20 minutes. The commercial samples were cooled to room temperature, whereas the camelina samples were cooled to room temperature, and then were refrigerated overnight. Gel strength was measured using a TA-TX Plus Texture Analyzer (Stable Micro Systems LTD, Surrey, UK) with a 100 mm diameter probe, 5 mm/s test speed, and a target distance of 0.5 mm from the plate. The maximum force (in Newtons) needed to rupture the gel was expressed as the gel strength.

2.7.3 Water Holding Capacity

Water holding capacity (WHC) was measured according to the method outlined by Boyle et al. (2018), with modifications. Solutions were prepared, in triplicates, at 15% or 20% protein (w/v), adjusted to pH 7 using 4 M NaOH, and stirred for 2 hours. Aliquots (1 mL) were transferred to a microcentrifuge tube using a positive displacement pipette. The weight of the protein solution was recorded before heating the samples at 95°C for 15 or 20 minutes. Samples were cooled to room temperature. Camelina protein samples were further cooled in refrigerated conditions overnight. After recording the weight, the cooled samples were centrifuged at 1,000 x g for 5 minutes. The tubes were then inverted for 10 minutes to drain the water expelled from the gel, followed by recording the final weight. The WHC was determined as the percentage of water that remained trapped in the gel matrix using the following equation (sample calculations can be found in Appendix J):

$$Water\ Holding\ Capacity = 100 \times \left(\frac{T_3 - T_1}{T_2 - T_1}\right)$$

Where,

T_1 = weight of protein solution before heating

T_2 = weight of protein solution + microcentrifuge tube after cooling

T_3 = weight of protein solution + microcentrifuge tube after draining excess water

2.7.4 Emulsification Capacity

Emulsification capacity (EC) was measured according to the method reported by Boyle et al. (2018), with some modifications. Samples were prepared, in triplicate, at 1% or 2% protein (w/v), adjusted to pH 7 with 4 M NaOH, and stirred for 2 hours. Corn oil dyed with Sudan Red 7B was titrated into a 5 mL aliquot of protein solution at a steady flow rate of 2 mL/minute for the first 3 minutes and then increased to 6 mL/minute for the remainder of the titration while blending using a homogenizer (IKA® RW 20 Digital, IKA Works Inc., Wilmington, NC, US) with a 4 blade, 50 mm diameter shaft (IKA® R 1342) rotating at 860 - 870 rpm. The oil titration and simultaneous homogenization was continued until a phase inversion occurred. Based on amount of oil titrated, EC was calculated using the following equation (sample calculations can be found in Appendix K):

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

2.7.5 Emulsion Stability and Activity

Emulsion stability (ES) and emulsification activity index (EAI) were determined following the method reported by Boyle et al. (2018), with modifications. Solutions were prepared, in triplicate, at 0.1% protein (w/v), adjusted to pH 7 with 4 M NaOH and stirred for 2 hours. An aliquot (5 mL) was dispensed into a glass vial containing 1.67 mL of corn oil, followed by immediate homogenization using IKA T-25 Ultra-turrax high shear homogenizer at 18,000 rpm for one minute. An aliquot (50 µL) of the homogenized emulsion was immediately added to 5 mL of 0.1% SDS solution, to accelerate creaming. After vortexing for 5 seconds, the initial absorbance was read at 500 nm using a UV/VIS spectrophotometer (Beckman Coulter DU 800, Brea, CA, USA). After 10 minutes, the absorbance of the emulsion was recorded again. The ES (min) and EAI (m^2/g) were calculated as reported by Boyle et al. (2018) and Cameron et al. (1991), respectively, using the following equations (sample calculations can be found in Appendix L):

$$ES\ (min) = \frac{A_0}{A_0 - A_{10}} \times 10\ min$$

$$EAI\left(\frac{m^2}{g}\right) = \frac{2T}{(1-\phi)C}$$

Where:

A_0 = initial absorbance at 500 nm

A_{10} = final absorbance at 500 nm

C = weight of protein per volume of aqueous phase

ϕ = volume fraction of oil

T = turbidity of oil at 500 nm = $\frac{2.303 \times A_0}{l}$

l = path length of the cuvette

2.8 Nutritional Quality of Camelina Protein Isolates

2.8.1 Amino Acid Composition

The total amino acid analysis of camelina protein isolates was performed by Eurofins (Madison, WI, USA) using the methods reported by Schuster (1988), Henderson *et al.* (2000), Barkholt and Jensen (1989), and Henderson and Brooks (2010). Tryptophan was analyzed by Eurofins using the official AOAC method (AOAC 988.15), and as reported by Schuster (1988), Henderson *et al.* (2000), and Henderson and Brooks (2010).

2.8.2 Protein Digestibility-Corrected Amino Acid Score (PDCAAS)

In vitro digestibility of the proteins was determined by Joseph Eggers, a graduate student in the Department of Food Science and Nutrition at the University of Minnesota, using a commercial kit (K-PDCAAS) from Megazyme (Bray, Ireland), following manufacturer instructions.

PDCAAS was then calculated as follows:

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

where

amino acid score

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

where the reference amino acid pattern is that required for children (6 months to 3 years) as defined by FAO/WHO Expert Consultation (1991).

2.9 Statistical Analysis

Analysis of variance was performed using IBM SPSS Statistics software version 27.0 for Windows (SPSS Inc., Chicago, IL, USA). Tukey–Kramer Honest Significant Difference (HSD) multiple means comparison test was used to test for significant differences ($P \leq 0.05$) among the means of at least three different samples. A student's unpaired t-test was used to determine significant differences ($P \leq 0.05$) between the means of two different samples.

Chapter 3: Results and Discussion

3.1 Screening of Camelina Lines

Camelina lines with differences in genotypes and origins were screened for differences in their protein profile. Protein profiling using SDS-PAGE allows visualization of the relative distributions of protein fractions and subunits. Among the 40 camelina lines that were screened (Appendix M, **Figure 9** and **Figure 10**), 10 camelina lines (**Figure 4**), including the abundant Joelle (winter) line showed pronounced differences in protein bands.

Under non-reducing conditions, the major protein bands in all camelina lines were just under 50 kDa and 15 kDa (**Figure 4A**), which have been identified as cruciferin and napin, respectively, in previous studies on camelina and other *Brassicaceae* seeds (Aluko and McIntosh, 2001; Wu and Muir, 2008; Boyle *et al.*, 2018). Under reducing conditions, the disulphide linkages between cruciferin subunits were cleaved, and the acidic and basic subunits of cruciferin can be observed at ~30 kDa and ~20 kDa, respectively (**Figure 4B**). Similarly, under reducing conditions, the disulphide linkages in napin were cleaved and the resulting subunits appear below 10 kDa.

Four camelina lines, namely Ascn 289, Ascn 413, Ascn 210, and Ascn 259 (**Figure 4A**, Lanes 2-5) had an additional less intense band above the cruciferin band. In these four lines, under reducing conditions, two cruciferin subunits near 30 kDa (corresponding to acidic subunits) and a single subunit near 20 kDa (corresponsing to basic subunits) were observed. In the other lines, only a single major subunit near 30 kDa and two subunits near 20 kDa were observed (**Figure 4B**). It has been reported that cruciferin from *Brassicaceae* species have several subunits between 18 kDa and 53 kDa observed under reducing conditions (Wanasundara, 2011). Genetic variation could lead to differences in amino acid composition, resulting in acidic and basic polypetides with different molecular weights (Wanasundara, 2011). Different combinations of such acidic and basic polypeptides are linked by disulfide bond to form heterogeneous monomers that assemble into the hexameric cruciferin protein. Previous research has identified cruciferin isoforms with varying number and type of amino acid residues in their acidic and basic subunits for *Brassica napus* (rapeseed), and for *Arabidopsis thaliana*, a genetic model plant with close

similiarity to *Camelina sativa* (Wanasundara, 2011; Withana-Gamage *et al.*, 2011). Thus, the observed differences in acidic and basic subunits in the different camelina lines potentially indicate the presence of genetic variants of cruciferin.

Ascn 289, Ascn 413, Ascn 210, and Ascn 259 (**Figure 4**, Lanes 2-5) also showed pronounced subunit near 30 kDa under non-reducing conditions. The presence of a similar band with a molecular weight of 29.5 kDa has been reported and identified as one of the disulfide-linked polypetides in canola cruciferin (Wu and Muir, 2008). Another notable difference, particularly among these four lines, is the presence of a rather high intensity subunit under reducing conditions at 15 kDa in Ascn 413 (**Figure 4B**, Lane 3) and complete absence of this band in Ascn 210 and Ascn 259 (**Figure 4**B, Lane 4 and 5). Aluko and McIntosh (2001) have reported the presence of low molecular bands at 11 kDa and 13 kDa in canola meals under reducing conditions, which were not observed under non-reducing conditions. However, there seems to be no clear information on the identification of these proteins in the literature. Further proteomic analysis using mass-spectroscopy is needed to identify and annotate this protein band. Since the protein profile of Ascn 210 and Ascn 259 were mostly similar and no other unique subunits were noted in Ascn 289, only Ascn 413 and Ascn 259 were selected for further protein evaluation.

The other four camelina lines (**Figure 4**, Lane 6, 7, 8, 10) showed similar cruciferin subunits as that of Joelle (**Figure 4**, Lane 11), whereas Ascn 355 (**Figure 4**, Lane 5) had slight differences. While there were no noticeable differences under non-reducing conditions, Ascn 355 had an additional low intensity subunit above the ~30 kDa cruciferin subunit under reducing conditions. This low intensity subunit was absent in Joelle and the other camelina lines. Ascn 355 most likely contains an additional genetic variant of the acidic subunit of cruciferin, which is different from the other acidic subunit variants in Ascn 289, Ascn 413, Ascn 210, and Ascn 259 (**Figure 4**, Lanes 2-5). Moreover, Ascn 355 contained a prominent protein subunit between 15 kDa and 20 kDa, which was more intense than that in most other camelina lines. The presence of this protein subunit under both reducing and non-reducing conditions indicate that it is not stabilized by disulfide linkages. Based on the molecular weight, this protein subunit could be oleosin, which is a low molecular weight basic protein associated with oil bodies (Katavic *et al.*, 2006). However, proteomics analysis is needed to accurately identify this subunit. The protein

profile of M4 54-1, M5 220-1, Ascn 418, and Ascn 311 (**Figure 4**, lanes 6, 7, 8, and 10) were fairly similar. Therefore, along with Ascn 355, M5 220-1 was selected for further protein characterization. It would be interesting to investigate if these differences in protein subunits among the camelina lines remain post protein extraction and whether they influence the structural and functional properties of the final protein isolates.

The four selected lines, namely Ascn 413, Ascn 259, M5 220-1, and Ascn 355, were planted in the greenhouse for production of seeds in amounts sufficient for protein extraction and characterization. Only Ascn 355 (spring line), however, produced seeds in sufficient amounts. The two protein extraction methods optimized using Joelle (winter line), as will be discussed in sections 3.2 and 3.3, were used to produce camelina protein isolates from both Joelle (winter line) and Ascn 355 (spring line). Camelina protein isolates from both winter and spring lines (WL-CPI and SL-CPI) were used to investigate the structural characteristics and functional properties of camelina protein as influenced by varietal differences and by the method of protein extraction.

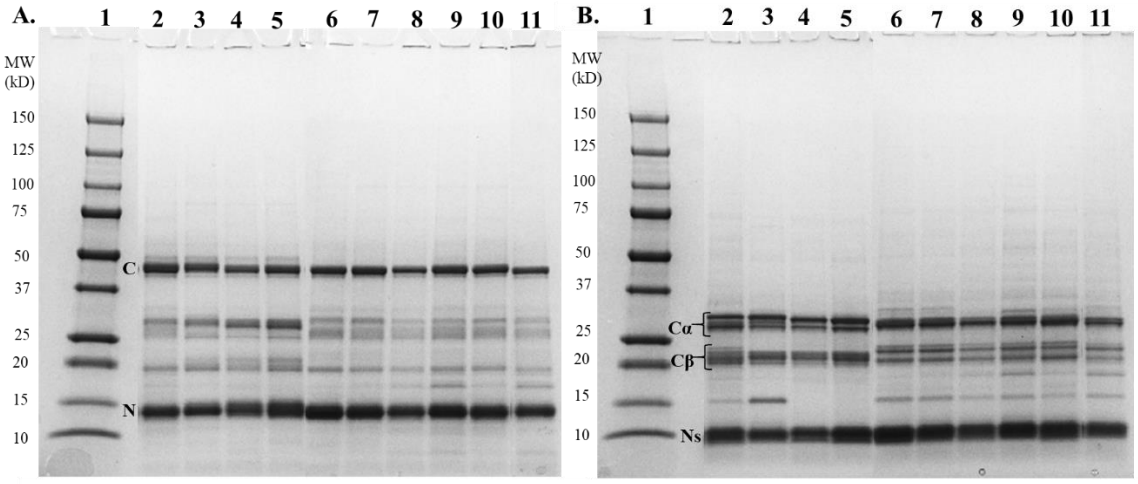

Figure 4. Visualization of protein profile among different camelina lines under non-reducing (A) and reducing (B) conditions. Lane 1: molecular weight (MW) marker; Lane 2: Ascn 289; Lane 3: Ascn 413; Lane 4: Ascn 210; Lane 5: Ascn 259; Lane 6: M4 54-1; Lane 7: M5 220-1; Lane 8: Ascn 418; Lane 9: Ascn 355; Lane 10: Ascn 311; Lane 11: Joelle. C: Cruciferin; N: Napin; Cα: acidic subunits of cruciferin; Cβ: basic subunits of cruciferin; Ns: subunits of napin.

3.2 Optimization of pH Extraction of Camelina Protein from the Winter Line (Joelle)

Because of its poor solubility at neutral pH, camelina protein was solubilized at pH 11. However, under such alkaline conditions, present phenolic compounds are oxidized to quinones, which then bind to proteins (Matheis and Whitaker, 1984; Pourcel *et al.*, 2007). These protein-phenol complexes usually result in the formation of brown-colored polymers that contribute to the dark color of the protein isolates. Reducing agents can be used during protein extraction to mitigate oxidation of phenolic compounds and thus prevent darkening of protein isolates. Sodium sulfite has been documented to inhibit the oxidation of phenols and to reduce quinones to form sulfo-phenolics, thus preventing the binding between quinones and proteins (Narvaez-Cuenca *et al.*, 2011; Queiroz *et al.*, 2011; Amer *et al.*, 2021). In this study, different concentrations of sodium sulfite were tested to improve the color of the camelina protein isolates, while maximizing the protein purity and yield.

Prior to alkaline extraction of camelina proteins, the defatted camelina meal (DCM) was degummed and water-soluble camelina proteins (WCP) were separated from soluble gums and polysaccharides by precipitation at pH 3. Since the degumming step and recovery of WCP was the same among the different extraction treatments, no significant difference was observed in the protein purity and yield of the WCP fractions (**Table 2**). Further, the degummed meal was solubilized at pH 11 with sodium sulfite at different concentrations (0, 0.05, and 0.1%) and the alkali-soluble camelina proteins (ACP) were precipitated at pH 5. Addition of sodium sulfite at 0.05% and 0.1% concentrations did not impact the protein purity of the ACP fractions but a significantly higher yield was noted at 0.1% sodium sulfite compared to ACP extracted without and with 0.05% sodium sulfite (**Table 2**). At 0.1% concentration, sodium sulfite may have sufficiently prevented polyphenol-protein crosslinking and consequent polymerization and aggregation, enabling the protein to be better solubilized, thus enhancing the yield.

Table 2. Protein extraction purities (%) and yields (%) of the pH-CPI, gums, supernatant, and pellet fractions from pH extractions under different extraction conditions, as well as ash content (%) of each pH-CPI.

Extraction Treatment	WCP[1]		ACP[2]		pH-CPI[3]			Discarded Gums[4]		Discarded Pellet[5]		Discarded Supernatant[6]	
Sodium sulfite (%)	Protein Purity (%)	Protein Yield (%)	Protein Purity (%)	Protein Yield (%)	Protein Purity (%)	Protein Yield (%)	Ash (%)	Protein (%)	Protein Lost (%)	Protein (%)	Protein Residue (%)	Protein (%)	Protein Lost (%)
0	76.6$^{a\wedge}$	20.4^{a}	80.4^{a}	32.4^{b}	79.4^{c}	53.1^{c}	3.6^{b}	20.0^{a}	10.2^{a}	18.4^{a}	17.4^{a}	33.1^{a}	12.2^{a}
0.05	77.9^{a}	20.4^{a}	83.0^{a}	34.4^{b}	80.9^{b}	54.8^{b}	4.1^{a}	18.1^{b}	8.9^{b}	17.4^{b}	17.2^{a}	28.4^{b}	11.2^{a}
0.1	79.0^{a}	21.3^{a}	83.3^{a}	41.2^{a}	83.0^{a}	63.4^{a}	4.1^{a}	18.3^{b}	9.0^{b}	16.6^{b}	16.6^{a}	24.6^{c}	9.5^{a}

[1]WCP - Water-soluble camelina protein fraction. [2]ACP - Alkali-soluble camelina protein fraction. [3]pH-CPI – pH extracted camelina protein isolate. [4]Gums discarded after degumming. [5]Pellet discarded after alkaline solubilization. [6]Supernatant discarded after isoelectric precipitation; Protein yield (%) represents the amount of protein extracted relative to the total amount of protein in the starting defatted camelina meal (DCM); Protein residue (%) represents the amount of protein left in the discarded pellet relative to the total amount of protein in the starting DCM; Protein lost (%) represents the amount of protein lost to the discarded supernatant/gums relative to the total amount of protein in the starting DCM. [^]Means in each column with different lowercase letters indicate significant differences across extraction treatments according to the Tukey-Kramer multiple means comparison test ($P < 0.05$).

The two lyophilized protein fractions, WCP and ACP, were combined to form pH extracted camelina protein isolate (pH-CPI). The protein purity and yield of pH-CPI was highest for 0.1% sodium sulfite compared to pH-CPI without and with 0.05% sodium sulfite (**Table 2**). The relatively higher purity value for ACP extracted with 0.1% sodium sulfite, though not significantly different, as compared to that for ACP extracted with 0.05% sodium sulfite, may have resulted in the significantly higher purity of pH-CPI at 0.1% sodium sulfite compared to 0 and 0.05% sodium sulfite. Addition of sodium sulfite also resulted in higher ash content in pH-CPI as compared to pH-CPI extracted without sodium sulfite (**Table 2**).

The protein content of the residual pellet after pH extraction was significantly lower for 0.05% and 0.1% sodium sulfite as compared to those for 0% sodium sulfite. This difference could have been the result of higher protein solubilization in the presence of sodium sulfite. The protein content of the discarded supernatant, produced after precipitation of the proteins, was also significantly lower for 0.05% and 0.1% sodium sulfite as compared to those for 0% sodium sulfite. This observation is consistent with the previous discussion and confirms that camelina protein was more effectively precipitated in the presence of sodium sulfite.

Color analysis of ACP fractions and pH-CPIs was performed to evaluate the change in color upon the addition of sodium sulfite at different concentrations. Both ACP fractions extracted using 0.05% and 0.1% sodium sulfite showed significant increase in lightness (L*) and significant increase in yellow color compared to ACP extracted without sodium sulfite (**Table 3**). Unlike ACP, WCP fractions were not subjected to alkaline conditions, and therefore had a relatively lighter color as confirmed by visual assessment. Therefore, after the addition of WCP fractions to corresponding ACP fractions with 0%, 0.05%, and 0.1% sodium sulfite concentrations, the lightness of the combined pH-CPI significantly increased ($P < 0.05$). Although pH-CPI extracted using 0.05% sodium sulfite had significantly higher lightness than pH-CPI extracted with 0.1% sodium sulfite, the difference was numerically very small. Both pH-CPIs extracted using 0.05% and 0.1% sodium sulfite were similar in color, but lighter than pH-CPI extracted without sodium sulfite.

Table 3. Color (L* a* b*) of alkali-soluble camelina protein fractions (ACP) and pH extracted camelina proteins (pH-CPI)

Extraction Treatment	Alkali-soluble camelina protein fraction (ACP)				pH extracted camelina protein isolate (pH-CPI)			
Sodium sulfite (%)	**L***	**a***	**b***	**ΔE[1]**	**L***	**a***	**b***	**ΔE[1]**
0	$60.2^{c\wedge}$	3.6^{c}	14.2^{c}	-	69.9^{c}	1.8^{b}	12.7^{c}	-
0.05	64.1^{a}	4.1^{b}	17.4^{a}	5.1^{*}	72.1^{a}	2.5^{a}	16.8^{a}	4.7*
0.1	62.8^{b}	4.4^{b}	16.6^{b}	3.6	71.7^{b}	2.3^{a}	14.7^{b}	2.8

[1]ΔE: Total color difference between protein sample with sodium sulfite (0.05 or 0.1% concentration) and protein sample without sodium sulfite; ^Means (n=3) in each column with difference lowercase letters indicate significant differences across extraction treatments according to the Tukey-Kramer multiple means comparison test ($P < 0.05$). An asterisk (*) indicates a significant difference in ΔE as tested by the student's two-sample unpaired t-test ($P < 0.05$).

Under non-reducing conditions, WCP fraction (**Figure 5A,** lane 2) showed intense cruciferin bands near 50 kDa and less intense napin bands near 15 kDa. On the other hand, ACP fractions (**Figure 5A,** lanes 3-5) obtained from extractions using 0%, 0.05%, and 0.1% sodium sulfite concentrations revealed intense napin bands at 15 kDa and several other bands between ~15-50 kDa, most likely corresponding to different cruciferin subunit variants (Wanasundara, 2011). Under reducing conditions, WCP fractions (**Figure 5B**, lane 2) showed more intense acidic and basic subunits of cruciferin at ~20 and ~30 kDa, respectively, as compared to ACP fractions (**Figure 5B**, lanes 3-5). Conversely, more intense napin subunits were observed below 10 kDa in ACP fractions (**Figure 5B**, lanes 3-5) as compared to WCP fractions (**Figure 5B**, lane 2). Based on these observations, cruciferin in camelina appeared to be more water soluble, while napin, a highly basic protein, needed high alkalinity to be effectively extracted from the DCM. The protein profile of ACP was similar regardless of the level of sodium sulfite used, showing no sign of polymerization after the addition of sodium sulfite. Consequently, the protein profile for pH-CPI (**Figure 5**, lanes 6-8), which contained the protein subunits from both WCP and ACP, was similar for all extraction treatments.

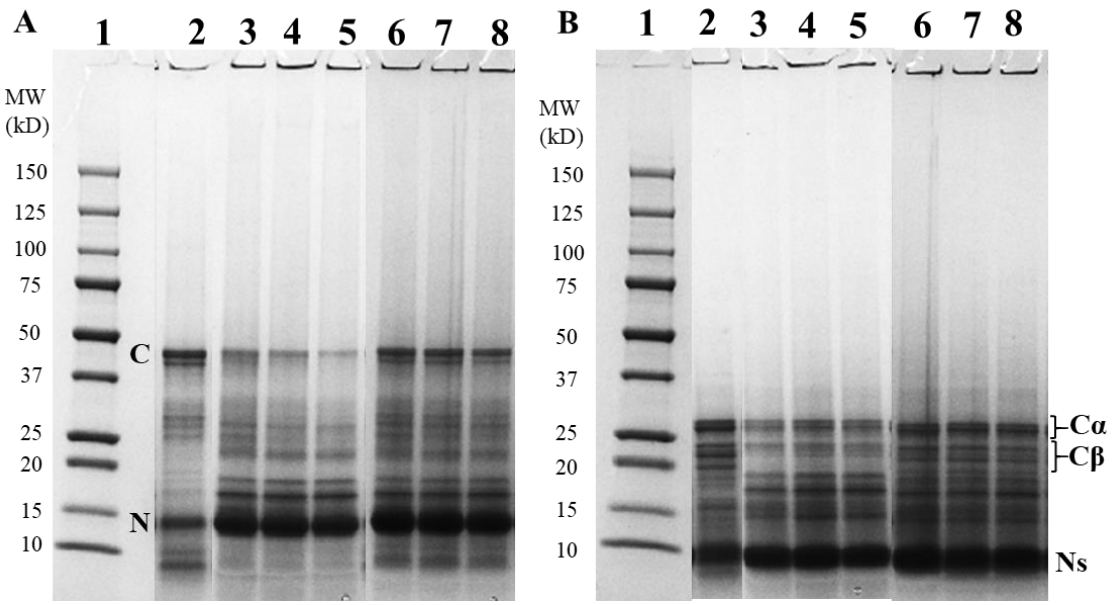

Figure 5. Visualization of the protein profiles of alkali-soluble (ACP), water-soluble (WCP), and pH extracted camelina protein isolates (pH-CPI) in the presence of different levels of sodium sulfite under non-reducing (A) and reducing (B) conditions. Lane 1: Molecular weight marker; Lane 2: WCP; Lane 3: ACP- 0% sodium sulfite; Lane 4: ACP- 0.05% sodium sulfite; Lane 5: ACP- 0.1% sodium sulfite; Lane 6: pH-CPI - 0% sodium sulfite; Lane 7: pH-CPI - 0.05% sodium sulfite; Lane 8: pH-CPI - 0.1% sodium sulfite. C: Cruciferin; N: Napin; Cα: acidic subunits of cruciferin; Cβ: basic subunits of cruciferin; Ns: subunits of napin.

Based on above findings, 0.1% sodium sulfite was determined to be the optimum level as it resulted in relatively high protein yield and lighter color, without impacting the protein profile (**Table 2**, **Figure 5**). Taha *et al.* (1981) have also reported the utilization of 0.1% sodium sulfite during pH extraction for the production of white, bland sunflower protein isolates with high purity. Boyle *et al.* (2018) used a similar pH extraction method to produce camelina protein concentrates with protein purity of 69-71% and protein yield of 37-38%. Proteins were extracted at pH 12 with no sodium sulfite, and a nitrogen to protein conversion factor of 5.30 was used instead of 6.25 used in this study, contributing to the differences in protein purity. Lower solubilization pH in this study was targeted to reduce protein denaturation to some extent and enhance protein functionality. Moreover, double solubilization was performed to increase extraction efficiency, and proteins lost in the degumming step were recovered, both steps contributing to enhanced protein yields. Additionally, the use of sodium sulfite further enhanced the yield and reduced browning.

These extraction conditions can be feasibly scaled up resulting in a food grade isolate with desirable organoleptic quality, facilitating its utilization in various food applications.

3.3 Optimization of Salt Extraction of Camelina Protein from the Winter Line (Joelle)

Salt extraction of plant proteins is less researched as compared to pH extraction and is not commonly used in the industry. Traditionally, a precipitation step with high salt concentration is used to isolate and purify the salt-solubilized protein from other small soluble polysaccharides. However, the excessive salt needs to be removed using several water washes. The large amount of water needed, and generation of salty wastewater makes the process commercially inefficient. Therefore, utilizing salt solubilization followed by ultrafiltration for the purification of camelina protein was investigated. All extractions were performed at 50°C as protein solubility is enhanced at elevated temperature.

To optimize salt extraction of camelina protein for maximum protein purity and yield, the salt concentration needed for the solubilization of camelina protein was tested. There was no significant difference in the protein purity and yield of salt extracted camelina protein isolate (salt-CPI) between 0.5 M and 0.75 M NaCl concentrations (**Table 4**). The ash content of 0.75 M NaCl treatment was also not significantly different from that of 0.5 M NaCl, indicating that ultrafiltration coupled with dialysis were effective in reducing the residual salt even at higher salt concentration. However, as higher amount of water is required to remove the higher salt content, 0.5 M NaCl was determined as the optimal salt concentration for the solubilization of camelina protein.

Boyle et al. (2018) used salt precipitation to purify the solubilized protein and produce camelina protein concentrates with a protein yield of 35-42%. The lower yield compared to our findings could be attributed to losses during the salt precipitation step. While ultrafiltration has been used following pH extraction, it is not well explored as a protein concentration step following salt solubilization. There is one reported study that employed ultrafiltration to reduce phenolic content in camelina protein concentrate, though a fairly low protein yield of ~11% was reported (Sarv, Trass and Diosady, 2017). Thus, the successful production of CPIs with high yield achieved in this study makes this optimized salt solubilization coupled with membrane filtration a feasible and scalable method.

Table 4. Camelina protein extraction purities (%), yields (%), and ash (%) of fractions from salt extractions testing NaCl concentrations.

Extraction Treatment	Salt-CPI[1]			Discarded Gums[2]		Discarded Pellet[3]	
NaCl concentrations (M)	Protein Purity (%)	Protein Yield (%)	Ash (%)	Protein (%)	Protein Lost (%)	Protein (%)	Protein Residue (%)
0.5	83.8	54.7	7.6	18.1*	8.7*	18.0	25.0
0.75	83.5	54.7	7.9	19.4	9.7	15.7*	24.7

[1]Salt-CPI – salt extracted camelina protein isolate. [2]Gums discarded after degumming. [3]Pellet discarded after salt solubilization; Protein yield (%) represents the amount of protein extracted relative to the total amount of protein in the starting defatted camelina meal (DCM); Protein residue (%) represents the amount of protein left in the discarded pellet relative to the total amount of protein in the starting DCM; Protein lost (%) represents the amount of protein lost to the discarded gums relative to the total amount of protein in the starting DCM. An asterisk (*) designates a significant difference in each column as tested by the student's two-sample unpaired t-test ($P < 0.05$).

The combination of bench-top ultrafiltration and dialysis could translate to the potential use of ultrafiltration coupled with diafiltration on a pilot or industrial scale. Utilizing membrane filtration to purify and isolate the proteins instead of salt precipitation can reduce the use of salt and water as well as create less waste. Nevertheless, it is important to investigate the organoleptic, structural, functional and nutritional properties of the CPIs produced using both pH and salt extraction methods to evaluate their acceptability and applicability in food applications.

3.4 Color of Camelina Protein Isolates (CPI)

The color of the protein isolates can influence their utilization and acceptability in food products. Lightness of pH-CPI was significantly lower than that of salt-CPI for both winter (WL) and spring lines (SL) (**Table 5**). This observation is not surprising since the pH extraction utilized high alkalinity for protein solubilization, which can cause oxidation of phenolic compounds and subsequent formation of protein-phenol complexes that are dark in color. The co-extraction of these complexes with the protein is responsible for the lower L* of the pH-CPI as compared to salt-CPI. The pH-CPI samples also had higher redness and lower yellowness than the salt-CPI samples (**Table 5**). Based on visual

assessment, it can be concluded that low redness and high yellowness is associated with an overall positive effect on the appearance of CPI.

Table 5. Color (L* a* b*) of pH and salt extracted camelina protein isolates (pH-CPI and salt-CPI) from winter and spring lines (WL and SL).

Protein Isolate	L*	a*	b*
WL-pH-CPI	$71.7^{d\wedge}$	2.3^{a}	14.7^{d}
WL-salt-CPI	75.4^{c}	0.8^{c}	22.7^{a}
SL-pH-CPI	78.0^{b}	1.1^{b}	17.1^{c}
SL-salt-CPI	81.6^{a}	0.5^{d}	17.8^{b}

$^{\wedge}$Means (n=3) in each column with difference lowercase letters indicate significant differences in L*, a*, and b* across protein isolates according to the Tukey-Kramer multiple means comparison test ($P < 0.05$).

Irrespective of the protein extraction method, the SL-CPI samples showed overall better color with significantly higher lightness, lower redness, and higher yellowness than those from WL-CPI (**Table 5**). These results could in part be attributed to the difference in the storage time between the two camelina lines before they were processed. Seeds from the winter line, harvested in 2019, were stored for longer time, thus may have had higher content of oxidized polyphenols in the starting material. On the other hand, seeds from the spring line, harvested in 2020, were stored only for a few months before being processed, and therefore most likely contained less oxidized polyphenols in the starting material. Overall, CPI samples had higher lightness, lower redness, and comparable yellowness to the reported values for canola protein isolates (Xu and Diosady, 2000b), thus indicating their potential acceptability in food products.

3.5 Structural Characterization of CPI as impacted by Variety and Extraction Conditions

3.5.1 Protein Profile

The protein profiles of commercial soy (cSPI) and pea protein isolates (cPPI), and of DCM and CPI from winter and spring lines were evaluated using SDS-PAGE under reducing and non-reducing conditions (**Figure 6**). Both cSPI and cPPI showed higher proportion of large molecular weight proteins (>75 kDa) than all camelina samples. The major storage proteins in soy and pea are 11S legumin and 7S vicilin ranging in molecular

weight of 300-400 kDa and 150-200 kDa, respectively (Danielsson, 1948; Derbyshire, Wright and Boulter, 1976; Gatehouse *et al.*, 1981; Prak *et al.*, 2005; Tzitzikas *et al.*, 2006; Boye, Zare and Pletch, 2010; Fukushima, 2011). Along with the high molecular weight protein bands, intense smearing was apparent in the lanes of the commercial samples under non-reducing conditions, indicating protein polymerization (**Figure 6A**, lanes 2 and 3). Even under reducing conditions, some streaking was apparent in the upper part of the lanes of commercial samples (**Figure 6**B, lanes 2 and 3), especially that of cPPI, indicating irreversible polymerization induced by covalent linkages other than disulfide linkages. During commercial production, proteins are subjected to extreme conditions (pH, temperature, pressure) during extraction, pasteurization, homogenization and spray drying, which result in protein denaturation (unfolding) and polymerization. Compared to commercial protein isolates, CPI samples were extracted under relatively milder conditions to minimize the formation of such large protein aggregates.

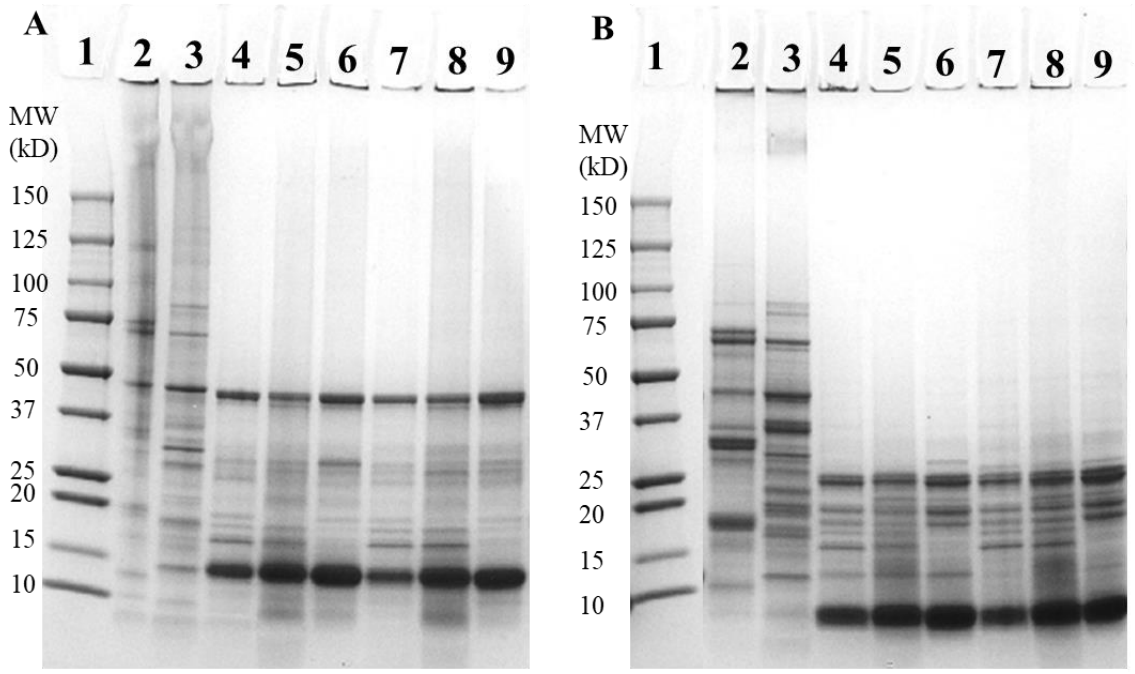

Figure 6. Visualization of the protein profiles of samples under non-reducing (A) and reducing (B) conditions. Lane 1: Molecular weight marker; Lane 2: cSPI; Lane 3: cPPI; Lane 4: WL-DCM; Lane 5: WL-pH-CPI; Lane 6: WL-salt-CPI; Lane 7: SL-DCM; Lane 8: SL-pH-CPI; Lane 9: SL-salt-CPI

Under non-reducing conditions, DCM and CPI of both winter and spring lines showed cruciferin bands just below 50 kDa and napin bands at 15 kDa (**Figure 6A**, lanes

4-9). Under reducing conditions, the acidic and basic subunits of cruciferin were observed at ~30 kDa and ~20 kDa, respectively, whereas the subunits of napin were observed below 10 kDa (**Figure 6B**, lanes 4-9). The SL-pH-CPI and SL-salt-CPI showed slightly more intense bands for acidic (~ 30 kDa) and basic (~ 20 kDa) subunits of cruciferin, under reducing conditions, compared to WL-pH-CPI and WL-salt-CPI, respectively (**Figure 6B**, lanes 5, 6, 8, 9). This observation indicated higher cruciferin to napin ratio in SL-pH-CPI and SL-salt-CPI as compared to WL-pH-CPI and SL-pH-CPI, respectively. A high variation in the levels of cruciferin and napin contents have been reported in rapeseed cultivars, which was correlated with the differences in their genetic composition (Malabat *et al.*, 2003). Moreover, DCM and CPI samples from spring line showed an additional but less intense band above the intense bands corresponding to acidic subunits of cruciferin (**Figure 6B**, lanes 7-9). This observation is consistent with the results from preliminary screening of camelina lines (**Figure 4**, lanes 5 and 11), indicating that the difference in the profile of camelina protein between spring and winter line was retained post protein extraction. As discussed before, this additional band in the spring line is due to the presence of a cruciferin variant arising most likely from genetic variations in amino acid composition and length of its acidic and basic subunits (Wanasundara, 2011). Another prominent difference observed between the two lines under reducing conditions is the presence of a band around 11-12 kDa in DCM and CPIs from winter line, more intense than that in DCM and CPIs from spring line (**Figure 6B**, lanes 4-6). This band was not apparent during preliminary screening of camelina lines (**Figure 4**), most likely because this protein subunit was not solubilized in the protein extraction solvent (50 mM Tris-Cl, 100 mM EDTA, 100 mM NaCl) utilized for the screening. To the best of our knowledge, there seems to be no clear information on the identity of this protein band in literature. Further proteomics analysis using mass-spectroscopy is needed to identify and annotate this protein. These differences in the protein profile among the isolates may cause differences in their structural characteristics.

The WL-salt-CPI had a distinct band of high intensity at ~30 kDa (**Figure 6A**, lane 6), which was absent in WL-DCM (**Figure 6A**, lane 4). The 30 kDa band was less apparent under reducing conditions (**Figure 6B**, lane 4), indicating that disulfide linkages were involved in the formation of this protein. Thermodynamic and structural analysis of napin

have reported that napin dimers with a molecular weight of ~30 kDa are formed at temperatures beyond 40°C (Jyothi *et al.*, 2007). The solubilization of protein at 50°C during salt extraction may be responsible for the formation of napin dimer in WL-salt-CPI. However, this protein subunit was less apparent in SL-salt-CPI (**Figure 6A**, lane 9), indicating potential differences in napin variants between the two lines. The amino acid residues on the surface of the napin variant of spring line may not be favorable to those responsible for the formation of the napin dimer observed in WL-salt-CPI.

Glutelin-type polypeptide (~15-20 kDa), which was present in DCM from both spring and winter lines, was present in the pH-CPI samples but was absent in the salt-CPI samples, under both reducing and non-reducing conditions (**Figure 6A and Figure *6*B**, lanes 4-9). Glutelins are soluble under high alkaline conditions and are insoluble in salt solutions (Li *et al.*, 2014, 2015; Wanasundara *et al.*, 2017). On the other hand, globulins (cruciferin), which are mostly soluble in salt solutions, were present in both pH-CPI and salt-CPI samples (**Figure 6**, lanes 4-9). Similar observations have been reported previously with canola and camelina globulins (Aluko and McIntosh, 2001; Boyle *et al.*, 2018), indicating that globulins, while mostly salt-soluble, can be extracted under strong alkaline conditions. However, the bands corresponding to cruciferin (~50 kDa), under non-reducing conditions, and cruciferin subunits (~30 kDa and ~20 kDa), under reducing conditions, in salt-CPI samples from both camelina lines had relatively higher intensity than those in pH-CPI samples. Accordingly, due to having preferential solubility in salt solutions, salt-CPI samples had a higher ratio of cruciferin to napin proteins than pH-CPI samples. Another notable difference between the two extraction methods was the lane smearing under non-reducing conditions in the upper region of the gel for the pH-CPI samples from both camelina lines (**Figure 6A**, lanes 5 and 8). The high alkalinity employed during pH extraction may have led to protein denaturation and polymerization. The increased lane smearing for pH-CPI samples was evident under reducing conditions as well, but it had migrated towards a lower region of the gel (**Figure 6B**, lanes 5 and 8), indicating that the polymerization was mostly caused by disulfide linkages. The smearing in the SL-pH-CPI and SL-salt-CPI, under both non-reducing and reducing conditions, was slightly more intense than that of WL-pH-CPI and WL-salt-CPI, respectively (**Figure 6**, lanes 5, 6, 8, and 9), indicating a relatively higher degree of polymerization in spring line. It is likely

that the protein variants in the spring line have more tendency to form polymers as compared to those in the winter line.

3.5.2 Protein Denaturation State

Protein extraction conditions could lead to protein denaturation, either partially or completely. Denaturation states of cSPI, cPPI, and CPI samples were evaluated using DSC. Both cPPI and cSPI did not show any endothermic peaks (**Table 6**), indicating that the proteins were completely denatured. This observation complements the SDS-PAGE results that showed excessive polymerization in commercial protein samples (**Figure 6**), which is caused by protein denaturation and exposure of hydrophobic residues and free sulfhydryl groups. Other studies have also reported similar protein denaturation states of commercial protein samples (Lee, Ryu and Rhee, 2003; Shand *et al.*, 2007).

All CPI samples had two endothermic peaks, which corresponded to cruciferin and napin with denaturation temperatures in the range of ~84-88°C and ~98-106°C, respectively. The observed denaturation temperature for cruciferin is in the range reported previously (Wu and Muir, 2008). For both winter and spring lines, the pH-CPI had significantly lower enthalpy of denaturation for cruciferin compared to salt-CPI. This observation is attributed to the high alkalinity employed during pH extraction that caused protein unfolding. Moreover, in relatively moderate concentrations, salt is known to have a protective effect against protein denaturation by increasing surface charges and thus enhancing interaction with water, contributing to higher protein denaturation temperature and enthalpy (Kinsella, 1979a; Hermansson, 1986; Shand *et al.*, 2007). Complete denaturation of camelina cruciferin was observed upon pH extraction when solubilization was done at pH 12, resulting in poor functionality as compared to salt extracted protein (Boyle *et al.*, 2018). Compared to extraction at pH 12, camelina protein extraction at pH 11 was slightly less denaturing and may result in slightly enhanced functionality of pH-CPI.

Table 6. Denaturation temperatures and enthalpy, surface hydrophobicity, surface charge, and secondary structure of commercial soy protein reference (cSPI), commercial pea protein reference (cPPI), and pH extracted, and salt extracted camelina protein isolates from winter (WL-pH-CPI, WL-salt-CPI) and spring lines (SL-pH-CPI, SL-salt-CPI).

Sample	Denaturation Temperature and Enthalpy				Surface Properties		Secondary Structure			
	Denaturation Temperature	Enthalpy of Denaturation	Denaturation Temperature	Enthalpy of Denaturation	Surface Hydrophobicity	Surface Charge	α Helix	β Sheet	β Turn	Random Coil
	Td, °C	ΔH, J g-1	Td, °C	ΔH, J g-1	RFI	mV	Relative Percentage			
	β-conglycinin		***glycinin***							
cSPI	N/A[†]	N/A	N/A	N/A	11025$^{b\wedge}$	-30.4^{b}	18.2^{c}	43.0^{a}	26.0^{ab}	12.7^{a}
	Vicilin		***Legumin***							
cPPI	N/A	N/A	N/A	N/A	18863^{a}	-31.9^{b}	18.6^{c}	44.0^{a}	26.9^{a}	11.2^{ab}
	Cruciferin		***Napin***							
WL-pH-CPI	84.7^{c}	0.2^{c}	105.3^{a}	0.3^{b}	9136^{c}	-41.7^{c}	24.3^{ab}	38.9^{bc}	23.2^{b}	12.3^{a}
WL-salt-CPI	87.8^{a}	1.4^{a}	102.7^{b}	0.1^{c}	5657^{d}	-16.3^{a}	27.5^{a}	36.0^{c}	26.4^{ab}	10.2^{bc}
SL-pH-CPI	86.8^{b}	0.2^{c}	105.1^{a}	0.5^{a}	8336^{c}	-43.0^{c}	23.5^{b}	41.4^{ab}	28.0^{a}	7.1^{d}
SL-salt-CPI	85.5^{c}	0.7^{b}	98.6^{c}	0.6^{a}	8210^{c}	-18.0^{a}	25.7^{ab}	38.3^{bc}	27.3^{a}	8.6^{cd}

[†]N/A represents no peak of denaturation observed; [^]Means (n = 3) in each column with lowercase letters indicate significant differences between samples according to the Tukey-Kramer multiple means comparison test ($P < 0.05$).

The temperature and enthalpy of denaturation for cruciferin in SL-salt-CPI was slightly but significantly lower than that in WL-salt-CPI. It is likely that the genetic variant of cruciferin in the spring line has lower thermal stability than that in the winter line. This speculation is supported by the differences in the thermal stability of cruciferin isomers predicted by Withana-Gamage *et al.* (2011) based on its amino acid composition and molecular structures.

The denaturation temperatures for napin were observed in the range of ~98-106°C, close to the reported value of 110°C for napin (Wu and Muir, 2008). The denaturation temperatures for napin were slightly but significantly higher in pH-CPI than in salt-CPI samples. This finding could be attributed to differences in the ash content as well as the type of salt present in the samples. Sodium chloride, which was more abundant in salt-CPI, has been reported to impact the napin structure and hence its thermal stability (Jyothi, Singh and Appu Rao, 2007).

The SL-CPI had higher enthalpy of denaturation for napin than that of WL-CPI, irrespective of the protein extraction method. Similar to cruciferin, napin isoforms in canola have also been reported in the protein database (Wanasundara *et al.,* 2016). The differences in the number and type of amino acids in the napin subunits arising due to genetic differences may result in structural differences among napin isoforms, thus influencing their structural stability. Thus, the DSC results for napin indicate that the napin in the spring camelina line is more resistant to thermal denaturation than that in the winter line. The degree of denaturation and resultant protein conformation of CPI could influence their functional properties.

3.5.3 Protein Surface Properties

Upon protein unfolding, the hydrophobic residues buried within the interior moiety of the globular proteins are exposed, resulting in increased surface hydrophobicity. Extraction and processing conditions influence the degree of protein unfolding as seen thus far and thus impact the surface hydrophobicity of the protein. The cSPI and cPPI had significantly higher surface hydrophobicity than all CPI samples (**Table 6**). This result is not surprising as both cSPI and cPPI were completely denatured as discussed earlier.

Comparing the CPI samples from the winter line, the WL-pH-CPI had significantly higher surface hydrophobicity than WL-salt-CPI, similar to previous research on camelina

protein (Boyle *et al.*, 2018). These results are in agreement with the observed degree of denaturation (**Table 6**). Conversely, there was no significant difference in surface hydrophobicity between SL-pH-CPI and SL-salt-CPI although cruciferin in SL-pH-CPI was more denatured than that in SL-salt-CPI. This observation could be attributed to attractive forces between hydrophobic groups on the surface of SL-pH-CPI causing polymerization and reduced measurement of surface hydrophobicity (Rickert, Johnson and Murphy, 2004). Protein polymerization in SL-pH-CPI was also confirmed upon protein profiling by SDS-PAGE (**Figure 6**), which showed more intense smearing than other CPI samples.

Comparable values for surface hydrophobicity for WL-pH-CPI and SL-pH-CPI could be attributed to similar denaturation enthalpies for cruciferins in these isolates (**Table 6**). On the other hand, SL-salt-CPI demonstrated significantly higher surface hydrophobicity than WL-salt-CPI, owing to the higher degree of cruciferin denaturation in SL-salt-CPI. These differences in surface hydrophobicity as impacted by extent of denaturation and the genetic variance will in turn impact protein intermolecular interactions, contributing to differences in protein functionality.

Surface charge is another important surface property that influences protein interactions and functional properties. All protein samples carried a net negative charge, as the zeta potential was measured at pH 7, which is above their average isoelectric point. The net surface charge for cSPI and cPPI was lower than that of pH-CPI samples but higher than that of salt-CPI samples (**Table 6**). As mentioned earlier, during the production of commercial isolates, the proteins most likely have undergone complete denaturation and extreme polymerization. Upon denaturation, the electrostatic interactions, hydrogen bonding, and hydrophobic interactions that stabilize the native globular protein structure are disrupted, resulting in changes in exposed amino acids, thus affecting the overall surface charge (Foegeding and Davis, 2011). Reduced surface charge and relatively high surface hydrophobicity of these commercial samples (**Table 6**) result in stronger attractive forces namely hydrophobic interactions leading to the observed polymerization (**Figure 6**).

The pH-CPI samples had a higher (more negative) net surface charge than salt-CPI samples (**Table 6**). Previous research on legume protein showed similar results, where protein isolates produced using alkaline solubilization followed by isoelectric precipitation

had a higher (more negative) surface charge than those produced using salt solubilization (Karaca, Low and Nickerson, 2011). These results could be explained by the differences in protein composition and isoelectric points of isolates. During isoelectric precipitation, the pH-CPI samples were precipitated at pH 5, where they have zero net charge. As noted by protein profiling (**Figure 6**), salt-CPI samples had relatively higher cruciferins as compared to pH-CPI samples. Cruciferins have an isoelectric pH of ~7.2 (Schwenke *et al.*, 1980). Therefore, the overall pI of salt-CPI samples is likely higher than pH 5. Protein carry higher charges at pH values farther away from their isoelectric point, which explains the higher surface charge in pH-CPI samples as compared to salt-CPI samples. Moreover, the lower (less negative) net surface charge of salt-CPI samples could also be attributed to the presence of higher residual salt in the salt-CPI samples than pH-CPI, based on the ash content of the isolates ($P < 0.05$). The salt ions shield the charges on the surface of the protein, resulting in decreased net surface charge (Duong-Ly and Gabelli, 2014).

There was no significant difference in surface charge between the WL-CPI and SL-CPI samples (**Table 6**). However, considering the significant differences in surface hydrophobicity between WL-salt-CPI and SL-salt-CPI, there is a difference in the balance between surface hydrophobicity and hydrophilicity between these samples, which may influence their functional properties.

3.5.4 Protein Secondary Structures

The relative abundance of α helix, β sheet, β turn, and random coil in the protein isolates was analyzed to evaluate protein secondary structure differences as impacted by varietal differences and by the method of extraction . Among the tested samples, both cSPI and cPPI showed the least relative amount of α helix and significantly higher amounts of β sheet (**Table 6**). The potentially severe processing conditions involved in the production of these commercial samples could be responsible for unfolding and polymerization, resulting in higher detectable level of β sheet and random coil (Kato and Takagi, 1988; Usoltsev *et al.*, 2019). Higher levels of β sheet structure has been associated with enhanced gelling and emulsification properties (Cao and Mezzenga, 2019). These commercial samples had the lowest ratio of α helix to β sheet ($P < 0.05$), indicating higher protein denaturation at the secondary structure level as compared to CPI samples. These results are

complementary to those of protein profiling, denaturation, and surface properties (**Figure 6**, **Table 6**).

While there was no varietal impact on the relative abundance of α helix and β sheet in CPI, the extraction method resulted in some noted differences. WL-pH-CPI had a lower ratio of α helix to β sheet ($P < 0.05$) than that of WL-salt-CPI and a comparable level of random coil to those in commercial samples. Similarly, although not significantly different, SL-pH-CPI had lower ratio of α helix to β sheet than SL-salt-CPI. These results indicate higher protein secondary structure denaturation in pH-CPI than salt-CPI caused due to the high alkalinity employed during pH extraction. These differences in the ratio of α helix to β sheet among protein isolates may lead to differences in their functional properties.

3.6 Functional Properties of CPI as impacted by Variety and Extraction Conditions

3.6.1 Protein Solubility

Solubility of the protein isolates was measured at pH 3.4 and pH 7 and at 1 and 5% protein concentration under both non-heated and heated (80°C for 30 min) conditions to evaluate their potential performance in acidic and neutral beverage systems (**Table 7**). At 1% protein, cSPI showed the highest solubility among all protein samples under all tested conditions. This observation could be attributed to the relatively high level of polar and charged amino acids on the surface of soy protein (Lampart-Szczapa, 2001).

At pH 7 under non-heated conditions, the solubility of WL-salt-CPI was higher than that of WL-pH-CPI, which can be attributed to the lower surface hydrophobicity (**Table 6**) of WL-salt-CPI. On the other hand, although SL-salt-CPI had comparable surface hydrophobicity and lower surface charge than SL-pH-CPI, SL-salt-CPI showed higher solubility than SL-pH-CPI. The charged groups in proteins bind more water molecules as compared to uncharged polar residues, which in turn bind more water molecules than nonpolar groups (Damodaran, 2017). Thus, this observation indicates that the proteins in SL-salt-CPI probably contained relatively high levels of uncharged polar residues that may have contributed to enhanced protein-water interactions (**Figure 6**).

Table 7. Solubility, gel strength, water holding capacity, emulsification capacity, emulsion stability, emulsification activity index of commercial soy protein reference (cSPI), commercial pea protein reference (cPPI), and pH extracted, and salt extracted camelina protein isolates from winter (WL-pH-CPI, WL-salt-CPI) and spring lines (SL-pH-CPI, SL-salt-CPI).

Sample	Solubility pH 3.4 (1% protein)		Solubility pH 7 (1% protein)		Solubility pH 3.4 (5% protein)		Solubility pH 7 (5% protein)		Gel Strength[2]	WHC[3]	EC[4]	ES[5]	EAI[6]
	%	%	%	%	%	%	%	%	N	%	g oil/g protein	min	m^2/g oil
	Non-Heated	Heated[1]	Non-Heated	Heated	Non-Heated	Heated	Non-Heated	Heated					
cSPI	49.4$^{a\wedge}$	61.7^{a*}	49.5^{a}	67.3^{a*}	24.9^{c}	39.1^{a*}	66.8^{a}	78.5^{a*}	25.7^{a}	99.9^{ab}	1240.0^{c}	14.5^{bc}	193.7^{d}
cPPI	25.1^{e}	29.6^{cd}	40.9^{bc}	59.1^{b*}	12.3^{e}	17.4^{e*}	36.2^{c}	55.4^{b*}	12.7^{b}	99.9^{a}	818.4^{d}	12.5^{c}	221.0^{bcd}
WL-pH-CPI	31.9^{bc}	33.4^{c}	35.9^{cd}	32.0^{de}	17.5^{d}	19.5^{d}	23.3^{e}	23.1^{e}	8.0^{c}	99.6^{ab}	1531.4^{b}	18.3^{a}	242.1^{ab}
WL-salt-CPI	37.1^{b}	40.6^{b}	41.7^{b*}	28.1^{e}	37.9^{a}	39.5^{a}	43.1^{b*}	34.1^{c}	-	-	1971.6^{a}	13.2^{bc}	233.3^{bc}
SL-pH-CPI	30.4^{cd}	29.8^{cd}	30.7^{d}	35.4^{cd*}	22.7^{c}	25.2^{c*}	27.5^{d}	28.0^{d}	2.5^{e}	99.5^{b}	1283.4^{c}	14.7^{b}	198.6^{cd}
SL-salt-CPI	25.3^{de}	26.0^{d}	47.7^{a*}	39.3^{c}	30.3^{b}	30.4^{b}	42.0^{b*}	27.0^{d}	4.6^{d}	99.7^{ab}	2120.4^{a}	14.3^{bc}	274.8^{a}

[1]Samples were heated at 80°C for 30 minutes; [2]cSPI, WL-pH-CPI, SL-pH-CPI and SL-salt-CPI gels were prepared at 15% protein (w/v) and heated for 10 minutes at 95°C. cPPI gels were prepared at 20% protein (w/v) and heated for 20 minutes at 95°C. WL-salt-CPI did not form a measurable gel; [3]WHC: Water holding capacity; [4]Emulsification capacity (EC) was measured at 1% for cSPI, pH-CPI, and salt-CPI whereas it was measured at 2% for cPPI; [5]ES: Emulsion stability; [6]EAI: Emulsification activity index; [^]Means (n = 3) in each column with different lowercase letters indicate significant differences according to the Tukey-Kramer multiple means comparison test (*P < 0.05);* An asterisk (*) indicates a significant difference among a not-heated and heated sample as tested by the student's two-sample unpaired t-test (*P < 0.05*).

All salt-CPI samples showed a decrease in solubility upon heating at pH 7. As discussed before, salt-CPI samples contained a higher level of cruciferins (pI~7.2) (**Figure 6**) and thus may have had a higher overall pI as compared to pH extracted samples. Thus, the closer proximity of pH 7 to the pI of salt-CPI samples resulted in lower surface charge and thermal stability, which may have caused the proteins to unfold upon heating and expose hydrophobic residues that were previously buried in the interior moiety of the protein, resulting in reduced solubility.

At pH 3.4, SL-salt-CPI showed lower solubility than WL-salt-CPI, which could be explained by the protein profile (**Figure 6**) and structural characteristics (**Table 6**). The cruciferin variant in SL-salt-CPI showed lower thermal stability and higher surface hydrophobicity than WL-salt-CPI. These results indicate that genetic variations in protein profile among camelina lines can lead to structural differences in the protein after processing, resulting in differences in functional properties.

With few exceptions, most samples tested at 5% protein concentration showed lower or comparable solubility as compared to those tested at 1% protein concentration. At higher protein concentrations, protein molecules are in close proximity to each other, allowing them to interact more readily via hydrophobic forces. The resultant formation of protein aggregates contributes to lower solubility. However, cSPI at pH 7 showed significantly higher solubility at 5% protein concentration than that at 1% protein concentration ($P < 0.05$). As noted in structural testing (**Table 6**), cSPI had relatively high surface hydrophobicity, which could have promoted hydrophobic interactions between protein molecules at high concentration, leading to the formation of soluble aggregates, thus potentially lowering surface hydrophobicity, which resulted in higher solubility of cSPI at 5% protein compared to 1% protein.

At 5% protein concentration, both salt-CPI samples showed higher or comparable solubility to cSPI and cPPI at pH 3.4. These results are in agreement with previous research that also reported higher solubility of salt extracted camelina protein compared to SPI at pH 3.4 (Boyle *et al.*, 2018). Based on these findings, salt-CPI shows potential to replace commonly used plant proteins in high protein acidic beverage applications.

3.6.3 Gel Strength and Water Holding Capacity

Thermally induced gelation requires heating the protein solution above the denaturation temperature to destabilize the secondary and tertiary protein structure and allow protein molecules to unfold (Shand *et al.*, 2007). The resultant exposure of hydrophobic and reactive sulfhydryl groups facilitates protein-protein interactions, leading to the formation of a 3D protein network that can trap water molecules (O'Kane *et al.*, 2004; Fukushima, 2011).

cSPI demonstrated the highest gel strength among all protein isolates (**Table 7**). Soy protein has a high legumin (glycinin) to vicilin (β-conglycinin) ratio (Shand *et al.*, 2007; Sun and Arntfield, 2011b; Tulbek *et al.*, 2016). The higher abundance of cysteine residues in legumin promote the formation of inter- or intra-molecular disulfide linkages, which contributes to the gel strength (Wolf, 1970). cPPI, on the other hand, has lower proportion of legumin compared to cSPI, and thus was not able to form a gel at 15% protein and even had a lower gel strength at 20% protein as compared to cSPI gels prepared at 15% protein.

All camelina protein gels showed significantly lower gel strength than cSPI at 15% protein. They were better in forming a gel than cPPI, as cPPI did not form a gel or formed a weak gel at 15% protein. As observed through protein profiling (**Figure 6**), cSPI contain proteins with higher molecular weight, which associate at more sites and thus form more extensive and stronger gel network than smaller proteins (Oakenfull, Pearce and Burley, 1997). Furthermore, these results are in agreement with the study by Cao and Mezzenga (2019), who found that higher β sheet content, as observed for cSPI (**Table 6**), leads to improved gelation properties.

WL-salt-CPI did not form a gel under the tested conditions. This observation may be explained by the more intact cruciferin in WL-salt-CPI as compared to other CPI samples. Heating temperature and heating rate influence the unfolding of the protein and hence the protein-protein interactions desirable for the formation of a well-structured gel network (Renkema and Van Vliet, 2002). Thus, the heating conditions used in this study may not have been sufficient or favorable to those needed for the optimum unfolding and association of cruciferin in WL-salt-CPI to form a gel. Furthermore, lower surface hydrophobicity (**Table 6**) and thus lower protein-protein interactions in WL-salt-CPI as

compared to other CPI samples may have hindered gel formation in WL-salt-CPI (Panyam and Kilara, 1996). The ability of other CPI samples to form a gel at the tested conditions could be attributed to their partially denatured cruciferin, and/or higher surface hydrophobicity (**Table 6**) compared to WL-salt-CPI, thus facilitating better protein-protein interactions.

Conversely, SL-salt-CPI formed a gel with a strength higher than that of SL-pH-CPI (**Table 7**). The proteins in SL-pH-CPI were partially denatured and aggregated as compared to those in SL-salt-CPI (**Table 6** and **Figure 6**). Upon heating, the proteins that are less denatured, as in those in SL-salt-CPI, will initially denature and then aggregate, resulting in a more ordered protein network and a better gel as compared to proteins that are already denatured and polymerized (Tombs, 1974; Hermansson, 1979). Moreover, cruciferin has been reported to show better gelling properties due to its higher molecular weight than napin (Schwenke, Dahme and Wolter, 1998). Accordingly, the higher cruciferin to napin ratio in SL-salt-CPI compared to SL-pH-CPI (**Figure 6**), may also have contributed to the higher gel strength.

Even with lower cruciferin to napin ratio in WL-pH-CPI, it showed significantly higher gel strength than SL-pH-CPI. This observation could be attributed to higher protein aggregation in SL-pH-CPI than WL-pH-CPI (**Figure 6**). The heat treatment utilized to induce gelation may have caused the aggregated proteins to form bigger and random aggregates, resulting in a coagulum-type gel, which are generally weak (Damodaran, 2017). Overall, the gelation properties of camelina protein are inferior to those of soy protein. However, varietal differences contributed to different gelation properties. Therefore, further breeding initiatives or modification strategies are needed to alter the structural properties of camelina protein, and thus make it suitable to be utilized in food applications requiring gelation ability.

Water holding capacity (WHC) refers to the ability of the proteins to trap water in a gel network. There were no significant differences in the WHC among CPI samples except for WL-salt-CPI (**Table 7**), whose WHC could not be measured since it did not form a gel. The CPI samples showed WHC higher than 99%, comparable to those of cSPI and cPPI. CPI samples apparently had appreciable levels of exposed hydrophilic residues that are required for maintaining the protein-water interactions, thus facilitating water

retention. Based on these findings, camelina protein shows potential to be used as an alternative plant protein ingredient in food applications such as meat or meat analogues, yogurt, and baked goods, which require high water retention and no syneresis.

3.6.4 Emulsification Capacity (EC), Emulsion Stability (ES) and Emulsification Activity Index (EAI)

A relatively flexible structure and a good hydrophilic/lipophilic balance (HLB) is required for the protein to interact with both water and oil phases and thus have a good emulsification capacity (EC). Emulsion activity index (EAI) is associated with the ability of the protein to migrate to the oil:water interface and unfold to reorient its hydrophobic residues to the oil phase and hydrophilic residues to the aqueous phase. At the interface, proteins create a thick, continuous film around the oil droplets to reduce the interfacial tension and act as a physical barrier to prevent the coalescence of oil droplets, thus providing emulsion stability (ES) (Kinsella, 1979b).

All CPI samples showed comparable or higher emulsifying properties than cSPI and cPPI (**Table 7**). This observation indicates that camelina protein has better HLB as compared to commercial samples. Native soy protein is also known to have a good balance of surface hydrophobicity and polar and charged residues on its surface (Lampart-Szczapa, 2001). However, its commercial production may have caused changes to its protein structure, thus impacting its emulsifying properties.

Salt-CPI showed higher EC than pH-CPI (**Table 7**). This observation could be attributed to the differences in solubility and surface hydrophobicity among the samples. Salt-CPI had higher solubility at pH 7 and lower surface hydrophobicity than the pH-CPI. Accordingly, the salt extracted proteins interacted effectively with both the oil and water phases, thus resulting in high EC. These results are also in agreement with a study that reported a decrease in emulsification properties with an increase in extraction pH of canola protein (Pedroche *et al.*, 2004).

There was no significant difference in the EC between WL-salt-CPI and SL-salt-CPI. The higher solubility of SL-salt-CPI compared to WL-salt-CPI at pH 7 (for 1% protein concentration) (**Table 7**) most likely offsetted its higher surface hydrophobicity (**Table 6**), resulting in similar surface properties and thus comparable EC. SL-pH-CPI, however, showed significantly lower EC than WL-pH-CPI. As observed from protein profiling

(**Figure 6**), SL-pH-CPI had more protein aggregation as compared to WL-pH-CPI. The presence of aggregates may reduce the flexibility of the protein hindering its migration and adsorption at the oil:water interface.

The WL-pH-CPI showed significantly higher ES and EAI among all tested samples (**Table 7**). The higher ES could be attributed to the higher net negative charge of WL-pH-CPI at neutral pH (**Table 6**), which causes repulsion among adsorbed proteins on the interface and thus delays coalescence and phase separation (Kinsella, 1979b; Dagorn-Scaviner, Gueguen and Lefebvre, 1986). Although SL-pH-CPI showed comparable surface charge to WL-pH-CPI, it had lower ES, most likely due to its slightly higher protein polymerization (**Figure 6**). Protein aggregation can reduce the flexibility of the proteins, thus preventing them from forming a thick protective layer around the oil droplets. As discussed above, inflexible structure prevents the migration of proteins to the oil:water interface and hinders the unfolding of the protein to expose their hydrophobic and hydrophilic residues to the oil phase and the water phase, respectively, which resulted in low EAI of SL-pH-CPI.

There were no significant differences in ES between WL-salt-CPI and SL-salt-CPI (**Table 7**). Although SL-salt-CPI had higher surface hydrophobicity than WL-salt-CPI (**Table 6**), the higher solubility of SL-salt-CPI compared to WL-salt-CPI at pH 7 under non-heated conditions at 1% protein concentration (**Table 7**) may have resulted in a balanced protein-protein interactions and protein-water interactions, thus delaying the coalescence of oil droplets.

SL-salt-CPI showed significantly higher EAI than all tested samples. Proteins with flexible structures and good solubility can efficiently migrate to the oil:water interface and result in high EAI (Nakai, 1983; Barac *et al.*, 2010; Feyzi, Milani and Golimovahhed, 2018). Accordingly, the high solubility of SL-salt-CPI at pH 7 under non-heated conditions at 1% protein concentration (**Table 7**) could have contributed to its high EAI. Moreover, the high cruciferin to napin ratio in SL-salt-CPI (**Figure 6**) might have also contributed to superior emulsifying properties since napin is known to have a detrimental effect on emulsifying properties (Wu and Muir, 2008). Napin is abundant in basic amino acids, which favor electrostatic interactions and enhanced interaction with the aqueous phase, leading to poor emulsification properties. Studies on canola protein have reported

comparable EAI values among different cultivars, and have attributed their observations to various factors such as protein solubility, protein conformation, and protein-protein interactions that may be balancing out each other (Hailing, 1981; Kinsella, Damodaran and German, 1985; Naczk, Diosady and Rubin, 1985; Aluko and McIntosh, 2001). Overall, the comparable or superior emulsifying properties of CPI to cSPI and cPPI indicate that it can potentially be utilized in food applications involving emulsion systems.

3.7 Amino Acid Composition and Nutritional Quality of the Different CPI Samples

The estimation of protein content of isolates is influenced by the nitrogen to protein conversion factor. The nitrogen to protein conversion factor can be defined as the ratio of total protein content to total nitrogen content in the sample (Mossé, 1990). In literature, the average nitrogen content in proteins has been reported to be 16%, resulting in a conversion factor of 6.25 (100% protein/16% nitrogen), which is mostly used in the food industry for protein content calculations. However, depending on the amino acid composition of specific proteins, there could be deviation in nitrogen content from the average nitrogen content, resulting in a different conversion factor than 6.25 (Morr, 1981).

The amino acid composition shown in **Table 8** was used to calculate the nitrogen to protein conversion factor for each CPI. The calculated conversion factors for WL-pH-CPI, WL-salt-CPI, SL, pH-CPI, and SL-salt-CPI were 5.5, 5.6, 5.6, and 5.7 respectively. This indicates that the CPI samples have relatively high proportion of basic amino acids, which leads to high nitrogen content than average and thus a low conversion factor. Using these calculated conversion factors will result in lower calculated protein content ranging from 72.2 to 74.7% in the CPI samples produced in this study compared to 80.6 to 83.1% when using 6.25 conversion factor. Higher protein content is of a higher economical value, which explains why in industry a conversion factor of 6.25 is often used regardless of the actual factor. However, using specific conversion factors based on each protein isolate's amino acid composition for the determination of protein content would give us more accurate information about the nutritional quality of camelina protein in food products.

Table 8. Amino acids content (mg/g protein) of WL-pH-CPI, WL-salt-CPI, SL-pH-CPI, and SL-salt-CPI.

Amino acids	WL-pH-CPI	WL-salt-CPI	SL-pH-CPI	SL-salt-CPI
Aspartic Acid	67.9	62.0	67.4	65.4
Threonine	32.7	27.1	32.4	28.3
Serine	38.8	34.2	37.6	34.3
Glutamic Acid	137	152	130	175
Proline	42.8	46.8	41.8	47.1
Glycine	42.3	42.7	39.9	37.6
Alanine	38.5	32.4	37.1	34.5
Valine	42.9	39.1	42.8	40.7
Isoleucine	30.9	28.6	31.6	29.4
Leucine	57.3	50.2	57.9	52.2
Tyrosine	25.1	20.6	25.3	21.6
Phenylalanine	37.2	33.1	37.7	35.6
Lysine	34.9	31.7	37.9	34
Histidine	19.4	18.8	20.3	19.6
Arginine	77.4	77.9	70.4	75.4
Cystine	16.1	20.7	15.5	21.1
Methionine	15.4	14.1	15.6	14.7
Tryptophan	9.91	8.47	9.61	8.31

The nutritional quality of the protein is determined by its essential amino acid content and digestibility. Based on the reference amino acid requirements (FAO/WHO Expert Consultation, 1991), lysine was found to be the first limiting amino acid in all camelina isolates. The first limiting amino acid score is expressed by comparing the amount of the first limiting amino acid in the protein to its amount in the recommended requirement pattern. The amino acid scores for camelina isolates were found to be in the range of 0.68-0.79 (**Table 9**).

The protein quality is often assessed using the protein digestibility-corrected amino acid score (PDCAAS), which is calculated by correcting the first limiting amino acid score with the digestibility of the protein. Joseph Eggers, a graduate student in the Department of Food Science and Nutrition at the University of Minnesota, performed in vitro digestibility assay and calculated PDCAAS values for CPI samples. The PDCAAS of camelina protein ranged between 0.70-0.77 (**Table 9**).

Table 9. Amino acid score, *in vitro* digestibility and protein digestibility-corrected amino acid score (PDCAAS) of WL-pH-CPI, WL-salt-CPI, SL-pH-CPI, and SL-salt-CPI.

Sample	Amino Acid Score[1]	*In vitro* Digestibility[2]	PDCAAS[2]
WL-pH-CPI	0.724	1.00[b^]	0.73[b]
WL-salt-CPI	0.678	1.03[a]	0.70[c]
SL-pH-CPI	0.794	0.97[c]	0.77[a]
SL-salt-CPI	0.715	0.97[c]	0.70[c]

[1]Calculated using the recommended amino acid scoring pattern for children (6 months to 3 years) (FAO/WHO Expert Consultation, 1991); [2]Reported by Joseph Eggers, a graduate student in the Department of Food Science and Nutrition, University of Minnesota; [^]Means (n = 2) in digestibility and PDCAAS columns with lowercase letters indicate significant differences between samples according to the Tukey-Kramer multiple means comparison test ($P < 0.05$).

The pH-CPI showed higher amino acid scores and PDCAAS than corresponding salt-CPI for both SL and WL (**Table 9**). These results indicate that proteins with high levels of lysine are lost during salt extraction. SL-CPI had higher lysine content than WL-CPI, most likely because SL-CPI contained higher levels of cruciferin (**Figure 6**), which is rich in lysine (Bos *et al.*, 2007). Thus, even with a lower *in vitro* digestibility, SL-pH-CPI showed significantly higher PDCAAS than WL-pH-CPI (**Table 9**).

Methionine is the first limiting amino acid in pea protein with a score of 0.77 (Keith, Youngs and McLaughlan, 1977). A PDCAAS in the range of 0.50-0.82 has been reported for pea protein depending on its cultivar and processing conditions (Arntfield and Maskus, 2011; Nosworthy and House, 2017). Based on the results from this study (**Table 9**), camelina protein show comparable PDCAAS to pea protein. Thus, camelina protein and pea protein can potentially be utilized as complementary proteins to balance the levels of lysine and methionine in the diet. The amino acid and PDCAAS scores for camelina protein are higher than those for wheat protein, which is also deficient in lysine with a score of 0.47 and PDCAAS of 0.42 (Jood, Kapoor and Singh, 1995; Schaafsma, 2000). Thus, based on these findings, camelina protein is a potential alternative to wheat protein, which is one of the known allergens. Soy protein, which also has an allergenic status, is the most widely used plant protein in the market. It is limiting in methionine, and has an amino acid score of 0.96 and PDCAAS of 0.91 (Schaafsma, 2000; Friedman and Brandon, 2001). Such evaluation of nutritional quality of proteins is useful in balancing the amino acid

composition of proteins that are deficient in one or more essential amino acids and incorporating complementary proteins in the diet to meet the body's protein requirements. Nutritional quality of the protein ingredient is one of the main determining factors for protein choice in the formulation of food products.

Chapter 4: Overall Conclusions, Implications, and Recommendations

The isolation and characterization of protein from camelina was investigated in this study. Protein profiling of 40 camelina lines revealed differences in cruciferin and napin subunits as well as in the abundance of other protein polypeptides, mostly due to genetic variance or environmental differences among the growing locations. Further, a comprehensive evaluation of two camelina lines for protein quality traits relevant for food use was performed. Isolation of camelina protein from two selected camelina lines (Winter line, Joelle, and Spring line, Ascn 355) was achieved through pH and salt extraction.

The conditions for pH and salt extraction were optimized to maximize protein purity and yield of camelina protein isolates (CPI) and to minimize any potential protein denaturation during the extraction process. Moreover, feasible and scalable techniques for purification of camelina protein were explored. The optimized methods produced CPI with competitive or superior protein purity and yield compared to those reported previously for camelina protein extractions. Furthermore, pH-CPI with acceptable color was produced, thus enhancing its organoleptic quality and utilization for food applications.

The protein profile and structural characteristics of CPI were linked to their functional properties. Salt extraction coupled with membrane filtration resulted in a higher ratio of cruciferin to napin and lower protein aggregation in CPI as compared to pH extraction. The differences in extraction conditions also resulted in protein structural differences between pH-CPI and salt-CPI. The salt-CPI were generally less denatured than pH-CPI samples and thus had better solubility. Moreover, the solubility of salt-CPI at acidic pH was comparable or higher than that of cSPI, indicating its potential utilization for high protein acidic beverages.

The impact of extraction conditions on degree of denaturation, protein aggregation, and surface properties varied between the two camelina lines based on the structural characteristics and stability of their protein variants. These structural differences in CPI samples led to differences in their functional properties. WL-pH-CPI showed comparable or inferior solubility but superior gelation and emulsifying properties than SL-pH-CPI. On the other hand, SL-salt-CPI had better or comparable gelation and emulsifying properties

than WL-salt-CPI. These results imply that the selection of suitable camelina lines as well as extraction method is important for utilizing CPI in targeted food applications.

CPI showed comparable or superior emulsifying properties to cSPI and cPPI, thus can potentially be employed as effective emulsifier in food applications. On the other hand, the gel strength of CPI, though superior than those of cPPI, was inferior to those of cSPI, due to the relatively small molecular weight of camelina proteins. Future work should explore protein modification techniques, such as targeted enzymatic hydrolysis, glycation, or non-thermal techniques such as high pressure, ozone treatment, pulsed electric field, and cold plasma, as they have the potential to induce structural changes that can enhance specific functional properties. Considering the current non-allergenic and non-GMO status of camelina protein, such targeted functionality enhancement of camelina protein will enable the utilization of CPI to replace SPI in different applications.

Furthermore, the functionality of camelina protein can also be enhanced through breeding programs to promote the expression of genetic variants associated with superior protein quality and functionality. The natural variations in protein profile among existing camelina lines explored in this study provided baseline data to initiate such breeding strategies. However, evaluation of more camelina lines and comprehensive identification of genetic variants associated with different protein functional properties is needed. Further investigation of these variants using proteomics will facilitate the development of genetic markers and tools, thus enabling targeted enhancement in protein functionality in current and future camelina breeding populations.

Finally, camelina protein showed promising nutritional quality to be utilized as a complementary protein source in the diet. However, the successful incorporation of CPI in food products will also depend upon its flavor profile. Thus, it is crucial to characterize and address any undesirable aromas or tastes in camelina that may be carried over to the final food products. Future investigations on modification, crop diversity, and flavor of camelina protein, coupled with the finding related to the extraction and evaluation of protein from several camelina lines reported in this study, will promote the advancement of camelina protein as a novel plant protein ingredient for food applications.

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Appendix A: Identification and agronomic information of camelina lines

Table 10. UMN identification number, public database identification number, growth habit, variety, growing location, and harvest year for camelina lines

UMN Identification Number	Public Database Identification	Growth Habit	Origin	Growing Location	Harvest Year
2020 Cam STP 02 Jol. 24.1	Joelle	Winter		Saint Paul Fields	2020
RMTEF 92.3.3	RMT-EF-9	Winter	UMN Mutant Population	Saint Paul Fields	2020
M4 21-1	15-21-1	Winter	UMN Mutant Population	Saint Paul Fields	2020
M4 28-5	15-28-5	Winter	UMN Mutant Population	Saint Paul Fields	2020
M4 54-1	15-54-1	Winter	UMN Mutant Population	Saint Paul Fields	2020
M4 76-1	15-76-1	Winter	UMN Mutant Population	Saint Paul Fields	2020
M4 62-1	15-62-1	Winter	UMN Mutant Population	Saint Paul Fields	2020
M5 220-1	15-220-1	Winter	UMN Mutant Population	Saint Paul Fields	2020
M5 77.1	15-77-1	Winter	UMN Mutant Population	Saint Paul Fields	2020
M5 133-1	15-133-1	Winter	UMN Mutant Population	Saint Paul Fields	2020
M5 94.1	15-94-1	Winter	UMN Mutant Population	Saint Paul Fields	2020
M5 336.1	STP-EF-336	Winter	UMN Mutant Population	Saint Paul Fields	2020
Ascn 418	IHAR502792	Winter	IHAR, Poland	Saint Paul Fields	2020
Ascn 226	PI650168	Winter	United States	Saint Paul Fields	2020
Ascn 399	IHAR247046	Winter	IHAR, Poland	Saint Paul Fields	2020
Ascn 432	444 Bashkiria	Spring	N.I. Vavilov All-Russian Institute of Plant Genetic Resources	Saint Paul Fields	2017

Table 10. continued

UMN Identification Number	Public Database Identification	Growth Habit	Origin	Growing Location	Harvest Year
Ascn 417	IHAR500884	Spring	IHAR, Poland	Saint Paul Fields	2017
Ascn 359	CN 113757	Spring	Plant Gene Resources of Canada	Saint Paul Fields	2017
Ascn 355	CN 113753	Spring	Plant Gene Resources of Canada	Saint Paul Fields	2017
Ascn 311	CN 113709	Spring	Plant Gene Resources of Canada	Saint Paul Fields	2017
Ascn 289	CN 113687	Spring	Plant Gene Resources of Canada	Saint Paul Fields	2017
Ascn 39	CJ11X-59	Spring	Dr. Johann Vollmann, Universität für Bodenkultur Wien (BOKU), Vienna, Austria	Saint Paul Fields	2017
Ascn 122	CJ11X-123	Spring	Dr. Johann Vollmann, Universität für Bodenkultur Wien (BOKU), Vienna, Austria	Saint Paul Fields	2017
Ascn 123	CJ12X-130	Spring	Dr. Johann Vollmann, Universität für Bodenkultur Wien (BOKU), Vienna, Austria	Saint Paul Fields	2017
Ascn 227	PI 652885	Spring	US National Plant Germplasm System	Saint Paul Fields	2017

Table 10. continued

UMN Identification Number	Public Database Identification	Growth Habit	Origin	Growing Location	Harvest Year
Ascn 430	405 Volgograd	Spring	N.I. Vavilov All-Russian Institute of Plant Genetic Resources	Saint Paul Fields	2017
Ascn 157	CMUT-555/4	Spring	Dr. Johann Vollmann, Universität für Bodenkultur Wien (BOKU), Vienna, Austria	Saint Paul Fields	2017
Ascn 159	CMUT-838/2	Spring	Dr. Johann Vollmann, Universität für Bodenkultur Wien (BOKU), Vienna, Austria	Saint Paul Fields	2017
Ascn 225	PI650167	Winter	Poland, Przemysl	Saint Paul Fields	2020
Ascn 264	CN113662	Winter	Former Soviet Union	Saint Paul Fields	2020
Ascn 262	CN113660	Winter	Poznan, Poland	Saint Paul Fields	2020
Ascn 216	PI650158	Winter	Poland	Saint Paul Fields	2020
Ascn 413	IHAR500033	Winter	IHAR, Poland	Saint Paul Fields	2020
Ascn 294	CN113692	Winter	IPK, Germany	Saint Paul Fields	2020
Ascn 193	PI311736	Winter	Poland	Saint Paul Fields	2020
Ascn 210	PI650152	Winter	Germany	Saint Paul Fields	2020
Ascn 293	CN113691	Winter	IPK, Germany	Saint Paul Fields	2020
Ascn 259	CN113657	Winter	Germany	Saint Paul Fields	2020
Ascn 215	PI650157	Winter	Former Soviet Union	Saint Paul Fields	2020
Joelle	Ames 33292	Winter	US National Plant Germplasm System	Rosemount Research Station in Rosemount, MN	2019

Appendix B: Optimization of fat extraction from camelina seeds with hexane

Table 11. Optimization of number of cycles and duration of hexane treatment to reduce fat content of winter line camelina meal

Defatting duration (min)				Fat content before milling (%)	Fat content in WL-DCM[1] (%)
Cycle 1	Cycle 2	Cycle 3	Cycle 4		
60				12.21[a^]	
30				12.86[a]	
30	30			6.16[b]	
30	30	60			5.02[a]
30	30	30			5.29[a]
30	30	30	30		3.12[b]

[1]WL-DCM – Defatted camelina meal from winter line; [^]Means (n =3) in each column with different lowercase letters indicate significant differences across defatting durations according to the Tukey-Kramer multiple means comparison test ($P < 0.05$).

Appendix C: Preliminary screening of solubilization pH and solubilization temperature for camelina protein extraction

Table 12. Protein yield in supernatant for different solubilization pH and solubilization temperature conditions during pH extraction of camelina protein

Solubilization pH	Solubilization Temperature (°C)	Protein Yield[1] in Supernatant (%)
10	25	54.12[b^]
11	25	64.61[a]
11	50	66.28[a]
12	25	69.34[a]

[1]Protein yield (%) represents the amount of protein extracted in supernatant relative to the total amount of protein in the starting Joelle winter line defatted camelina meal (WL-DCM); [^]Means (n =3) in each column with different lowercase letters indicate significant differences across extraction treatments according to the Tukey-Kramer multiple means comparison test ($P < 0.05$).

Appendix D: Sample calculation for determining protein yields using mass balance of pH extraction optimization

Calculating protein yield/loss/residue of each fraction in pH extraction optimization:

$$\%\ protein\ yield\ in\ WCP = \frac{WCP\ mass\ (g) \times WCP\ protein\ purity\ (\%)}{DCM\ mass\ (g) \times DCM\ protein\ purity\ (\%)}$$

$$\%\ protein\ yield\ in\ ACP = \frac{ACP\ mass\ (g) \times ACP\ protein\ purity\ (\%)}{DCM\ mass\ (g) \times DCM\ protein\ purity\ (\%)}$$

$$\%\ protein\ yield\ in\ pH\text{-}CPI = \frac{pH\text{-}CPI\ mass\ (g) \times pH\text{-}CPI\ protein\ purity\ (\%)}{DCM\ mass\ (g) \times DCM\ protein\ purity\ (\%)}$$

$$\%\ protein\ lost\ in\ gums = \frac{gums\ mass\ (g) \times gums\ protein\ purity\ (\%)}{DCM\ mass\ (g) \times DCM\ protein\ purity\ (\%)}$$

$$\%\ protein\ residue\ in\ pellet = \frac{pellet\ mass\ (g) \times pellet\ protein\ purity\ (\%)}{DCM\ mass\ (g) \times DCM\ protein\ purity\ (\%)}$$

$$\%\ protein\ lost\ in\ supernatant = \frac{supernatant\ mass\ (g) \times supernatant\ protein\ purity\ (\%)}{DCM\ mass\ (g) \times DCM\ protein\ purity\ (\%)}$$

Calculating protein yields of each fraction for optimized pH extraction (0.1% sodium sulfite):

$$\%\ protein\ yield\ in\ WCP = \frac{0.4828\ g \times 79.0781\ \%}{5.0042\ g \times 37.1500\ \%} = 20.5367\ \%$$

$$\%\ protein\ yield\ in\ ACP = \frac{0.9165\ g \times 84.8500\ \%}{5.0042\ g \times 37.1500\ \%} = 41.8303\ \%$$

$$\%\ protein\ yield\ in\ pH\text{-}CPI = \frac{4.2572\ g \times 83.0469\ \%}{5.0042\ g \times 37.1500\ \%} = 63.879\ \%$$

$$\%\ protein\ lost\ in\ gums = \frac{0.9129\ g \times 18.0644\ \%}{5.0042\ g \times 37.1500\ \%} = 8.8709\ \%$$

$$\%\ protein\ residue\ in\ pellet = \frac{1.8697\ g \times 16.5941\ \%}{5.0042\ g \times 37.1500\ \%} = 16.6890\ \%$$

$$\%\ protein\ lost\ in\ supernatant = \frac{0.7397\ g \times 25.5119\ \%}{5.0042\ g \times 37.1500\ \%} = 10.1507\ \%$$

Table 13. Mass balance for camelina protein pH-extraction optimization using 0.1% sodium sulfite (rep 1)

	Mass (g)	Protein Purity (%)	Mass of Protein (g)	Protein Yield (%)
DCM	5.0	37.2	1.9	[redacted]
WCP	0.5	79.1	0.4	20.5
ACP	0.9	84.9	0.8	41.8
pH-CPI	4.3	83.0	3.5	63.4
Gums	0.9	18.1	0.2	8.9
Pellet	1.9	16.6	0.3	16.7
Supernatant	0.7	25.5	0.2	10.2
Sum of Fractions	4.9	[redacted]	1.8	[redacted]
Recovered Material (%)	98.3			98.1

Appendix E: Preliminary screening of solubilization solvent for camelina salt extraction

Table 14. Protein yield in supernatant for different solubilization solvent during salt extraction of camelina protein

Solubilization Solvent	Protein Yield[1] in Supernatant (%)
0.05 M Phosphate Buffer, pH 8, 0.5 M NaCl	57.33[c^]
0.5 M NaCl	61.01[bc]
0.75 M NaCl	67.70[a]
1 M NaCl	64.24[ab]

[1]Protein yield (%) represents the amount of protein extracted in supernatant relative to the total amount of protein in the starting defatted camelina meal (DCM); [^]Means (n =3) in each column with different lowercase letters indicate significant differences across extraction treatments according to the Tukey-Kramer multiple means comparison test ($P < 0.05$).

Appendix F: Sample Calculation for Determining Protein Yields Using Mass Balance of Salt Extraction Optimization

Calculating protein yield/lost/residue of each fraction in salt extraction optimization

$$\%\ protein\ yield\ in\ salt\text{-}CPI = \frac{salt\text{-}CPI\ mass\ (g) \times salt\text{-}CPI\ protein\ purity\ (\%)}{DCM\ mass\ (g) \times DCM\ protein\ purity\ (\%)}$$

$$\%\ protein\ lost\ in\ gums = \frac{gums\ mass\ (g) \times gums\ protein\ purity\ (\%)}{DCM\ mass\ (g) \times DCM\ protein\ purity\ (\%)}$$

$$\%\ protein\ residue\ in\ pellet = \frac{pellet\ mass\ (g) \times pellet\ protein\ purity\ (\%)}{DCM\ mass\ (g) \times DCM\ protein\ purity\ (\%)}$$

Calculating protein yields of each fraction for optimized salt extraction (0.5M NaCl)

$$\%\ protein\ yield\ in\ salt\text{-}CPI = \frac{1.2358\ g \times 83.7219\ \%}{5.0038\ g \times 37.1500\%} = 55.6581\ \%$$

$$\%\ protein\ lost\ in\ gums = \frac{0.9225\ g \times 18.1019\%}{5.0038\ g \times 37.1500\ \%} = 8.9832\ \%$$

$$\%\ protein\ residue\ in\ pellet = \frac{2.5515\ g \times 18.1947\ \%}{5.0038\ g \times 37.1500\ \%} = 24.9736\ \%$$

Table 15. Mass balance for camelina protein salt-extraction using 0.5M NaCl (rep 1)

	Mass (g)	Protein Purity (%)	Mass of Protein (g)	Protein Yield (%)
DCM	5.0	37.2	1.9	[redacted]
Pellet	2.6	18.2	0.5	25.0
Gums	0.9	18.1	0.2	9.0
Salt-CPI	1.2	83.7	1.0	55.7
Sum of Sample Fractions	4.7	[redacted]	1.7	[redacted]
Recovered Material (%)	94.1			89.6

Appendix G: Sample Calculation for Determining Surface Hydrophobicity Index

Net Relative Fluorescence Intensity (RFI) at a single protein concentration:

$\text{Net RFI} = \text{RFI}_{final} - \text{RFI}_{initial}$

$\text{RFI}_{initial} = \text{Sample}_{initial} - \text{Blank}_{initial}$

$\text{RFI}_{final} = \text{Sample}_{final} - \text{Blank}_{final}$

Where:

$\text{Sample}_{initial}$ = fluorescence emission of protein sample before ANS probe is added

$\text{Blank}_{initial}$ = fluorescence emission of buffer blank before ANS probe is added

Sample_{final} = fluorescence emission of protein sample after ANS probe is added and 15-minute incubation at room temperature

Blank_{final} = fluorescence emission of buffer blank after ANS probe is added and 15-minute incubation at room temperature

Example calculation for WL-pH-CPI at 0.05% protein:

$\text{RFI}_{initial} = 28 - 15.5 = 12.5$

$\text{RFI}_{final} = 504 - 18.17 = 485.83$

$\text{Net RFI} = 485.83 - 12.5 = 473.33$

Surface Hydrophobicity Index:

Net RFI values for all concentrations of protein solution (0.05%, 0.025%, 0.02%, 0.015%, 0.01%, and 0.005% protein) are plotted against protein concentration, as seen in **Figure 7.**

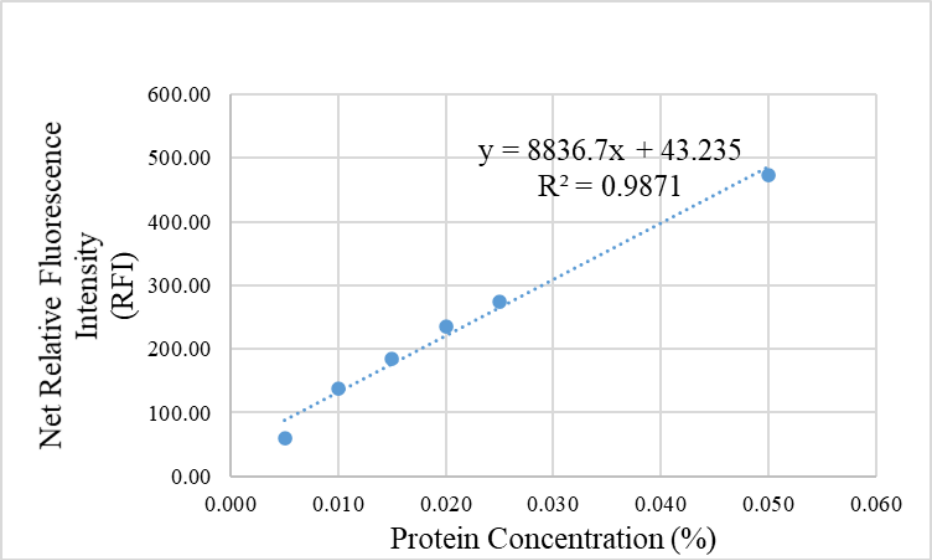

Figure 7. Net Relative Fluorescence Intensity (RFI) plotted against protein concentration (%) for WL-pH-CPI to determine surface hydrophobicity index.

The slope of the trendline in **Figure 7** is the surface hydrophobicity index 8836.7, r^2=0.9871.

The final value for surface hydrophobicity index is the average of three replicates.

Appendix H: Sample Spectrum for Determining Protein Secondary Structure

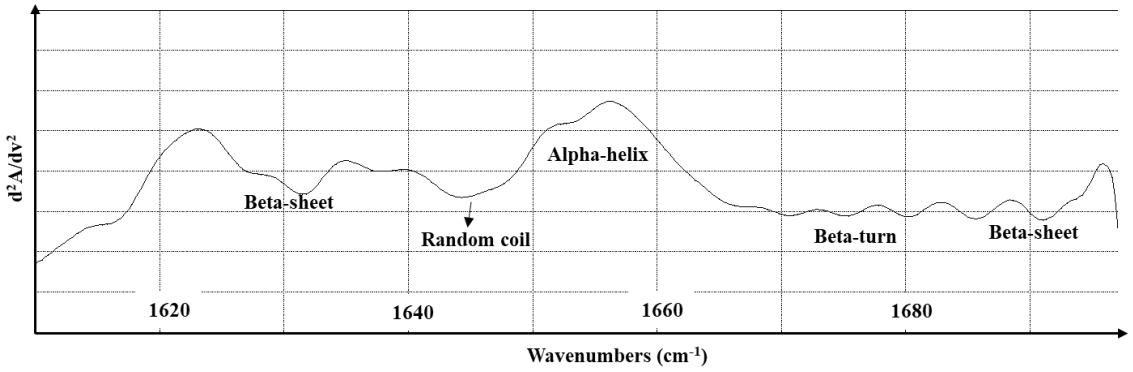

Figure 8. Second-derivative Spectrum of WL-pH-CPI

Second derivative of Amide I band (1600cm^{-1} -1700 cm^{-1}) (as illustrated in **Figure 8**) were obtained by PeakFit v. 4.12 to identify alpha-helix, beta-sheet, beta-turn, and random coil, according to the range of 1648-1660 cm^{-1}, 1612-1641 cm^{-1} and 1684-1694 cm^{-1}, 1662-1684 cm^{-1}, and 1640-1650 cm^{-1}, respectively.

Appendix I: Sample Calculation for Determining Protein Solubility

Protein Solubility of WL-pH-CPI:

$$\%\ protein\ solubility = \frac{\%\ supernatant\ protein}{\%\ initial\ protein} \times 100\%$$

$$\%\ protein\ solubility = \frac{0.37}{1.03} \times 100\% = 36.08\ \%$$

% initial protein and % supernatant protein were determined by Dumas method, before and after centrifugation (15,682 x g for 10 minutes).

Appendix J: Sample Calculation for Determining Water Holding Capacity (WHC)

Water Holding Capacity of WL-pH-CPI:

$$Water\ Holding\ Capacity = 100 \times \left(\frac{T_3 - T_1}{T_2 - T_1}\right)$$

$$Water\ Holding\ Capacity = 100 \times \left(\frac{1.6475 - 0.782}{1.6488 - 0.782}\right) = 99.85\%$$

Where,

T_1 = weight of protein solution before heating

T_2 = weight of protein solution + microcentrifuge tube after cooling

T_3 = weight of protein solution + microcentrifuge tube after draining excess water

Appendix K: Sample Calculation for Determining Protein Emulsification Capacity

Emulsification Capacity of WL-pH-CPI:

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

$$EC = \frac{83\ mL \times 0.93\ (\frac{g}{mL})}{0.05\ g} = 1543.8 \frac{g\ oil}{g\ protein}$$

Where:

0.93 g/mL = density of corn oil

0.05 g = grams of protein in 5 mL of a 1% protein solution

Appendix L: Sample Calculation for Determining Emulsion Stability and Emulsion Activity Index

Emulsion Stability (ES):

$$ES\ (min) = \frac{A_0}{A_0 - A_{10}} \times 10\ min$$

$$ES\ (min) = \frac{0.4067}{0.1856 - 0.4067} \times 10\ min = 18.39\ min$$

Where:

A_0 = absorbance at 0 min

A_{10} = absorbance at 10 min

Emulsion Activity Index (EAI):

$$EAI\left(\frac{m^2}{g}\right) = \frac{2T}{(1-\phi)C} = \frac{2(2.303 \times A_0)}{l(1-\phi)C}$$

$$EAI\left(\frac{m^2}{g}\right) = \frac{2(2.303 \times 0.4067)}{0.01\ m\,(1-0.25)1\ g/m^3} = 250\frac{m^2}{g}$$

Where:

C = weight of protein per volume of aqueous phase

= 0.1% protein solution = 0.1 g protein/100 mL = 1 g/m^3

ϕ = volume fraction of oil

= 1.67 mL oil in 5 mL of 0.1% protein solution

= (1.67 mL)/(1.67 mL + 5 mL) = 0.25

A_0 = initial absorbance at 500 nm

= 0.4067

l = path length of the cuvette

= 10 mm = 0.01 m

T = turbidity of oil at 500 nm

$$Turbidity\ of\ oil\ (T) = \frac{2.303 \times A_0}{l}$$

Appendix M: Screening of Camelina Lines using SDS-PAGE

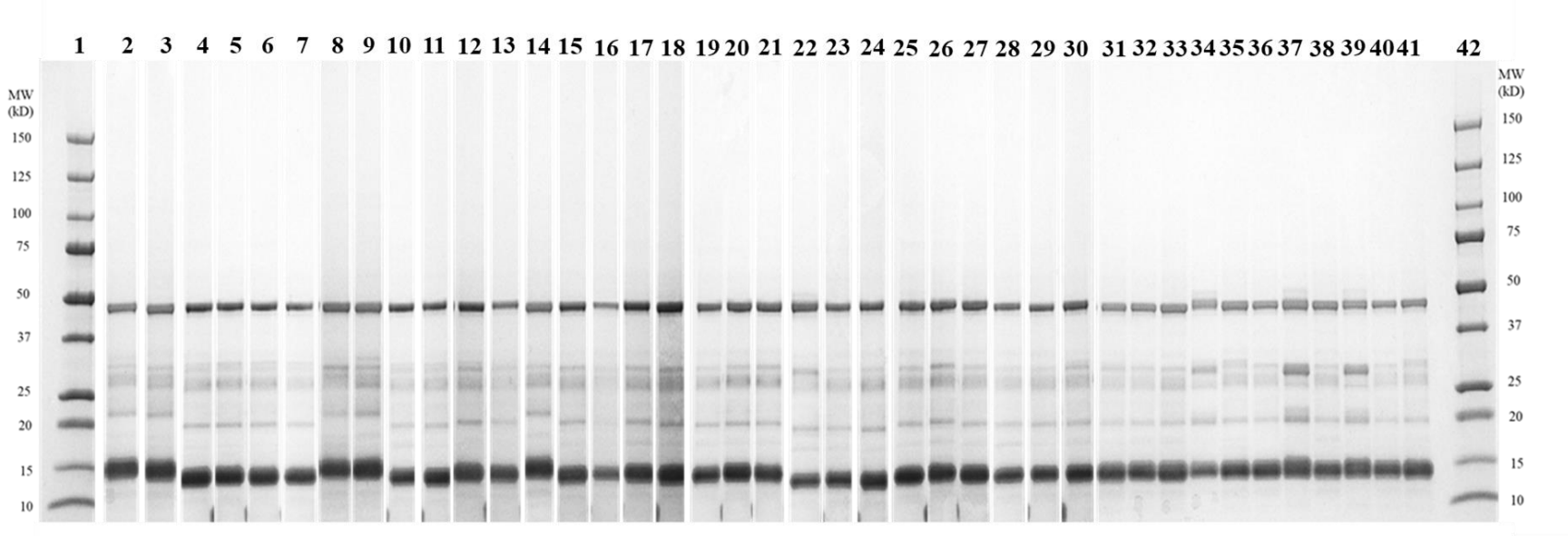

Figure 9. Visualization of the protein profiles of camelina lines under non-reducing conditions. Lane 1 and 42: Molecular weight marker; Lane 2: 2020 Cam STP 02 Jol. 24.1; Lane 3: RMTEF 92.3.3; Lane 4: M4 21-1; Lane 5: M4 28-5; Lane 6: M4 54-1; Lane 7: M4 76-1; Lane 8: M4 62-1; Lane 9: M5 220-1; Lane 10: M5 77.1; Lane 11: M5 133-1; Lane 12: M5 94.1; Lane 13: M5 336.1; Lane 14: Ascn 418; Lane 15: Ascn 226; Lane 16: Ascn 399; Lane 17: Ascn 432; Lane 18: Ascn 417; Lane 19: Ascn 359; Lane 20: Ascn 355; Lane 21: Ascn 311; Lane 22: Ascn 289; Lane 23: Ascn 39; Lane 24: Ascn 122; Lane 25: Ascn 123; Lane 26: Ascn 227; Lane 27: Ascn 430; Lane 28: Ascn 157; Lane 29: Ascn 159; Lane 30: Ascn 225; Lane 31: Ascn 264; Lane 32: Ascn 262; Lane 33: Ascn 216; Lane 34: Ascn 413; Lane 35: Ascn 294; Lane 36: Ascn 193; Lane 37: Ascn 210; Lane 38: Ascn 293; Lane 39: Ascn 259; Lane 40: Ascn 215; Lane 41: Joelle.

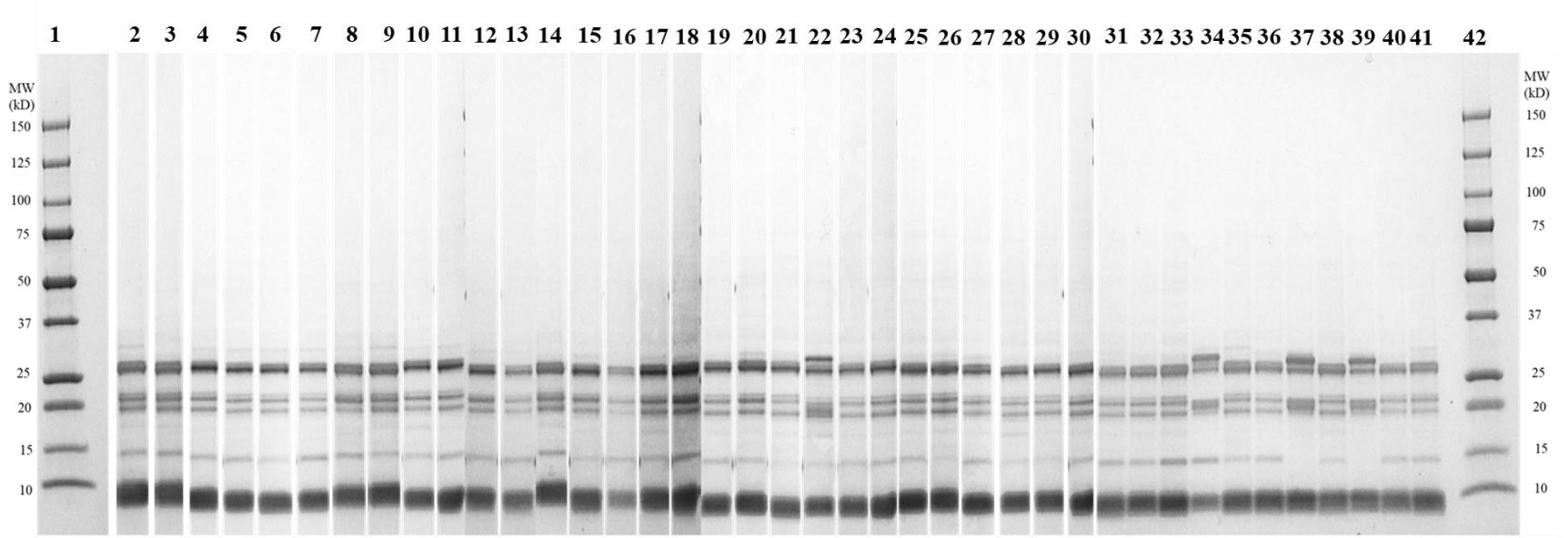

Figure 10. Visualization of the protein profiles of camelina lines under reducing conditions. Lane 1 and 42: Molecular weight marker; Lane 2: 2020 Cam STP 02 Jol. 24.1; Lane 3: RMTEF 92.3.3; Lane 4: M4 21-1; Lane 5: M4 28-5; Lane 6: M4 54-1; Lane 7: M4 76-1; Lane 8: M4 62-1; Lane 9: M5 220-1; Lane 10: M5 77.1; Lane 11: M5 133-1; Lane 12: M5 94.1; Lane 13: M5 336.1; Lane 14: Ascn 418; Lane 15: Ascn 226; Lane 16: Ascn 399; Lane 17: Ascn 432; Lane 18: Ascn 417; Lane 19: Ascn 359; Lane 20: Ascn 355; Lane 21: Ascn 311; Lane 22: Ascn 289; Lane 23: Ascn 39; Lane 24: Ascn 122; Lane 25: Ascn 123; Lane 26: Ascn 227; Lane 27: Ascn 430; Lane 28: Ascn 157; Lane 29: Ascn 159; Lane 30: Ascn 225; Lane 31: Ascn 264; Lane 32: Ascn 262; Lane 33: Ascn 216; Lane 34: Ascn 413; Lane 35: Ascn 294; Lane 36: Ascn 193; Lane 37: Ascn 210; Lane 38: Ascn 293; Lane 39: Ascn 259; Lane 40: Ascn 215; Lane 41: Joelle.

Appendix N: ANOVA Tables

Table 16. Analysis of variance on the effect of sodium sulfite concentration on WCP protein purity in pH-extraction.

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
WCP in pH extraction	Sodium Sulfite Concentration	2	4.320	3.377	0.104
	Error	6	1.280		

Table 17. Analysis of variance on the effect of sodium sulfite concentration on WCP protein yield in pH-extraction.

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
WCP in pH extraction	Sodium Sulfite Concentration	2	0.758	0.631	0.564
	Error	6	1.201		

Table 18. Analysis of variance on the effect of sodium sulfite concentration on ACP protein purity in pH-extraction.

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
ACP in pH extraction	Sodium Sulfite Concentration	2	7.556	2.208	0.191
	Error	6	3.422		

Table 19. Analysis of variance on the effect of sodium sulfite concentration on ACP protein yield in pH-extraction.

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
ACP in pH extraction	Sodium Sulfite Concentration	2	63.525	45.579	2.36e-4
	Error	6	1.394		

Table 20. Analysis of variance on the effect of sodium sulfite concentration on pH-CPI protein purity in pH-extraction.

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
pH-CPI in pH extraction	Sodium Sulfite Concentration	2	6.681	200.822	6.38e-4
	Error	3	0.033		

Table 21. Analysis of variance on the effect of sodium sulfite concentration on pH-CPI protein yield in pH-extraction.

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
pH-CPI in pH extraction	Sodium Sulfite Concentration	2	60.858	3648.854	8.33E-06
	Error	3	0.017		

Table 22. Analysis of variance on the effect of sodium sulfite concentration on pH-CPI ash content in pH-extraction.

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
pH-CPI in pH extraction	Sodium Sulfite Concentration	2	0.179	30.780	0.010
	Error	3	0.006		

Table 23. Analysis of variance on the effect of sodium sulfite concentration on protein content of discarded gums in pH-extraction.

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
Discarded gums in pH extraction	Sodium Sulfite Concentration	2	3.271	22.329	0.002
	Error	6	0.146		

Table 24. Analysis of variance on the effect of sodium sulfite concentration on protein lost to discarded gums.

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
Discarded gums in pH extraction	Sodium Sulfite Concentration	2	1.587	7.777	0.022
	Error	6	0.204		

Table 25. Analysis of variance on the effect of sodium sulfite concentration on protein content of discarded pellet in pH-extraction.

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
Discarded pellet in pH extraction	Sodium Sulfite Concentration	2	2.677	18.995	0.003
	Error	6	0.141		

Table 26. Analysis of variance on the effect of sodium sulfite concentration on pellet protein residue in pH-extraction.

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
Discarded pellet in pH extraction	Sodium Sulfite Concentration	2	0.515	1.584	0.280
	Error	6	0.325		

Table 27. Analysis of variance on the effect of sodium sulfite concentration on protein content of discarded supernatant in pH-extraction.

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
Discarded supernatant in pH extraction	Sodium Sulfite Concentration	2	54.646	38.119	3.88e-4
	Error	6	1.434		

Table 28. Analysis of variance on the effect of sodium sulfite concentration on protein lost to discarded supernatant in pH-extraction.

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
Discarded supernatant in pH extraction	Sodium Sulfite Concentration	2	5.693	4.281	7.00e-2
	Error	6	1.330		

Table 29. Analysis of variance on the effect of sodium sulfite concentration on lightness (L*) of ACP.

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
ACP in pH extraction	Sodium Sulfite Concentration	2	11.589	930.413	3.32e-8
	Error	6	0.012		

Table 30. Analysis of variance on the effect of sodium sulfite concentration on red and green color (a*) of ACP.

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
ACP in pH extraction	Sodium Sulfite Concentration	2	0.539	25.028	1.23e-3
	Error	6	0.022		

Table 31. Analysis of variance on the effect of sodium sulfite concentration on yellow and blue color (b*) of ACP.

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
ACP in pH extraction	Sodium Sulfite Concentration	2	8.573	921.808	3.41e-8
	Error	6	0.009		

Table 32. Analysis of variance on the effect of sodium sulfite concentration on lightness (L*) of pH-CPI.

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
pH-CPI in pH extraction	Sodium Sulfite Concentration	2	4.102	546.893	1.62e-7
	Error	6	0.008		

Table 33. Analysis of variance on the effect of sodium sulfite concentration on red and green color (a*) of pH-CPI.

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
pH-CPI in pH extraction	Sodium Sulfite Concentration	2	0.316	24.661	1.28e-3
	Error	6	0.013		

Table 34. Analysis of variance on the effect of sodium sulfite concentration on yellow and blue color (b*) of pH-CPI.

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
pH-CPI in pH extraction	Sodium Sulfite Concentration	2	12.858	180.055	4.40e-6
	Error	6	0.071		

Table 35. Analysis of variance on the effect of camelina protein isolate type on lightness (L*).

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
WL-pH-CPI, WL-salt-CPI, SL-pH-CPI, SL-salt-CPI	Camelina Protein Isolate Type	3	51.809	8278.365	2.65e-14
	Error	8	0.006		

Table 36. Analysis of variance on the effect of camelina protein isolate type on red and green (a*) color.

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
WL-pH-CPI, WL-salt-CPI, SL-pH-CPI, SL-salt-CPI	Camelina Protein Isolate Type	3	1.747	366.440	6.69e-9
	Error	8	0.005		

Table 37. Analysis of variance on the effect of camelina protein isolate type on yellow and blue (b*) color.

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
WL-pH-CPI, WL-salt-CPI, SL-pH-CPI, SL-salt-CPI	Camelina Protein Isolate Type	3	34.406	2392.060	3.78e-12
	Error	8	0.014		

Table 38. Analysis of variance on the effect of plant protein isolate type on thermal denaturation temperature for first peak on DSC.

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
WL-pH-CPI, WL-salt-CPI, SL-pH-CPI, SL-salt-CPI*	Plant Protein Isolate Type	3	5.454	50.641	1.51e-5
	Error	8	0.108		

*cSPI and cPPI were denatured before analysis.

Table 39. Analysis of variance on the effect of plant protein isolate type on enthalpy of denaturation for first peak on DSC.

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.

WL-pH-CPI, WL-salt-CPI, SL-pH-CPI, SL-salt-CPI*	Plant Protein Isolate Type	3	0.996	366.536	6.68e-9
	Error	8	0.003		

*cSPI and cPPI were denatured before analysis.

Table 40. Analysis of variance on the effect of plant protein isolate type on thermal denaturation temperature for second peak on DSC.

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
WL-pH-CPI, WL-salt-CPI, SL-pH-CPI, SL-salt-CPI*	Plant Protein Isolate Type	3	29.587	176.033	1.21e-7
	Error	8	0.168		

*cSPI and cPPI were denatured before analysis.

Table 41. Analysis of variance on the effect of plant protein isolate type on enthalpy of denaturation for second peak on DSC.

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
WL-pH-CPI, WL-salt-CPI, SL-pH-CPI, SL-salt-CPI*	Plant Protein Isolate Type	3	0.141	64.882	5.88e-6
	Error	8	0.002		

*cSPI and cPPI were denatured before analysis.

Table 42. Analysis of variance on the effect of plant protein isolate type on surface hydrophobicity.

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
cSPI, cPPI, WL-pH-CPI, WL-salt-CPI, SL-pH-CPI, SL-salt-CPI	Plant Protein Isolate Type	5	62954614	208.85	3.11e-11
	Error	12	301435		

Table 43. Analysis of variance on the effect of plant protein isolate type on surface charge.

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
cSPI, cPPI, WL-pH-CPI, WL-salt-CPI, SL-pH-CPI, SL-salt-CPI	Plant Protein Isolate Type	5	390.461	258.046	8.87e-12
	Error	12	1.513		

Table 44. Analysis of variance on the effect of plant protein isolate type on the relative percentage of α helix on IR spectra.

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
cSPI, cPPI, WL-pH-CPI, WL-salt-CPI, SL-pH-CPI, SL-salt-CPI	Plant Protein Isolate Type	5	42.788	22.500	1.03e-5
	Error	12	1.902		

Table 45. Analysis of variance on the effect of plant protein isolate type on the relative percentage of β sheet on IR spectra.

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
cSPI, cPPI, WL-pH-CPI, WL-salt-CPI, SL-pH-CPI, SL-salt-CPI	Plant Protein Isolate Type	5	28.248	15.154	7.94e-5
	Error	12	1.864		

Table 46. Analysis of variance on the effect of plant protein isolate type on the relative percentage of β turn on IR spectra.

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
cSPI, cPPI, WL-pH-CPI, WL-salt-CPI, SL-pH-CPI, SL-salt-CPI	Plant Protein Isolate Type	5	8.162	5.730	6.27e-3
	Error	12	1.424		

Table 47. Analysis of variance on the effect of plant protein isolate type on the relative percentage of random coil on IR spectra.

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
cSPI, cPPI, WL-pH-CPI, WL-salt-CPI, SL-pH-CPI, SL-salt-CPI	Plant Protein Isolate Type	5	14.271	26.922	3.95e-6
	Error	12	0.530		

Table 48. Analysis of variance on the effect of plant protein isolate type on the ratio of α helix to β sheet.

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
cSPI, cPPI, WL-pH-CPI, WL-salt-CPI, SL-pH-CPI, SL-salt-CPI	Plant Protein Isolate Type	5	0.056	25.090	5.78e-6
	Error	12	0.002		

Table 49. Analysis of variance on the effect of plant protein isolate type on protein solubility at pH 3.4 at 1% protein concentration for not-heated samples.

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
cSPI, cPPI, WL-pH-CPI, WL-salt-CPI, SL-pH-CPI, SL-salt-CPI	Plant Protein Isolate Type	5	249.539	69.293	1.98e-8
	Error	12	3.601		

Table 50. Analysis of variance on the effect of plant protein isolate type on protein solubility at pH 3.4 at 1% protein concentration for heated samples.

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
cSPI, cPPI, WL-pH-CPI, WL-salt-CPI, SL-pH-CPI, SL-salt-CPI	Plant Protein Isolate Type	5	517.833	100.733	2.26e-9
	Error	12	5.141		

Table 51. Analysis of variance on the effect of plant protein isolate type on protein solubility at pH 7 at 1% protein concentration for non-heated samples.

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
cSPI, cPPI, WL-pH-CPI, WL-salt-CPI, SL-pH-CPI, SL-salt-CPI	Plant Protein Isolate Type	5	150.152	33.399	1.22e-6
	Error	12	4.496		

Table 52. Analysis of variance on the effect of plant protein isolate type on protein solubility at pH 7 at 1% protein concentration for heated samples.

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
cSPI, cPPI, WL-pH-CPI, WL-salt-CPI, SL-pH-CPI, SL-salt-CPI	Plant Protein Isolate Type	5	756.856	130.896	4.88e-10
	Error	12	5.782		

Table 53. Analysis of variance on the effect of plant protein isolate type on protein solubility at pH 3.4 at 5% protein concentration for non-heated samples.

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
cSPI, cPPI, WL-pH-CPI, WL-salt-CPI, SL-pH-CPI, SL-salt-CPI	Plant Protein Isolate Type	5	249.683	388.710	7.77e-13
	Error	12	0.642		

Table 54. Analysis of variance on the effect of plant protein isolate type on protein solubility at pH 3.4 at 5% protein concentration for heated samples.

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
cSPI, cPPI, WL-pH-CPI, WL-salt-CPI, SL-pH-CPI, SL-salt-CPI	Plant Protein Isolate Type	5	270.410	542.161	1.07e-13
	Error	12	0.499		

Table 55. Analysis of variance on the effect of plant protein isolate type on protein solubility at pH 7 at 5% protein concentration for non-heated samples.

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
cSPI, cPPI, WL-pH-CPI, WL-salt-CPI, SL-pH-CPI, SL-salt-CPI	Plant Protein Isolate Type	5	707.155	766.070	1.36e-14
	Error	12	0.923		

Table 56. Analysis of variance on the effect of plant protein isolate type on protein solubility at pH 7 at 5% protein concentration for heated samples.

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
cSPI, cPPI, WL-pH-CPI, WL-salt-CPI, SL-pH-CPI, SL-salt-CPI	Plant Protein Isolate Type	5	1410.004	1540.746	2.07e-16
	Error	12	0.915		

Table 57. Analysis of variance on the effect of plant protein isolate type on gel strength.

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
cSPI, cPPI, WL-pH-CPI, SL-pH-CPI, SL-salt-CPI*	Plant Protein Isolate Type	4	256.265	1129.330	3.15e-13
	Error	10	0.227		

* WL-salt-CPI did not form a gel.

Table 58. Analysis of variance on the effect of plant protein isolate type on WHC.

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
cSPI, cPPI, WL-pH-CPI, SL-pH-CPI, SL-salt-CPI*	Plant Protein Isolate Type	4	0.099	4.271	0.028
	Error	10	0.023		

* WL-salt-CPI did not form a gel.

Table 59. Analysis of variance on the effect of plant protein isolate type on EC.

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
cSPI, cPPI, WL-pH-CPI, WL-salt-CPI, SL-pH-CPI, SL-salt-CPI	Plant Protein Isolate Type	5	712309	225.980	1.95e-11
	Error	12	3152		

Table 60. Analysis of variance on the effect of plant protein isolate type on ES.

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
cSPI, cPPI, WL-pH-CPI, WL-salt-CPI, SL-pH-CPI, SL-salt-CPI	Plant Protein Isolate Type	5	12.321	21.809	1.22e-05
	Error	12	0.565		

Table 61. Analysis of variance on the effect of plant protein isolate type on EAI.

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
cSPI, cPPI, WL-pH-CPI, WL-salt-CPI, SL-pH-CPI, SL-salt-CPI	Plant Protein Isolate Type	5	2702.552	16.304	5.50e-05
	Error	12	165.758		

Table 62. Analysis of variance on the effect of camelina protein isolate type on *in vitro* digestibility.

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
WL-pH-CPI, WL-salt-CPI, SL-pH-CPI, SL-salt-CPI	Camelina Protein Isolate Type	3	0.002	45.667	0.001
	Error	4	3.750e-5		

Table 63. Analysis of variance on the effect of plant protein isolate type on PDCAAS.

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
WL-pH-CPI, WL-salt-CPI, SL-pH-CPI, SL-salt-CPI	Camelina Protein Isolate Type	3	0.002	44.000	0.002
	Error	4	5.000e-05		

Table 64. Analysis of variance on the effect of number of cycles and duration of hexane treatment on fat content of camelina meal before milling.

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
Camelina meal treated with hexane	Number of Cycles and Duration of Hexane Treatment	2	41.006	112.999	1.73e-5
	Error	6	0.363		

Table 65. Analysis of variance on the effect of number of cycles and duration of hexane treatment on fat content of WL-DCM.

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
Camelina meal treated with hexane	Number of Cycles and Duration of Hexane Treatment	2	4.183	73.644	6.00e-5
	Error	6	0.057		

Table 66. Analysis of variance on the effect of solubilization pH and temperature on protein yield in supernatant during pH extraction of camelina protein.

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
Supernatant during pH extraction of camelina protein	Solubilization pH and Temperature	3	256.717	11.102	2.35e-4
	Error	18	23.123		

Table 67. Analysis of variance on the effect of solubilization solvent on protein yield in supernatant during salt extraction of camelina protein.

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
Supernatant during salt extraction of camelina protein	Solubilization Solvent	3	118.104	7.035	0.002
	Error	20	16.789		