Optimization of Hemp (*Cannabis sativa* L.) Protein Extraction and Characterization of Protein Structure, Function, and Nutritional Quality across Different Cultivars

A THESIS
SUBMITTED TO THE FACULTY OF THE
UNIVERSITY OF MINNESOTA
BY

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IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF
MASTER OF SCIENCE

Baraem P. Ismail, PhD

January 2022

Acknowledgements

First and foremost, I would like to thank my advisor, Dr. Baraem (Pam) Ismail, for giving me the opportunity to pursue my master's degree with her, and for the countless hours she has spent teaching and mentoring me. I feel fortunate to have studied under someone who is so well-respected and accomplished, and is truly an expert in her field. I admire her strength, her dedication to her students (so much so that she would spend a large part of her Christmas and New Year's holidays reading and editing this thesis), and her kindness. My sincere thanks to the other members of my committee – Dr. Tom Michaels and Dr. Serpil Metin. Thank you to Dr. Michaels for his many contributions to this research project, and especially for his insightful questions, entertaining stories, and infectious enthusiasm for hemp. Thank you to Dr. Metin for her valuable industry perspective on this research and for the sincerity in which she made the time to get to know me as a person.

A huge thank you to everyone who helped on this project, especially to Dr. Jim House, Adam Franczyk, and Jason Neufeld for conducting the nutritional analyses and answering all my nutrition-related questions. Thank you also to Riley Gordon and AURI, Daniel Garcia and Bühler, Donn Vellekson, Carl Anfinson, and Roger Wippler for their help with dehulling and separation of the hemp seeds. Thank you to Sam Adler and Maddi Johnson for their help with defatting and milling the hemp samples, to Rachel Mitacek for running the samples for FTIR, and to Fan Bu for her help analyzing the FTIR data. And last but certainly not least, my sincere thanks to the Plant Protein Innovation Center (PPIC) for graciously funding this research.

This project also would not have been possible without the support of my wonderful lab family, of which there are now so many that it would be impossible to individually thank each one of you. I hope you know how grateful I am to have met and worked alongside you all throughout this degree. I want to especially thank Amy Mathiowetz, Chelsey Hinnenkamp, Rachel Mitacek, Fan Bu, Lucy Hansen, Ben Millis, and Maneka Malalgoda, for training me on the methods needed to conduct this research, and Leslie Loehr, for learning methods alongside me. To my original desk buddy, Allie Schneider – thank you for your friendship, for being there for me since day one, and for

your tolerance of my spontaneous hugs and enthusiasm. To my roommate, Rachel Mitacek – living with you this last year of grad school has been way more fun that I could have imagined. From the hours spent plant shopping, repotting plants, caring for plants, watching college football, and discussing science and life, I could not have gotten through this degree without you. I am so thankful for yours, Allie's, and the close friendships and many memories made with Amy, Chelsey, Fan, Leslie, Holly Husband, and Claire Boyle over the past couple years. You all make my life better by being in it.

To my family and friends for their endless love and support, thank you. To my parents, for being there for me throughout my degree but especially during the writing process – you both are the best parents I could have asked for. To my family in Minnesota – Christopher, Kathryn, Ollie, Uncle Tim, Aunt Sandy, Uncle David, Aunt Donna, Meg, and Tyler – for being there for me and for making Minnesota feel like home. To my lifelong friends who at this point are basically family – Molly, Mary, Sarah, and Catherine – for being a supportive and positive part of my life. To my friend and fellow RDN, Lindsay MacNab, for her encouragement on this thesis when I needed it most. To my fellow grad students – Loren Avalos and Gretchen Gustafson – for your friendship and for being such a fun group to work with on our product development competition entry.

It has been a journey, and I can't believe it's coming to an end. I thank God for this season of my life, and most especially for the people He put there.

Dedication

This thesis is dedicated to my mom and dad, who have been a loving and constant support system throughout my entire life, and in loving memory of my grandma Marilyn, who never let me forget how proud she was of me.

Abstract

The global population is predicted to reach 9.7 billion people in 2050, presenting a significant challenge of producing enough nutritious food in a sustainable way. Specifically, the demand for protein is increasing, with marked increase in the demand for plant protein ingredients. Current plant protein sources (soy, wheat, pea) have limitations with respect to consumer perspective or from a functional or flavor perspective, necessitating exploration of novel sources.

Hemp (*Cannabis sativa* L.), which has been cultivated for thousands of years, is an environmentally friendly crop. However, legal restrictions due to the presence of the psychotropic component delta-9 tetrahydrocannabinol (THC) have antagonized the hemp market for decades. In 2018, hulled hemp seeds, hemp seed protein powder, and hemp seed oil achieved "generally recognized as safe" GRAS status in the U.S. Hemp seeds contain high amounts of oil (~ 30%) and protein (~25%). Research on feasible production of hemp protein isolate (HPI) and on the functional properties for food applications is minimal. Determining optimal protein extraction procedures to produce HPI will be instrumental in the adoption as a desirable protein ingredient. Additionally, identifying differences in seed protein characteristics in different cultivars is needed to initiate breeding strategies to improve the prospects for food applications.

Therefore, the objectives of this work were to 1) optimize protein extraction from hemp seed, following alkaline solubilization coupled with isoelectric precipitation and salt solubilization coupled with membrane filtration, to produce a protein isolate with acceptable color, purity, yield, structural and functional characteristics, and nutritional quality, and 2) evaluate HPI produced from four industrial cultivars for differences in color and protein structural, functional, and nutritional properties.

Whole hemp seeds from one cultivar (CFX-2) harvested in 2016 were dehulled using an impact dehuller and further separated using sieves, an aerator, a gravity separator table, and manual separation. Dehulled seeds were pressed using a cold hydraulic press, ground, defatted with hexane, and milled to 50 mesh prior to protein extraction. Two methods of protein extraction were tested – alkaline solubilization coupled with isoelectric precipitation and salt solubilization coupled with membrane

filtration. Optimal protein extraction conditions were determined by evaluating different parameters including solubilization pH, precipitation pH, salt (NaCl) solubilization concentration, and heating and assessing protein purity and yield. HPIs produced using both methods (pH-HPI and salt-HPI) were characterized in comparison to commercial soy protein (cSPI) and pea protein isolates (cPPI). Color was measured using a colorimeter. Structural analysis was performed using SDS-PAGE for protein profile, DSC for protein denaturation, a spectrophotometric method for surface hydrophobicity, zeta potential for surface charge, and Fourier transform infrared spectroscopy (ATR-FTIR) for protein secondary structure. Protein functionality including solubility, gel strength, water-holding capacity, and emulsification and foaming properties were evaluated. Structural and functional testing were performed in water and in 0.5 M NaCl due to sedimentation of salt-HPI in water. Nutritional quality was determined by calculating the protein digestibility-corrected amino acid score (PDCAAS) based on amino acid analysis and the pH drop *in vitro* protein digestibility assay.

Whole hemp seeds from four industrial cultivars (CFX-2, Grandi, Joey, Picolo) harvested in 2019 were dehulled, separated, defatted, and milled, as described above. Four HPI samples were produced from the cultivars following the optimized pH extraction method. Color and protein structural, functional, and nutritional properties were characterized as described above. Structural and functional testing was only performed in water.

Both the optimized pH-assisted (solubilization at pH 11, precipitation at pH 5) and salt-assisted (solubilization in 0.75 M NaCl at 50°C followed by ultrafiltration/diafiltration) protein extraction methods produced HPI with high protein purities (87 – 88% protein) and remarkable yields (> 80%). The use of dehulling prior to defatting and protein extraction resulted in HPIs with desirable light and bland colors. Both extraction methods produced isolates with similar protein profiles, but pH-HPI exhibited some protein polymerization and was partially denatured. These structural differences appeared to improve HPI properties. Specifically, salt-HPI needed to be dispersed in a dilute salt solution to form a gel, while pH-HPI formed a gel in water at relatively low protein concentration (10% protein). Overall, HPI was less functional than cSPI and cPPI, but had similar solubility to cSPI at acidic pH in water, and superior

solubility ($P < 0.05$) and gel strength at neutral pH in 0.5 M NaCl. While the use of 0.5 M NaCl for solubilization improved HPI gel strength and solubility at neutral pH, it negatively impacted water holding capacity and foaming stability. The PDCAAS of HPI (0.58 for pH-HPI and 0.54 for salt-HPI) was within the range previously reported for whole hemp seeds, dehulled hemp seeds, and hemp seed meal (0.48 – 0.61). In general, alkaline solubilization coupled with isoelectric precipitation was determined to produce a more functional and nutritious HPI.

Minimal structural differences among HPI extracted from the four cultivars were observed, which contributed to only slight differences in functionality and nutritional properties. All HPIs had similar solubility to cSPI at acidic pH, and three cultivars (Grandi, Joey, Picolo) produced significantly stronger gels ($P < 0.05$) than cSPI. There were no significant differences in *in vitro* digestibility among the four HPIs, but differences in amino acid profile led to significant yet minor differences in PDCAAS.

This work demonstrated that protein can be successfully extracted from dehulled hemp seeds to produce an HPI with high protein purity, yield, and acceptable color. Protein extraction using pH produced an HPI with some promising functional attributes comparable or in some cases superior to cSPI and cPPI. This study was the first to optimize protein extraction parameters for hemp and to provide a comprehensive structural, functional, and nutritional comparison between pH-extracted and salt-extracted HPI. Additionally, this study was the first to examine the impact of cultivar on HPI properties. Since minimal differences among HPIs from four industrial cultivars were observed, more cultivars should be tested for differences that would warrant a hemp breeding program for improved functionality and nutritional quality. Future work is also needed to understand how HPI functions within a food matrix.

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Chapter 1: Literature Review

1.1 Introduction

The global population is predicted to reach 9.7 billion people in 2050, an increase of over 50% from 6.1 billion in 2000 (United Nations 2004, 2019). With this growth comes the challenge of feeding the population in a nutritious and sustainable way. Protein is one of the three macronutrients (fat, carbohydrates and protein) necessary for human health, and it is responsible for proper functioning of cells, tissues, organs, and systems throughout the body (Nadathur et al. 2017a). The health benefits associated with protein from animal sources are well-established, and consumer demand for these sources of proteins is growing (Ismail et al. 2020). However, demand for proteins from plant sources is also rapidly increasing (Grand View Research 2021).

Protein from plant sources provides many benefits when compared with animal protein. Cultivation of plant sources of protein requires fewer land and water resources, meaning that plant proteins can be produced at lower prices, providing economic benefits for food producers and consumers (Nadathur et al. 2017b; Grand View Research 2021). Consumers are increasingly following a vegan, vegetarian, or flexitarian diet due to concerns related to personal health, the environmental, or animal welfare (Grand View Research 2021). Additionally, animal-derived sources of proteins, such as eggs and dairy, are among the major allergens in the United States (FDA 2021). Limitations in the use of certain proteins create challenges for food producers who rely on proteins for providing functional properties, such as emulsification, gelation, water holding, and foaming, in food products (Ismail et al. 2020). Differences in protein functionality and nutritional quality among various sources of proteins are directly related to their inherent structural characteristics and processing conditions. Currently, soy and wheat are two major sources of plant proteins that provide desirable functional properties. However, they are among the “big nine” allergens. Additionally, over 90% of soy in the United States is genetically modified (GMO) (Laxmi 2020), a fact that deters consumers who prefer organic food. Therefore, it is necessary to search for novel plant protein sources that can provide comparable functionality and nutritional quality (Ismail et al. 2020). Novel plant protein sources currently being explored include chickpea, oat, canola, sunflower, and hemp.

Hemp (*Cannabis sativa* L.) is an ancient crop that has been used for food, fiber, and medicine for thousands of years (Callaway 2004). Hemp cultivation requires minimal to no use of herbicides, pesticides, and fungicides, limiting the amount of these harmful chemicals in the environment (Aluko 2017). Additionally, hemp has a deep rooting system that aerates the soil, ideal for crop rotation practices. Other than the environmental benefits, the hemp plant produces seeds that are high in protein and oil. Hemp seeds historically have been consumed raw, cooked, or roasted. Currently, hemp protein is considered to have low allergenicity (Wang and Xiong 2019). However, hemp has been underutilized as a crop due to legal restrictions because of its similarities to marijuana, which contains high amounts of the psychotropic component delta-9 tetrahydrocannabinol (THC) (Johnson 2018). In contrast to marijuana, hemp has been bred to contain less than 0.3% THC. In 2018, hulled hemp seeds, along with hemp seed protein powder and hemp seed oil, were granted "generally recognized as safe" (GRAS) status in the United States, paving the way for expansion of the use of hemp in foods (FDA 2018).

Currently, hemp seeds are pressed to extract the nutritious edible oil, leaving behind a meal with a protein content up to 50% (House et al. 2010). This protein-rich byproduct could be utilized as a novel source of plant protein in food products. Some work has been done examining hemp protein's structural properties and how they impact its functionality and nutritional quality. However, these three properties are impacted by processing and protein extraction conditions, which have yet to be optimized for hemp protein. Traditional methods of protein extraction rely on altering environmental conditions, such as pH or salt concentration, to promote protein solubilization and facilitate purification (Boye et al. 2010a). Overall, research on hemp protein is lagging behind other more established plant protein sources, such as soy and pea.

Another key area of research that has yet to be explored is the differences in hemp protein profile, structure, and functionality across different cultivars. Breeding of different industrial hemp cultivars has focused on end-use – seed, fiber, or cannabidiol (CBD) production, or for dual purposes. Additionally, research has demonstrated differences in seed protein content, oil content, and fatty acid profile across different cultivars (Vonapartis et al. 2015; Galasso et al. 2016; Irakli et al. 2019; Lan et al. 2019;

Schultz et al. 2020). However, breeding to improve functional and nutritional properties of hemp protein for human consumption remains unexplored (House et al. 2010; Cherney and Small 2016). Therefore, characterizing differences in hemp seed protein across cultivars would provide valuable information for breeders to improve hemp seed protein for food use.

Hemp seeds are a promising novel source of plant protein that could be used to help meet the growing consumer demand. Therefore, it is worth investigating how to produce a high protein hemp ingredient to deliver desirable functionality and nutrition, and how these properties differ across cultivars.

1.2 Hypothesis and Objectives

We hypothesize that extracting hemp seed protein using two different methods, alkaline solubilization with isoelectric precipitation and salt solubilization coupled with membrane filtration, will produce protein isolates with acceptable color, yield, and purity. We hypothesize that the use of salt solubilization coupled with membrane filtration will produce a hemp protein isolate (HPI) with a lighter color, better nutritional quality, and preserved structural characteristics, leading to better functionality. We hypothesize that HPI will provide comparable or superior functionality to commercially produced soy or pea protein isolates for certain applications. Additionally, we hypothesize that there will be significant differences in the structural, functional, or nutritional properties of HPI produced from different cultivars, which will warrant a breeding program for hemp protein for food use.

The overall objective of this project was to investigate the impact of cultivar and protein extraction conditions on hemp protein structural, functional, and nutritional properties. Specific objectives were:

1. Optimize protein extraction from hemp seed, following alkaline solubilization coupled with isoelectric precipitation and salt solubilization coupled with membrane filtration, to produce a protein isolate with acceptable color, purity, yield, structural and functional characteristics, and nutritional quality.
2. Evaluate HPI produced from four industrial cultivars for differences in color and protein structural, functional, and nutritional properties.

1.3 Need for Novel, Plant Protein Ingredients

1.3.1 Consumer Demand for Plant Protein

The growing global population necessitates expansion of the capabilities of the current food system. A 50% increase in global food production between 2012 and 2050 is needed to prevent undernourishment of an estimated 637 million people (Sathe et al. 2018). One major challenge is the availability of high quality protein that can provide bioaccessible essential amino acids in sufficient amounts to meet nutritional requirements (House et al. 2010; Sathe et al. 2018). Animal protein sources, such as eggs, dairy milk, meat, and poultry provide high quality proteins; however, production of animal proteins is costly (Sathe et al. 2018). Large amounts of water, land, and energy are needed to grow feed for animals and to raise those animals for human consumption (Nadathur et al. 2017a). In contrast, plant protein sources require less land, water, and energy use to produce and can be produced at a lower cost (Henchion et al. 2017; Nadathur et al. 2017a). Therefore, plant proteins have been identified as an important protein source to help feed the growing global population.

Consumer preferences are also driving the demand for plant proteins. Overall protein demand has grown due to increased consumer recognition of its health benefits, including weight maintenance, satiety, muscle development and maintenance, and healthy aging (Sathe et al. 2018; Ismail et al. 2020; Grand View Research 2021). Plant-based proteins are perceived to be healthier than animal-derived proteins (Grand View Research 2021), contributing to the increase in vegan, vegetarian, and flexitarian diets. Additionally, consumers are concerned about environmental issues and sustainability. The food industry is responsible for an estimated 26% of greenhouse gas emissions produced by humans, with livestock production contributing more than half of these emissions (Gaillac and Marbach 2021). Greenhouse gases, particularly carbon dioxide, are associated with global climate change. Current animal production practices have been deemed unsustainable, as animal production requires more of limited resources (land, energy, and water) than plant protein sources (Nadathur et al. 2017a). Consumers are also concerned for animal welfare, contributing to a decreased consumption or avoidance of animal proteins.

Protein allergenicity is another considerable challenge facing the food industry. Animal sources of protein, including milk and eggs, and two common sources of plant proteins, soy and wheat, are on the Food and Drug Administration's (FDA) list of "Big 9" allergens (Sathe et al. 2018; FDA 2021). These four protein sources commonly provide desirable functionality in food products, necessitating their use to achieve sensory and structural goals (Ismail et al. 2020). While culturally acceptable sources of animal proteins are limited, many diverse crops exist as potential sources of proteins that could provide desired functional attributes. Investigation into novel plant protein sources is necessary to meet consumers' needs and keep up with market trends.

1.3.2 Plant Protein Market Trends

In 2020, the global protein ingredient market was valued at $38.5 billion and is expected to reach $85.5 billion in 2028 (Grand View Research 2021). While 70% of the global revenue came from animal protein ingredients in 2020, plant proteins are expected to take part of this market share in the future (Ismail et al. 2020; Grand View Research 2021). During the COVID-19 pandemic, plant proteins experienced a sharp increase in sales due to shortages and price increases of animal proteins (Kamp 2020). Even after the market recovers from the effects of the pandemic, the plant protein market is expected to keep growing (Kamp 2020).

Key market trends include: 1) consumer avoidance of certain protein ingredients, such as dairy, soy, and wheat, 2) a shift toward purchasing practices that align with personal beliefs, and 3) a desire for more variety in plant proteins. Globally, 16% of new food and beverage product launches in 2019 featured low, no, or reduced allergen claims, and 14% featured gluten-free claims (Schofield 2020). Consumers report avoiding dairy, wheat, soy, and eggs as a lifestyle choice for health or ethical reasons, and/or due to medically diagnosed allergies or intolerances. Additionally, consumers want the products they purchase to align with their personal beliefs regarding environmental issues. Mintel found that 61% of respondents want to be connected with brands that match their values (Kamp 2021). Lastly, consumers are choosing plant proteins to expand their palate. Almost half of consumers who are choosing more plant-based proteins are doing so out of a desire for variety in their diets (Formanski 2021). Therefore, expanded use of protein

ingredients that align with consumer trends is important for the industry to continue growing.

1.3.3 Current Plant Protein Sources

Currently, protein ingredients produced from soy and wheat dominate the plant protein market. Soy is used in meat and dairy alternatives such as tofu and soymilk (Deak et al. 2008). Soy protein ingredients provide functional properties, such as gelling, emulsification, and water holding, for processed meat, meat alternative, baking, convenience, and other food applications (Deak et al. 2008; Ismail et al. 2020). Robust research was dedicated to characterize soy protein properties, providing information that has helped food producers understand and incorporate this plant protein into various products (Ismail et al. 2020). Additionally, soy protein has similar nutritional quality to animal sources, and has been linked to beneficial health properties, such as a reduction in the risk of heart disease, cancer, and osteoporosis (Potter 1998; Deak et al. 2008; Hughes et al. 2011). However, soy's allergenicity and the negative consumer perceptions regarding the use of GMO ingredients has caused food producers to look for alternatives (Ismail et al. 2020).

Wheat, the other popular source of protein, contains gluten forming proteins, which impart unique viscoelastic properties to foods, such as baked products and meat alternatives (Ismail et al. 2020). In baking applications gluten protein creates an extensible dough structure that entraps air to result in desired volume and structural characteristics (Nadathur et al. 2017a). However, producers are shifting away from using wheat due to gluten allergenicity and sensitivity, and due to consumer avoidance of gluten for non-medical reasons (Srivastava 2021).

Peas is a source of plant protein that is experiencing a steep market growth (Grand View Research 2021). Similar to soy protein, pea protein can be used to impart functional properties such as emulsification, gelling, and water holding capacity in foods (Ismail et al. 2020). Pea protein aligns well with industry needs, as it currently has low allergenicity, low cost, non-GMO, and wide availability (Lam et al. 2018). It also fits within consumer demands for plant proteins with environmental benefits, as peas are a short season crop that can replenish the soil, while increasing revenue for growers (Ismail

et al. 2020). However, pea protein has functional, nutritional, and flavor limitations compared to soy protein (Lam et al. 2018). Therefore, it is important to explore other plant proteins that can provide functional benefits, help nourish a growing population, and align with consumer trends.

1.3.4 Novel Plant Protein Sources

Novel plant sources of protein ingredients include pulses, such as lentils, chickpeas, and beans; grains, such as wheat, oats, rice, corn, and ancient grains; and oilseeds, such as canola, sunflower, flax, and hemp (Nadathur et al. 2017b; Galves et al. 2019; Ismail et al. 2020). Oilseeds in particular are being explored for their high contents of two food ingredients – oil and protein – where cultivation could result in at least two sources of revenue for growers. Typically, the protein-rich meal remaining after oil extraction has been underutilized for these crops, however, research is being done to explore their potential to produce high protein ingredients (Arntfield 2011).

While less developed, hemp as an oilseed has vast potential as a source of protein (*Cannabis sativa* L). Hemp belongs to the *Cannabaceae* family, which includes marijuana and hops (Small 2015; Farinon et al. 2020). Each part of the hemp plant – leaves, flowers, stalk, and seeds – can be used in different industrial applications (Farinon et al. 2020). Hemp seeds are of interest as they contain approximately 30% oil and 25% protein depending on cultivar and growing conditions, with appreciable amounts of fiber, vitamins, and minerals (Aluko 2017; Farinon et al. 2020).

Currently, whole or dehulled hemp seeds are the main hemp ingredient used in new product launches globally, more so than the oil or protein-rich ingredients (House et al. 2010; Mattucci 2020). Hemp seed oil is sold as a niche edible oil for its desirable nutritional profile, while the remaining protein-rich press cake is milled and sold as hemp protein powder or hemp flour (Matthäus and Brühl 2008; House et al. 2010). Hemp seed protein is a viable plant protein alternative as it has an amino acid composition similar to that of egg white and soy protein, with the exception of lysine deficiency (Aluko 2017; Sathe et al. 2018). While, currently, hemp is non-allergenic, it also aligns with consumer trends around environmental benefits, as the hemp plant requires minimal to no use of herbicides, pesticides, and fungicides, and it returns nutrients to the soil after harvest

(Aluko 2017). Historically, due to being closely related to marijuana, hemp production has been hindered by legal challenges, especially in Canada and the United States, preventing its widespread adoption as a food ingredient (House et al. 2010; Cherney and Small 2016). With the removal of many of these legal barriers in the past two decades, renewed interest in hemp is driving exploration into its potential as a source of plant protein.

1.4 Hemp (*Cannabis sativa* L.)

Hemp (*Cannabis sativa* L.) is an annual plant that has been cultivated for thousands of years (Callaway 2004; Aluko 2017). Most parts of the hemp plant – stalk, flowers, leaves, and seeds – have industrial uses, making it a valuable and versatile crop (Potin and Saurel 2020; Farinon et al. 2020). Breeding efforts have produced cultivars with desirable traits based on end use, namely fiber production (Fike 2016). There is renewed interest in hemp cultivation, especially for food, due to the recent removal of legal restrictions on growing hemp and hemp products (Aluko 2017). These restrictions, which hindered the industry for decades, were implemented due to hemp's close relationship to the drug-producing plant, marijuana.

1.4.1 Differences Between Hemp and Marijuana

A common source of confusion when discussing hemp as a food crop is its similarities to marijuana. Both plants belong to the genus *Cannabis* and are commonly referred to as such (Cherney and Small 2016). However, marijuana contains the psychotropic compound delta-9-tetrahydrocannabinol (THC) in sufficient quantities (ranging from 1% to 20% or more in the leaves and flowering parts) to produce intoxicating effects, whereas hemp has been bred to legally contain less than 0.3% or 0.2% THC on a dry weight basis, depending on the country (Cherney and Small 2016; Fike 2016; Johnson 2018). Additionally, THC and other cannabinoids such as CBD are mostly found in the plant's leaves and flowers and are hardly present in hemp seeds unless cross-contamination during processing occurs (Farinon et al. 2020). Legal limits on THC in hemp seeds ranges from 10 parts per million (ppm) down to 0.005 ppm; therefore, the presence of THC in hemp seeds is not of concern (Small 2015). The

similarities between hemp and marijuana have caused hemp cultivation for food use to lag behind other crops.

1.4.2 Hemp History and Production

The cultivation of hemp can be traced back thousands of years and is believed to have originated in central Asia (Callaway 2004; Farinon et al. 2020). Traditionally, hemp was cultivated for food, medicine, and fiber using different parts of the plant (House et al. 2010). In China, raw, cooked, and roasted hemp seeds as well as hemp seed oil have been used as food and medicine for over 3,000 years (Callaway 2004). In the western world, hemp was primarily grown for fiber produced from the bast (stem), which was used in textiles and ropes, while hemp seeds were a byproduct used for animal feed (Cherney and Small 2016; Farinon et al. 2020).

Hemp is believed to have come to North America in 1606, where it was cultivated as an agricultural fiber crop for a few hundred years (Cherney and Small 2016). However, recognition and concerns about the presence of THC in the leaves, flowers, and resin of *Cannabis* plants, hindered hemp production in Europe and North America starting in the mid-1900s (Matthäus and Brühl 2008; House et al. 2010; Farinon et al. 2020). In the United States, the 1937 Marijuana Tax Act did not legally distinguish between marijuana and hemp, placing strict controls on growing hemp (Farinon et al. 2020). The following year, Canada made cultivation of *Cannabis* plants illegal with the Canadian Opioid and Narcotics Act (Cherney and Small 2016). However, hemp production in both countries enjoyed a brief boom during World War II as imports of fiber were disrupted. Production drastically decreased after the war due to reinstated legal restrictions and cheaper imported fibers (Cherney and Small 2016; Fike 2016). By the end of the 20th century, Canada and Europe re-permitted hemp cultivation, causing renewed interest in hemp in the United States (Fike 2016; Farinon et al. 2020). Legal restrictions in the United States were gradually lifted, and in 2018, GRAS status was granted to dehulled hemp seeds, hemp seed oil, and hemp seed protein powder for use in food products (Fike 2016; FDA 2018).

As of 2018, approximately 30 countries around the world allow farmers to grow hemp (Johnson 2018). The European Union leads global hemp production followed by

Canada, while China, South Korea, and Russia are also major producers (Johnson 2018; Farinon et al. 2020). Globally, the hemp fiber market has remained relatively steady in recent years, while the market for hemp seeds has been increasing due to the interest in hemp seeds for their nutritional properties (Johnson 2018; Farinon et al. 2020). In the U.S. alone, approximately two-thirds of hemp imports in 2017 were for hemp seeds, which does not include other hemp seed products such as oil or press cake or hemp fiber (Johnson 2018). In addition to hemp being a valuable agricultural crop, its production benefits the environment.

1.4.3 Industrial and Agronomic Benefits of Hemp Production

Hemp is an annual herbaceous plant than can grow one to six meters high depending on environmental conditions and cultivar (Fike 2016; Aluko 2017). Hemp is composed of a tall woody stalk covered with distinctive leaves. Flowers and seeds are produced at the top of the plant (Fike 2016; Farinon et al. 2020). Typically, hemp is a dioecious plant, meaning that there are distinctive male and female plants, although modern breeding has produced monoecious varieties, which have both male and female flowers (Small 2015; Fike 2016; Crescente et al. 2018). Male plants are usually slender and taller, and they flower earlier than female plants, but they die after pollination (Fike 2016; Pavlovic et al. 2019; Farinon et al. 2020). Wind carries pollen from the male plants to the female plants, causing the females to flower and produce hemp seeds (Crescente et al. 2018; Pavlovic et al. 2019). Resin is found in glandular trichomes, which are epidermal appendages most abundant in the flowering portion of the female plant (Cherney and Small 2016; Farinon et al. 2020).

One of the major benefits of hemp production is that many parts of the plant have industrial uses. As many as 50,000 uses for the hemp plant have been reported, although the uses that can generate significant market value are fiber, pharmaceutical, and oilseed products (Cherney and Small 2016). Hemp fiber, which comes from the stem, can be used in applications such as textiles, hemp-lime construction, and animal bedding. Pharmaceutical applications can utilize the resin for cannabinoids, which are unique substances with potential medicinal benefits such as anti-anxiety and anti-depression. The oil seeds have applications in food, nutritional supplements, cosmetics, and biofuel

industries (Cherney and Small 2016; Farinon et al. 2020). As such, hemp is a versatile crop that can provide value in multiple industries, minimizing waste streams from cultivation.

Apart from the industrial benefits, growing hemp has benefits for the environment. Due to its pest-resistance characteristic, the plant can be grown with little or no use of pesticides, herbicides, and fungicides, minimizing the amounts of these chemicals that remain in the environment and making hemp suitable for organic production (Callaway 2002; Small 2015; Cherney and Small 2016; Fike 2016; Aluko 2017). Additionally, hemp has a deep rooting system that aerates the soil and fertilizes it for crops planted after it, such as wheat (Bouloc and van der Werf 2013; Cherney and Small 2016). An increase in wheat yields were observed for up to two years when planted after hemp, suggesting that hemp could be used in rotation with wheat or other conventional crops (Zegada-Lizarazu and Monti 2011; Rupasinghe et al. 2020). When dried and composted in the field after harvest, hemp plants can return up to 60 – 70% of the nutrients it used back to the soil (Aluko 2017). Furthermore, some hemp products have environmental benefits. For instance, hemp-lime concrete used in building is considered carbon negative, while hemp biomass can be used as a source of energy (Cherney and Small 2016). Accordingly, hemp is considered an environmentally friendly and sustainable crop, providing benefits to the planet regardless of end use.

1.4.4 Hemp Cultivars

Hemp cultivars are bred for specific end uses – either seed, fiber, or CBD production, or dual purposes – all while consistently having a THC content below the legal limit (Matthäus and Brühl 2008; Cherney and Small 2016). For example, cultivars bred for seed production produce more flowers than fiber varieties, presenting the possibility of cultivation for seed and CBD (Cherney and Small 2016). Historically, breeding focused on fiber cultivars, where seeds were a byproduct. However, breeding efforts focused on improving seed cultivars have increased in the past two decades, resulting in over 40 seed cultivars (Cherney and Small 2016; Leonard et al. 2020). The goal of breeding has typically been to improve yields and plant traits for a more uniform

crop (Fike 2016). However, observed differences in the composition of hemp seeds suggests that potential exists to breed hemp with a focus on human food as the end use.

1.4.5 Hemp Seed Composition

Hemp seeds, technically achenes, are 2.5 – 3.5 mm in length and are the edible fruit of the hemp plant (Aluko 2017; Farinon et al. 2020). They are oval or spherically shaped with a brown-colored hull and a papery green layer called testa, surrounding a soft white seed commonly referred to as hemp heart (Crescente et al. 2018; Schultz et al. 2020). While exact values vary based on cultivars and growing conditions, whole hemp seeds contain approximately 30% oil; 25% protein; 30% total fiber, of which 10-15% is insoluble fiber; and 5% ash, which includes valuable minerals (Aluko 2017; Leonard et al. 2020; Farinon et al. 2020). The majority of the insoluble fiber is found in the seed hull, while dehulled hemp seeds contain about 1.5 times as much fat and protein by weight compared to non-dehulled seeds (House et al. 2010). The content of protein and fat varies amongst different hemp cultivars and accessions (**Table 1)**. A cultivar is a variety that can be grown for industrial use, while an accession is a collection of similar seeds gathered from a particular area, typically saved for their genetic material (Galasso et al. 2016). The nutritional properties of the oil and the structural, functional, and nutritional properties of the protein will be discussed in further detail in subsequent sections.

Table 1. Reported ranges in crude protein and oil of hemp seeds from different cultivars and accessions across different reports.

Author(s)	Publication Year	Crude Protein Range (%)	Crude Oil Range (%)
House et al.	2010	21.3 – 27.5	25.4 – 33.0
Russo and Reggiani	2015	33.1 – 35.1	Not reported
Vonapartis et al.	2015	23.8 – 28.0	26.9 – 30.6
Galasso et al.	2016	31.6 – 35.6	28.5 – 36.0
Irakli et al.	2019	12.2 – 25.4	8.5 – 29.2
Lan et al.	2019	24.3 – 28.1	32.8 – 35.9
Schultz et al.	2020	19.5 – 26.9	26.6 – 37.8

1.5 Hemp Seed Oil

Currently, hemp seeds are valued for their high edible oil content (~ 30%), which has a unique, nutritious fatty acid profile (Matthäus and Brühl 2008; Aluko 2017). Hemp seed oil is favored for non-thermal applications due to its low smoke point (Cherney and Small 2016). The extraction method affects the quality of the extracted oil and the properties of the protein-rich residual press cake or meal.

1.5.1 Composition and Health Benefits of Hemp Seed Oil

Hemp seed oil is high in unsaturated fatty acids, with polyunsaturated fatty acids (PUFAs) making up 70-80% of total fatty acids (Callaway 2004; Cherney and Small 2016; Aluko 2017). Across different hemp cultivars, linoleic acid was reported to be the predominant fatty acid, making up over 50% of the PUFAs, and α-linolenic was reported to be the second most prominent fatty acid (Matthäus and Brühl 2008; Youn et al. 2018; Lan et al. 2019). Gamma-linolenic acid, which is known for its anti-inflammatory and cardiovascular health properties, was also present in significant amounts (Youn et al. 2018; Lan et al. 2019). The ratio of linoleic (omega-6) to α-linolenic (omega-3) fatty acids ranges from 2.5:1 to 5.1:1 across different hemp cultivars (Callaway 2004; Da Porto et al. 2015; Lan et al. 2019). A high (>10:1) ratio has been associated with increased risk for cardiovascular and inflammatory diseases and cancer (Simopoulos

2011; Leonard et al. 2020). The typical omega-6 to omega-3 ratio in Western diets ranges from 15:1 to 17:1, largely contributed to by high consumption of oils high in omega-6 fatty acids such as soybean, corn, sunflower, safflower, and linseed oil (Simopoulos 2011; Leonard et al. 2020). Consumption of hemp seed oil could increase the amount of omega-3 fatty acids in the diet, helping to bring the ratio closer to the recommended 2:1 or 3:1 (Callaway 2004; Simopoulos 2011).

In addition to having a desirable fatty acid profile, hemp seed oil is higher in tocopherols than most vegetable oils, such as sunflower, sesame, corn, and linseeds oils (Leonard et al. 2020). Tocopherols are powerful antioxidants that help lower the risk for cancer, cardiovascular disease, and macular degeneration (Matthäus and Brühl 2008). While tocopherols also help combat the effects of oxidative degradation of oil, the high amount of unsaturated fatty acids in hemp seed oil creates challenges for processing and storing.

1.5.2 Oil Extraction Conditions

The predominant oil extraction methods for commercial use are mechanical pressing and solvent extraction, which can be used alone or in tandem (Kumar et al. 2000; Xu et al. 2020). The use of mechanical pressing without the use of heat is known as "cold-pressing" and is the most common oil extraction process used for hemp (House et al. 2010; Kostić et al. 2013). Cold-pressing using a hydraulic screw press can extract 60-80% of the total amount of oil in the hemp seeds (Matthäus and Brühl 2008; Aluko 2017). The low temperatures employed in mechanical pressing minimizes oil degradation, although oil yields are lower than when solvents or heat are used (Aluko 2017). Hexane is the main organic solvent used to extract oil from almost all oilseeds, including soybean and canola (Thrane et al. 2017). This method is cost-effective and can extract 95% or more of the total oil present, depending on factors such as extraction time, sample particle size, and seed to solvent ratio (Campbell et al. 2011; Kostić et al. 2013; Rezvankhah et al. 2019). However, hexane is flammable and explosive, posing a danger to plant personnel, and residual hexane must be removed as it is not safe for human consumption (Campbell et al. 2011; Cherney and Small 2016). Removal of hexane involves a desolventization step using steam with temperatures between 120 – 140°C to

evaporate residual hexane, which could be detrimental to the protein in the residual meal (White 1995; Arntfield 2011).

Other oil extraction methods currently being explored include the use of carbon dioxide (supercritical fluid), ultrasound, or microwave technology (Aiello et al. 2020; Xu et al. 2020). Supercritical carbon dioxide (CO_2) can be used instead of an organic solvent to extract the oil, leaving no solvent residue, as CO_2 leaves the system when returned to room temperature and pressure (Romano et al. 2014; Aiello et al. 2020). When applied to hemp, this oil extraction resulted in high yields similar to solvent extraction and produced a less oxidized oil compared to hexane extracted oil (Aiello et al. 2020). However, this method is has a high operating cost (Cherney and Small 2016; Aiello et al. 2020; Xu et al. 2020). Ultrasound technology has been employed to extract hemp seed oil with a similar fatty acid profile to that from solvent extraction, but yields (~ 91% of total oil extracted) still remain lower than reported for solvent extraction (Lin et al. 2012; Xu et al. 2020). Lastly, microwave technology has been studied in comparison to solvent extraction, and while extraction time is shorter, yields are lower (Rezvankhah et al. 2019; Xu et al. 2020). Therefore, mechanical pressing with solvent extraction remains the best choice for high yields, where pressing removes a large amount of oil while maintaining oil quality, and solvent extraction leaves very little residual oil (< 3%) in the meal (Boyle et al. 2018; Xu et al. 2020).

Due to the high amount of unsaturated fatty acids present, extracted hemp seed oil must be bottled quickly under nitrogen in opaque bottles and refrigerated to protect against oxidative degradation (Matthäus and Brühl 2008; Aluko 2017; Xu et al. 2020). Sometimes, antioxidants are added to extend shelf life (Aluko 2017). Hemp seed oil has a light to dark greenish color due to the amount of chlorophyll present, most likely due to the presence of seed hulls during the extraction (Callaway 2004; Matthäus and Brühl 2008; Aiello et al. 2020). Nevertheless, the unique properties of hemp seed oil make it suitable for several different applications.

1.5.3 Oil Applications

Hemp seed oil is currently a niche market, as about 90% of the world's edible oils are produced from soybean, canola, palm, and sunflower (Cherney and Small 2016).

Hemp seed oil is characterized by a fresh nutty taste and smell, and it works well as a salad oil, dipping oil for bread, or nutritional supplement (Deferne and Pate 1996; Callaway and Pate 2009; Cherney and Small 2016). Hemp seed oil is best consumed raw, as it has a relatively low smoke point of 165°C that makes it unsuitable for frying or cooking (Cherney and Small 2016). In addition to food uses, hemp seed oil is used in the cosmetics, pharmaceutical, nutraceutical, and biofuel industries (Pojić et al. 2014; Cherney and Small 2016; Farinon et al. 2020). While hemp seed oil is a valuable product in multiple industries, the remaining hemp press cake or meal after oil extraction is also gaining increased attention in the food industry for its nutritional value, specifically its high protein content.

1.5.4 Applications of Press Cake and Residual Meal

Currently, hemp seed oil is extracted from whole hemp seeds, leaving a press cake that contains both the hull and heart fractions (House et al. 2010). This press cake is mainly used for animal feed, but investigation into its use in human food applications is growing due to its attractive nutritional profile (Xu et al. 2020; Potin et al. 2021). Mechanical pressing results in a hemp press cake with a fat content between 8.4 – 15.5% and a protein content between 31.5 – 53.3%, depending on the cultivar, growing environment, and pressing conditions (Callaway 2004; House et al. 2010; Malomo et al. 2014; Teh et al. 2014; Potin et al. 2019). This press cake also contains high amounts of dietary fiber (42 – 43%), mostly due to the presence of seed hulls (Callaway 2004; Radocaj et al. 2014). The ground press cake has been investigated for use in applications such as energy bars and gluten-free crackers (Norajit et al. 2011; Radocaj et al. 2014). Further defatting of the press cake using solvent extraction results in a defatted meal, which is higher in protein (48 – 52.5%) as very low amounts of residual oil remain (1.5 – 2.8%) (Teh et al. 2014; Galves et al. 2019). The high protein content of residual hemp meal has garnered increased attention due to its potential as a novel plant protein source.

1.6 Hemp Protein

The protein content of hemp seed is similar to that of other plant protein sources, such as pea, but is slightly lower than soy (Singh et al. 2008; Boye et al. 2010a).

Accordingly, whole hemp seeds (21.3 – 27.5% protein) and dehulled hemp seeds (30.3 – 38.7% protein) contain relatively appreciable amounts of protein that can be increased through processing to produce high protein ingredients (House et al. 2010; Zając et al. 2019).

1.6.1 Hemp Protein Ingredients

Production of hemp protein ingredients is similar to that of other oilseeds, although dehulling, while done for soy, is not standard in hemp seed processing (Deak et al. 2008; House et al. 2010; Aluko 2017). In general, protein ingredients are classified based on their protein content as either flours (20 – 60%), concentrates (60 – 80%), or isolates (> 80%) (Swanson 1990; Aluko 2017; Leonard et al. 2020; Ismail et al. 2020).

Flours are produced by milling and sieving defatted press cake or meal, which for hemp results in a protein content around 38 – 44% (Malomo and Aluko 2015a; Multari et al. 2016; Zając et al. 2019). Typically, to produce a protein concentrate, aqueous alcohol, acid, or water washes are used to remove soluble components, such as sugars and soluble fiber (Singh et al. 2008; Thrane et al. 2017). Protein isolates are produced by removing solubles as well as insoluble fiber and starch if present. This is typically achieved by solubilization of the proteins at an alkaline pH, centrifugation to separate insoluble components, followed by precipitation of the proteins at acidic pH to separate it from other solubles. In some cases, this process produces a protein concentrate instead of an isolate, as conditions need to be optimized for each protein source to achieve a high protein purity (Ismail et al. 2020).

Commercially available hemp protein ingredients mainly include flours and concentrates with 70% protein or less, while commercial pea and soy protein isolates are readily available (Lee et al. 2003; Malomo and Aluko 2015a; Reinkensmeier et al. 2015). A deeper understanding of the unique properties of hemp seed protein could help in developing optimized processing conditions to produce ingredients with high protein content suited for a wide range of food applications.

1.6.2 Amino Acid Composition and Nutritional Quality

Proteins are made up of a unique sequence of amino acids, which is referred to as a protein's primary structure (Sathe et al. 2018). The amino acid composition and sequence impacts all other aspects of the protein's properties – structural, functional, and nutritional. From a nutritional perspective, proteins provide essential amino acids (histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine) that must be obtained from the diet (FAO/WHO Expert Consultation 1991; Farinon et al. 2020). Therefore, one of the main ways a protein's nutritional quality is evaluated is based on its essential amino acids content relative to the needs of people of all ages.

The amino acid composition of hemp protein compared to that of soy and pea is shown in **Table 2**. Hemp protein contains all of the essential amino acids (House et al. 2010). The amino acids present in the largest amount in hemp have been identified as glutamic acid, aspartic acid, and arginine (Callaway 2004; Wang et al. 2008; House et al. 2010; Ellison et al. 2021). While these amino acids are not considered essential, hemp protein's high arginine content has been identified as a nutritional benefit. Arginine is a precursor for nitric oxide, a vasodilator, which helps promote good blood flow and maintain normal blood pressure, suggesting that hemp protein may provide health benefits for the cardiovascular system (Wu and Meininger 2002; House et al. 2010; Shen et al. 2020a). Additionally, arginine content increases with dehulling, suggesting that this processing step may be necessary to produce a protein ingredient of higher nutritional quality (House et al. 2010; Shen et al. 2020a).

Hemp protein is also reported to be higher in the essential sulfur-containing amino acids, cysteine and methionine, than soy and pea proteins (Reinkensmeier et al. 2015). When evaluated against the nutritional recommendations, pea protein is limited in tryptophan and methionine and soy protein can be limited in methionine depending on processing (Wolf 1970; Singh et al. 2008; Hughes et al. 2011; Ma et al. 2017; Lam et al. 2018). Cysteine and methionine are of particular importance because they are abundant in animal sources of protein but are limited in many plant sources (Teh et al. 2014). Thus, hemp protein provides a unique edge as a plant protein.

Table 2. Amino acid composition (%) of hemp seed products, soy, and pea as currently reported in literature.

	House et al. 2010			Callaway 2004	Ma et al. 2017
Amino Acid	**Whole Hemp Seeds[a]**	**Dehulled Hemp Seed[a]**	**Hemp Seed Meal[a]**	**Soy[b]**	**Yellow Pea Flour[b]**
Alanine	0.96	1.52	1.61	1.39	0.87
Arginine	2.28	4.55	3.91	2.14	1.49
Aspartic acid	2.39	3.66	3.66	3.62	2.26
Cystine	0.41	0.65	0.70	0.54	0.25
Glutamic acid	3.74	6.23	6.03	5.89	3.21
Glycine	1.06	1.61	1.66	1.29	0.84
Histidine*	0.55	0.97	0.93	0.76	0.46
Isoleucine*	0.80	1.29	1.45	1.62	0.67
Leucine*	1.49	2.14	2.35	2.58	1.32
Lysine*	0.86	1.26	1.32	1.73	1.13
Methionine*	0.56	0.94	0.88	0.53	0.15
Phenylalanine*	1.03	1.43	1.62	1.78	0.86
Proline	0.90	1.62	1.59	1.65	0.29
Serine	1.19	1.70	1.73	1.54	0.99
Threonine*	1.01	1.27	1.35	1.35	0.72
Tryptophan*	0.23	0.38	0.39	0.41	--^
Tyrosine	0.68	1.28	1.15	1.14	0.45
Valine*	1.14	1.78	1.91	1.60	0.79

[a]Values reported on % as is basis
[b]Values reported in g per 100 g
*Denotes essential amino acid
^Value not reported

While hemp protein has an attractive amino acid profile, it has been widely reported to be deficient in the essential amino acid lysine (Wang et al. 2008; House et al. 2010). In contrast, soy protein is rich in lysine, and pea protein provides lysine in sufficient quantities to meet nutritional needs (Reinkensmeier et al. 2015). Processing of

hemp protein should focus on preservation of lysine to ensure that this amino acid is present in the highest amount possible.

In addition to amino acid composition, protein digestibility is the other important factor contributing to the overall protein nutritional quality. If the protein is not fully digested, essential amino acids may not be completely bioaccessible. The most common method for measuring protein digestibility is using *in vivo* studies involving rat bioassays. The official AOAC method 991.29 outlines the *in vivo* assay. The assay involves feeding rats a standardized diet containing a set amount of the test protein. A standardized diet containing a set amount of casein is commonly used as a reference (Nosworthy et al. 2017). Nitrogen intake and fecal nitrogen losses of the rats fed the protein-containing diets are compared with fecal nitrogen losses in rats fed a non-protein diet to calculate true protein digestibility (**Eq. 1**) (AOAC 2006).

$$\text{True digestibility (\%)} = \frac{\text{N intake - (fecal N - fecal metabolic N loss)}}{\text{N intake}} \times 100 \qquad \textbf{Eq. 1}$$

Other methods of analyzing protein digestibility are being investigated such as the digestible indispensable amino acid score (DIAAS) method and *in vitro* methods. The DIAAS method evaluates bioaccessibility of individual amino acids as determined at the end of small intestine, preferably in humans (FAO/WHO Expert Consultation 2013). The lowest score of all the amino acids is reported as the DIAAS, which is calculated using a slightly different reference amino acid pattern than used for PDCAAS (FAO/WHO Expert Consultation 2013; Nosworthy et al. 2017). However, DIAAS is not currently an official method in the United States. *In vitro* methods, on the other hand, are more desirable than *in vivo* methods due to the high cost and the ethical concerns of using live animals for analyses carried out frequently to establish food protein claims.

There are two types of *in vitro* methods – static and dynamic. Static methods can be performed in the lab, and they are relatively quick and inexpensive to perform, while yielding comparable results to *in vivo* methods (Wiggins et al. 2019). One such method is the static enzymatic pH drop method. This method uses a cocktail of three enzymes – chymotrypsin, trypsin, and protease or peptidase – to digest the protein sample while the

drop in pH from the initial pH 8.0 is monitored over 10 minutes (Hsu et al. 1977; Tinus et al. 2012). The pH after 10 minutes of digestion with these enzymes is highly correlated with *in vivo* digestibility (Hsu et al. 1977). The regression equation (**Eq. 2**) to determine *in vitro* digestibility was simplified by Tinus et al. (2012). Dynamic methods attempt to replicate the digestion conditions of the human gastrointestinal tract (Wiggins et al. 2019). For example, the *in vitro* dynamic TNO gastrointestinal model (TIM-1) is a complex system with four sections, representing the stomach, duodenum, jejunum, and ileum, connected via peristaltic valve pumps (Minekus 2015; Verwei et al. 2016). TinyTIM is a simplified version with a gastric chamber and only one section representing the intestine. Both methods require sophisticated technology to control conditions (e.g., pH, temperature, flow time, secretion of electrolytes, bile, and digestive enzymes), making them much more limited in applicability (Minekus 2015; Wiggins et al. 2019).

$$\textit{In vitro}\text{ protein digestibility} = 65.66 + (18.10 \times \Delta pH_{10\ min}) \qquad \textbf{Eq. 2}$$

Protein digestibility is used to calculate the protein digestibility-corrected amino acid score (PDCAAS), which is commonly used to calculate % daily value and establish protein content claims for nutrition labels (Wiggins et al. 2019). PDCAAS is determined using the protein's amino acid score (AAS), which is calculated using the first limiting amino acid, and the percent protein digestibility (**Eq. 3**) (Smith 2017). PDCAAS is reported on a scale 0-1.0, with a score less than 1.0 indicating that there is one or more amino acids that are not present in the amount required for human nutrition and/or that the protein is not fully digestible.

$$\text{PDCAAS} = \text{AAS of first limiting amino acid} \times \text{protein digestibility} \qquad \textbf{Eq. 3}$$

The PDCAAS of hemp protein (0.48 – 0.61) has been reported to be lower than that of soy and pea proteins (0.92 – 1.0 and 0.69 – 0.89, respectively); however, it is generally higher than that of wheat protein (0.25 – 0.51) (House et al. 2010; Ismail et al. 2020). Hemp protein digestibility is relatively high (~ 95% for dehulled hemp seeds),

suggesting that an improvement in hemp protein's lysine content could be a strategy for improving the PDCAAS.

1.6.3 Structure and Profile

Different sources of food proteins are made up of heterogeneous mixtures of proteins that vary in structural characteristics (molecular configuration, surface properties, and size), and in relative abundance (i.e. profile) (Ismail et al. 2020). Besides being important for nutritional considerations, a protein's primary structure (amino acid composition and sequence) also dictates its molecular configuration, size, and surface properties, and thus its behavior in a food system. Amino acids have side chains that are either 1) hydrophobic, 2) hydrophilic and uncharged, or 3) charged (basic or acidic) (Sathe et al. 2018). These side chains dictate how the protein will interact with itself and its environment, influencing molecular configuration and consequently functionality.

Molecular configuration refers to the folding of the primary structure into secondary, tertiary, and potentially quaternary structures. Protein secondary structure refers to how amino acids sequences fold at different parts of the polypeptide chain (Damodaran 2017). Certain sequences of amino acids produce either helical structures, such as α-helices; sheet-like structures, such as β-sheets and β-turns; or random structures, which lack a defined order. The secondary structure of a protein influences its thermal stability and can change during processing when the proteins are subjected to conditions that cause them to denature or unfold. Proteins fold further into 3-D structures, known as their tertiary structure. This folding is driven and stabilized by reaching an orientation where the total free energy value of the protein is the lowest possible. This folding often results in orienting most of the hydrophobic groups to the interior moiety of the protein, and most of the hydrophilic groups to the surface where they can interact with water. For plant proteins, this typically results in a globular structure. However, proteins with high amounts of hydrophobic amino acids (> 30%) will still have a relatively high percentage of hydrophobic residues on the surface. Hydrophobic interactions between different monomers will result in quaternary structures involving three or more monomers. Such molecular configurations results in rigid structures of relatively high molecular weights, imparting unique functional properties, desired for

some food applications, and not desired in others. Such molecular characteristics vary among different plant proteins.

Hemp protein is comprised of 65 – 85% globulins (also referred to as legumins), which are a type of salt-soluble globular proteins found in leguminous plants (Patel et al. 1994; Malomo and Aluko 2015b; Aluko 2017; Wang and Xiong 2019; Potin et al. 2021). The remaining hemp proteins are water-soluble albumins, which have a less compact structure and are more flexible than globulins (Patel et al. 1994; Malomo and Aluko 2015b; Aluko 2017; Wang and Xiong 2019; Xu et al. 2020). These proteins can be further classified by their sedimentation coefficient (S), where higher coefficients are correlated with quaternary protein structures of a relatively larger molecular weight (Boye et al. 2010a; Ponzoni et al. 2018).

The most abundant hemp globulins are the 11S legumin, known as edestin (300 – 340 kDa) and the 7S vicilin-like globulin (~ 150 kDa) (Aluko 2017; Sun et al. 2021). Edestin is made up of six monomers, linked together via hydrophobic, electrostatic, and hydrogen bonding. Each monomer consists of an acidic (33 kDa) and basic subunit (20 kDa) linked by a disulfide bond. The 7S vicilin-like globulin is made up of three subunits (47 – 48 kDa) linked together mostly via hydrophobic interactions, and does not contain any disulfide bonds (Tang et al. 2006; Mamone et al. 2019; Sun et al. 2021). Hemp albumins (< 20 kDa, 2S) are dimers linked by a disulfide bond (Mamone et al. 2019). In contrast to globulin proteins, albumins are non-functional proteins constituting mostly enzymes and enzyme inhibitors (Boye et al. 2010a). A summary of the major proteins in hemp seeds are presented in **Table 3,** and models of each protein are shown in **Figure 1**.

At neutral pH, hemp albumins have a higher abundance of α-helices and lower abundance of β-sheets than hemp globulins (Malomo and Aluko 2015b). Typically, β-sheets are more stable than α-helixes, indicating higher thermal stability of hemp globulins than albumins (Malomo and Aluko 2015b; Damodaran 2017). Additionally, hemp albumins have a higher relative abundance of unordered structure and less compact structure than globulins, which, along with their relatively small molecular weight, contribute to higher solubility (Malomo and Aluko 2015b; Sathe et al. 2018).

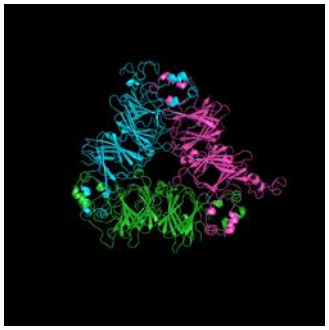
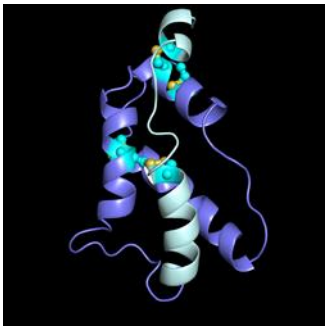
a)  b)  c) 

Figure 1. Models of major proteins found in hemp seeds - a) 11S edestin, b) 7S vicilin-like protein, c) 2S albumin (Picture credit: Fan Bu)

Table 3. Major proteins present in hemp seeds.

Protein Fraction	% of Total Protein	Protein	Dissolves in...	Structure
Globulins	60 – 85	11S edestin	Salt solution	Hexamer with acidic and basic subunits
	~ 5	7S vicilin-like protein		Trimer
Albumins	13 – 37	2S albumin	Water	Two polypeptide chains linked by a disulfide bond

Data Sources: (Tang et al. 2006; Aluko 2017; Wang and Xiong 2019; Shen et al. 2020a; Potin et al. 2021; Sun et al. 2021)

The hemp protein profile bears some similarity to soy and pea proteins. All three contain a hexameric legumin. In soy protein, legumin is specifically known as glycinin (11S), whereas in pea protein, it is known only as legumin (11S). Soy and pea proteins also contain 7S vicilin (in soy is known as β-conglycinin) and 2S albumins. Soy protein contains an additional globulin with a sedimentation coefficient of 15S, and pea protein contains an additional 8S globulin (known as convicilin) (Kinsella 1979; Tang et al. 2006; Barac et al. 2010; Singh et al. 2015; Sun et al. 2021). A key difference of hemp protein when compared with soy and pea protein is the ratio of 11S to 7S globulins and the relatively higher content of the non-functional 2S albumins. Hemp globulins are mostly 11S proteins with a low 7S content (11S to 7S ratio of 12 – 16) (Sun et al. 2021). Soy protein, on the other hand, contains 11S and 7S proteins in a more balanced proportion (11S to 7S ratio of 1.5 – 2.0) (Pesic et al. 2005), and pea protein typically contains a higher proportion of 7S to 11S (7S to 11S ratio of 1.6 – 4.8) (Tzitzikas et al.

2006). The ratio of 11S to 7S proteins has been extensively studied in soy and pea, as the difference in amino acid sequence, size, configuration, and surface properties of these two protein classes greatly impact functionality (Lam et al. 2018). 7S globulins have better emulsifying properties than 11S globulins, which in contrast form stronger heat-induced gels (Kinsella 1979; Lam et al. 2018). The large imbalance in the 11S to 7S ratio for hemp, and relatively high content of 2S albumins suggest that there will be differences in hemp protein functional properties compared to soy and pea proteins. Limited research has been done on the functional properties of hemp protein profile as it relates to its protein profile.

While protein profile (type and proportion of globulins and albumins) is innate to the plant source and variety (Tzitzikas et al. 2006; Sathe et al. 2018), protein structural characteristics are impacted by environmental conditions such as pH, heat, ionic strength, or the presence of organic solvents (Damodaran 2017). Under certain conditions the interactions within the protein can be disrupted leading to unfolding, i.e., denaturation. Denaturation can either improve or reduce functionality and nutritional quality. Protein denaturation temperature and enthalpy of denaturation are important indices in understanding a protein's stability and environment driven changes in structural characteristics, which in turn impact functional and nutritional attributes.

The denaturation temperature of hemp protein, specifically edestin, ranges between 92 - 95°C (Tang et al. 2006; Wang et al. 2008; Shen et al. 2020b). This range is close to that reported for 11S proteins found in soy and pea (Lee et al. 2003; Mo et al. 2006; Sun and Arntfield 2010; Withana-Gamage et al. 2011). No thermal transition has been reported for 7S vicilin-like hemp globulin, mostly likely due to either its inevitable denaturation during processing, or to the low abundance of this protein in hemp, preventing the detection of its denaturation indices (Wang et al. 2008). On the other hand, the energy released as the protein denatures, referred to as enthalpy of denaturation, is an indication of the extent of the proteins ordered structure. The more ordered the structure is, the higher is its enthalpy of denaturation (Tang et al. 2006). If the protein is partially denatured due to certain processing/environmental conditions, its measured enthalpy of denaturation will be reduced compared to its native form (Shen et al. 2021). The measured enthalpy of denaturation for hemp proteins ranged from 5.5 – 21.7 J/g,

depending on processing/environmental conditions (Yin et al. 2008; Hadnađev et al. 2018; Shen et al. 2020a, 2021).

Surface charge and surface hydrophobicity are two structural properties that play an important role in determining how protein molecules interact with each other and with water. Generally, if a protein exhibits a surface charge between +30 mV and -30 mV, at a specific pH, the protein will not be very soluble at that pH because the charge is insufficient for intermolecular repulsion (Long and Labute 2010). The surface charge of hemp protein falls in this range when measured between pH 3 – 7, resulting in poor solubility (Tang et al. 2006; Malomo et al. 2014; Hadnađev et al. 2018; Galves et al. 2019; Shen et al. 2020b). In comparison, the surface charge of soy and pea protein at pH 7 is approximately -40 to -45 mV (Ladjal-Ettoumi et al. 2016; Kaushik et al. 2016). At neutral pH, soy protein has excellent solubility (>80%), while pea protein has acceptable solubility (>60%), when not denatured (Fernández-Quintela et al. 1997; Tang et al. 2006). The lower solubility of pea protein compared to soy protein at neutral pH could be due to the differences in surface hydrophobicity. Hemp protein has higher surface hydrophobicity than pea protein, which in turn has higher surface hydrophobicity than soy (Tang et al. 2009; Karaca et al. 2011). While high surface hydrophobicity can drastically decrease protein solubility, some extent of surface hydrophobicity is needed to form emulsions and foams, as hydrophobic residues are needed to form a stable film around oil droplets or air bubbles (Lam et al. 2018). Additionally, protein intermolecular interactions facilitated by hydrophobic interactions are needed to form strong and elastic gels in applications such as meat or meat alternatives. More work is needed to characterize the surface properties of hemp protein to better understand its functionality compared to soy and pea protein.

1.6.4 Functionality

Protein profile and structural characteristics dictate how the protein molecules will interact with each other and with other components in a food system. Protein ingredients play specific functional roles in food applications, thus successful adaptation of new plant protein ingredients requires a thorough understanding of their unique functionality (Ismail et al. 2020). Desired functional attributes vary across different

products. For example, important functional characteristics to produce plant-based meat, dairy, and egg alternatives with desirable textural attributes include solubility, water-binding capacity, gelation, emulsification, and foaming (Singhal et al. 2016). These main functional attributes can be categorized as those involving protein-water interactions (solubility, water-binding capacity, and water-holding capacity), protein-protein interactions (gelation and texturization), and surface activity (emulsification and foaming).

1.6.4.1 Solubility, Water Binding Capacity, and Water Holding Capacity

The ability of a protein to interact with water to facilitate solubilization is arguably the most important functional attribute, since other functional attributes, such as emulsification and gelation, often require the protein to be dissolved in water (Gao et al. 2020; Shen et al. 2021). Protein solubility depends on intrinsic factors, such as molecular size and native surface charge and hydrophobicity, as well as extrinsic factors including processing/environmental conditions such as pH, temperature, and ionic strength (Karaca et al. 2011; Gao et al. 2020). When examining solubility over different pH, plant proteins display a u-shaped curve with moderate solubility at acidic pH that decreases as the pH approaches the isoelectric point before reaching its highest solubility in the alkaline pH range (Tang et al. 2006; Lam et al. 2018). Protein solubility is low close to the isoelectric point, where net charge is zero causing the proteins to aggregate, mainly via hydrophobic interactions, due to reduced intermolecular repulsion. At pH values lower or higher than the isoelectric point, the proteins regain a net positive or negative charge, respectively, inducing electrostatic repulsion and better protein/water interactions (Galves et al. 2019).

Both hemp and pea proteins exhibit the lowest protein solubility at pH 4 – 6, whereas soy protein exhibited its lowest solubility at pH 4 – 5 (Wolf 1970; Tang et al. 2006; Boye et al. 2010b; Lam et al. 2018). Food products produced commercially are either at neutral (pH 7) or acidic pH (< pH 5, specifically < pH 3.5 for ready-to-drink beverages). Therefore, it is important for proteins to display good solubility in at least one of these pH ranges (Paulsen 2009; Gao et al. 2020; Liu et al. 2021). Generally, hemp protein exhibits low solubility at neutral pH, although reported solubility varies depending on protein extraction and processing conditions (Malomo et al. 2014; Galves

et al. 2019; Shen et al. 2020a). Hemp protein demonstrated inferior solubility compared to both soy and pea protein at either acidic or neutral pH (Tang et al. 2006; Karaca et al. 2011; Hadnađev et al. 2018). Characterization and improvement of hemp protein solubility should be prioritized in future research.

While sometimes used interchangeably, water binding and water holding capacity describe two different ways that proteins interact with water. Water binding is the ability of a protein to chemically bind with water, whereas water holding capacity is the ability of a protein matrix (e.g., a gel network) to physically entrap and retain water (Damodaran 2017). Water binding capacity is dependent not only on environmental conditions, but also on the amino acid composition, as presence of charged residues will increase a protein's ability to bind water. At neutral pH hemp protein demonstrates inferior water binding capacity compared to soy protein and pea protein, likely related to its low surface charge (Tang et al. 2006; Galves et al. 2019). Water binding capacity is especially important in low- to moderate-moisture food applications as a loss of water could lead to sticky, dry, or brittle product (Singhal et al. 2016). In contrast, water holding capacity imparts juiciness and tenderness in meats, processed meats, and fish. Trapping water within a protein matrix improves mouthfeel, maintains freshness, and minimizes undesirable moisture loss (syneresis) in a food product (Wang and Xiong 2019; Shen et al. 2021). There is limited information in literature on the water holding capacity of hemp protein.

1.6.4.2 Gelation and Texturization

Protein-protein interactions are important for applications that involve gelation and texturization. The ability of proteins to interact with each other to produce a 3D network (e.g., a gel), entrapping water, is another desirable functional attribute for food applications such as meats and meat alternatives (Damodaran 2017). Food gels are most commonly thermally induced by heating above the protein's denaturation temperature, allowing proteins to unfold and interact with each other to form a network. Highly hydrophobic proteins will form opaque, coagulum-type gels, because they tend to aggregate through hydrophobic interactions when heated, while proteins with fewer hydrophobic groups form translucent gels. Gel networks stabilized primarily by

noncovalent bonds (mostly hydrogen bonding) can be thermally reversible, but gel networks stabilized primarily by hydrophobic interactions and disulfide linkages are mostly thermally irreversible. Other methods of forming a gel include enzymatic cross-linking (e.g., transglutaminase induced cross-linking), pH reduction to the isoelectric point of a denatured protein, or by the addition of divalent ions (Oztop et al. 2012; Schuldt et al. 2014; Damodaran 2017).

Environmental factors can affect the formation of a gel and the strength of the resulting gels. Such factors include the pH of the system, protein concentration, and the presence of salts or cations such as Ca^{2+} (Damodaran 2017). The minimum protein concentration (i.e., least gelation concentration) needed to produce a stable gel is relevant from an economical perspective. The least gelation concentration (LGC) of hemp protein was reported to be in the range of 12 – 22% depending on protein extraction and processing conditions (Malomo et al. 2014). Soy protein, on the other hand, has been reported to form a gel at concentrations as low as 12% (Boyle et al. 2018). Another measure of gelation is gel strength. Gel strength can be measured by producing heat-induced gels and measuring the force in Newtons needed to rupture the gel (Boyle et al. 2018). Overall, pea protein isolates have been reported to form less elastic and weaker gels than soy protein (Lam et al. 2018). Differences in the strength of gels formed by hemp proteins extracted under different conditions have been reported (Dapčević-Hadnađev et al. 2018). However, literature on gelation properties of hemp protein as impacted by various environmental and processing conditions is scarce.

Texturization is another protein property of particular interest for the use of plant protein in meat alternative applications (Zhang et al. 2019). Specifically, texturization of plant proteins using extrusion technology produces fibers that mimic meat fibers through heat, shear, pressure, and cooling (Zhang et al. 2019; Zahari et al. 2020). In this process, globular proteins unfold, associate, aggregate, and cross-link (Zhang et al. 2019). Disulfide linkages, hydrophobic interactions, and hydrogen bonding are instrumental in forming these protein fibers. High moisture conditions are often used to create meat analogs with unique physical properties including chewiness, elasticity, softness, and juiciness (Damodaran 2017). Limited research has been done on extrusion of hemp proteins; however, partial replacement of soy protein concentrate with hemp protein

concentrate have been shown to produce a high moisture meat analog with desirable physical qualities (Zahari et al. 2020).

1.6.4.3 Emulsification and Foaming

Proteins exhibit surface properties that allow them to interact with oil to create emulsions or with air to form foams. In food systems, emulsions (oil-in-water or water-in-oil) and air-in-water foams are common (Lam et al. 2018). Emulsions and foams are created following physical denaturation (e.g. shear, whipping), which causes the protein to adsorb to the oil-water or air-water interface to form a film around the oil droplets or air bubble (Amagliani and Schmitt 2017; Lam et al. 2018). In both cases, proteins must 1) be able to easily move to the oil-water or air-water interface, 2) be flexible enough to orient their hydrophobic residues towards the oil or air and their hydrophilic residues towards the water, and 3) be able to form a cohesive film around the oil droplet or air bubble via stable intermolecular protein interactions (Shen et al. 2021). Additionally, droplet/bubble size, net charge on the surface of the droplet or bubble, and viscosity of the continuous phase are key parameters for the stability of the emulsion or foam. Therefore, it is desired to have a balance between surface hydrophobicity and surface hydrophilicity to produce stable emulsions or foams. Protein surface properties, solubility, and concentration, as well as extrinsic factors including pH, ionic strength, and temperature impact emulsification and foaming properties (both capacity and stability) (Damodaran 2017). While emulsions and foams are influenced by similar factors, proteins that are good emulsifiers may not necessarily have good foaming properties, and vice versa.

When evaluating emulsions, emulsification capacity, stability, and activity are commonly measured. Emulsification capacity (EC) measures the amount of oil a gram of protein can emulsify, whereas emulsification stability (ES) evaluates how resistant the emulsion is to phase separation over a certain period of time (Lam et al. 2018). Emulsification activity index (EAI) estimates the amount of interfacial area that a gram of protein can emulsify. Soy protein has higher EC, ES, and EAI than pea protein (Karaca et al. 2011), while hemp protein has been reported to have an inferior EAI but a similar, if not slightly higher, ES than pea protein (Tang et al. 2006; Karaca et al. 2011). Reported

information on EC of hemp protein is scarce. The poor hemp protein emulsification properties have been attributed to its low solubility (Teh et al. 2014).

Foaming properties are valuable in products such as baked products and egg alternatives (Singhal et al. 2016). Foaming properties are commonly characterized by foaming capacity (FC), which is the volume of foam produced per gram of protein, and foaming stability (FS), which is the ability of the foam to hold its structure against gravitational forces over time (Lam et al. 2018). Protein solubility is an important precursor to FC, with the lowest FC being at a protein's isoelectric point (Shen et al. 2021). The importance of solubility is also has been illustrated by the finding that hemp albumins have higher solubility and thus higher FC than hemp globulins between pH 3 – 9 (Malomo and Aluko 2015b). The small, flexible open structure of albumins can easily reorient themselves to the air-water interface and encapsulate air compared to the larger inflexible globulins. However, globular proteins with more hydrophobic groups on the surface tend to produce stable foams due to the formation of relatively thick films around the air bubbles (Malomo and Aluko 2015b).

1.6.5 Potential Applications

Studies examining the use of hemp protein in food products have mainly focused on hemp seeds or hemp flour, while only a few applications have been explored for defatted hemp and hemp protein concentrate. Hemp flour and hemp seeds have been studied in gluten-free applications as they contain no starch and more fiber than other common gluten-free flours, such as corn (Leonard et al. 2020). Conflicting results exist as to the effects on bread loaf volume, hardness, and sensory properties. Using hemp flour to partially replace other flours in gluten-free crackers or breads increased protein and fiber content, making it more valuable to consumers looking to increase consumption of these nutrients (Radocaj et al. 2014; Korus et al. 2017). Other applications that have been explored are plant-based yogurts, liver pate, pork loaves, and energy bars (Leonard et al. 2020). Higher hardness was noted for meat products, but lower syneresis was noted for yogurt made with hemp compared with yogurt made with other plant sources (soy, wheat, and pumpkin seed protein). Inclusion or substitution using moderate amounts of hemp (10 – 20% of either substituted ingredient or total formula) appeared to have acceptable

effects on sensory properties. However, exploring HPI's potential applicability in foods remains an open research space. Further characterization of the structural and functional properties of hemp protein is needed to discover more potential food applications. Additionally, characterization of HPI produced following controlled and industry-feasible protein extraction methods is needed to facilitate hemp protein's adoption in the food industry as a novel and functional plant protein.

1.7 Extraction Methodology for the Production of HPI

Typically, the goal of protein extraction is to produce a protein isolate with high yield and purity and with desirable nutritional and functional attributes for food applications. The conditions used in protein extraction can affect protein structure, which dictates functionality and nutrition. The most common way plant protein isolates are produced is through alkaline solubilization with isoelectric precipitation. Other extraction methodologies have also been reported but have not been seen in industrial application.

1.7.1 Alkaline Solubilization with Isoelectric Precipitation

Alkaline solubilization coupled with isoelectric precipitation approach for protein isolation, commonly referred to as pH extraction, is based on manipulations in pH, along with the application of centrifugal force, to selectively separate proteins from dietary fiber, hydrocolloids, gums, other carbohydrates, as well as other constituents to produce a protein isolate with high purity (Paulsen 2009). Specifically, defatted meal, often milled into flour is dispersed in water, typically in a ratio between 1:10 and 1:20 (w/v) meal to water (Thrane et al. 2017). The pH is then adjusted to an alkaline pH suitable to maximize solubilization of a particular plant protein. Centrifugation follows to remove insoluble components, which mainly consist of dietary fiber and/or starch (Thrane et al. 2017). The pH of the soluble fraction is then adjusted to the proteins' isoelectric point, causing the proteins to precipitate out of solution, while solubles, including soluble dietary fiber and small molecular weight carbohydrates, stay in solution and are further separated through centrifugation. Redispersion in water along with neutralization of the separated protein fraction follows. Additional washes with water and diafiltration may be employed to further purify the protein fraction and remove most of the residual small

molecular weight components such as salts and sugars (Karaca et al. 2011). The resultant protein fraction is homogenized, pasteurized, and spray dried to produce a protein isolate in a powder form (Deak et al. 2008; Akharume et al. 2021).

While pH extraction is applied widely to different plant proteins, extraction conditions (e.g., solubilization pH, precipitation pH, temperature, centrifugation speed, diafiltration, and drying conditions) vary during the production of different plant protein isolates. These extraction conditions must be optimized for each protein source to ensure maximal structural integrity and functionality. Different pH extraction conditions have been reported for the production of HPI (**Table 4**). Typically, solubilization is carried out at pH 10 and precipitation at pH 5. Few studies have explored how different solubilization and precipitation pHs may affect the protein yield, structural, and functional characteristics of HPI. Recent work by Potin et al. (2019, 2021) showed a higher solubilization of hemp protein at pH 11 and 12 than pH 10. However, the authors did not use protein purification to produce a protein isolate that could be characterized and compared with other pH-extracted HPIs. Another recent work by Shen et al. (2020a, b) produced an HPI with higher protein purity and yield when solubilized at pH 10 and precipitated at pH 6 instead of pH 5. Given these inconclusive reports, more in depth research is needed to establish the effect of different solubilization and precipitation pHs on hemp protein purity and yield, and the resultant protein structural and functional characteristics.

While not thoroughly optimized, pH extraction has been successful in producing HPIs with high purity (>80%) and in some cases acceptable yield (~50-70%) (Tang et al. 2006; Wang et al. 2008; Yin et al. 2008; Lu et al. 2010; Malomo et al. 2014; Teh et al. 2014; Ren et al. 2016; Orio et al. 2017; Hadnađev et al. 2018; Wang et al. 2018; Mamone et al. 2019; Shen et al. 2020a, b). However, the reported investigation of different pH extraction conditions often lacked comprehensive information on the resultant protein structural and functional properties. Shen et al. (2020a, b) did highlight some differences in HPI produced by solubilization at pH 10 and precipitated at pH 6 instead of 5. The authors reported differences in thermal stability, which was attributed to differences in the proportion of edestin in each isolate and the consequent proportion of β-sheets. However, the authors found similar trends in solubility and surface charge between the

two isolates. Future research should further explore differences in structural and functional properties of HPIs as impacted by different solubilization and precipitation pHs.

Information is limited regarding structural characteristics such as surface hydrophobicity and secondary structure, which help in understanding changes in protein conformation as induced by different extraction conditions. Additionally, there is only scarce information related to functional properties, namely gelling properties and water holding capacity, as impacted by pH extraction conditions. With the growing interest in plant-based food products that rely on key protein functional properties, comprehensive evaluation of various properties as impacted by extraction conditions is warranted.

One drawback of pH extraction is the use of harsh alkaline conditions. High alkalinity can increase intramolecular electrostatic repulsions that cause the protein to swell and unfold, leading to partial or full denaturation, depending the length of time and pH used, thus exposing hydrophobic residues, which in turn lead to hydrophobicity driven polymerization (Damodaran 2017). Such polymerization is detrimental to the functionality of the protein. An alternative protein extraction method that avoids the use of harsh conditions is salt-assisted extraction (Hadnađev et al. 2018).

Table 4. Reported conditions used during hemp protein extraction following alkaline solubilization coupled with isoelectric precipitation.

			Solubilization				Precipitation	Neutralization		
Author(s) & Year	**Starting Material**	**Meal to Water Ratio (w/v)**	**pH**	**Time (hr)**	**Temp (°C)**	**Centrifugation**	**pH**	**pH**	**Conversion Factor**	**Dialysis**
(Tang et al. 2006)	Dehulled hemp seeds disintegrated and defatted with supercritical liquid	1:20	10	1+	35	30 min at 8,000 x g at 20°C[a] 10 min at 8,000 x g[b]	5	6.8	6.25	--^
(Yin et al. 2008)	Dehulled hemp seeds disintegrated and defatted with supercritical liquid	1:20	10	2	37	30 min at 8,000 x g at 20°C[a] 20 min at 5,000 x g at 20°C[b]	5	7	6.25	--^
(Wang et al. 2008)	Hempseed flour from company that was dehulled, disintegrated, and defatted with supercritical liquid	1:6.67	10	1	--^	30 min at 8,000 x g[a] 25 min at 6,500 x g[b]	5	7	6.25	Yes
(Lu et al. 2010)	Defatted hempseed meal from local company	1:20*	9	1.167	40	30 min at 8,000 x g at 25°C[a] 15 min at 5,000 x g at 25°C[b]	5	7	6.25	Yes
(Girgih et al. 2014)	Hempseed protein powder (50% protein) from Manitoba Harvest	1:20	10	2	37	60 min at 7,000 x g at 4°C[a] 40 min at 7,000 x g at 4°C[b]	5	7	--[c]	--^

[a] Centrifugation settings used after solubilization / [b] Centrifugation settings used after precipitation

[c] Protein determined using Lowry method

* Solubilized in 1M NaOH instead of water

^ Information not reported

Table 4 (continued).

			Solubilization				Precipitation	Neutralization		
Author(s) & Year	**Starting Material**	**Meal to Water Ratio (w/v)**	**pH**	**Time (hr)**	**Temp (°C)**	**Centrifugation**	**pH**	**pH**	**Conversion Factor**	**Dialysis**
(Malomo et al. 2014)	Defatted hemp protein meal from Manitoba Harvest	1:20	10	2	37	60 min at 7,000 x g at 4°C[a] 60 min at 7,000 x g at 4°C[b]	5	7	--[c]	--^
(Teh et al. 2014)	Hemp seed press-cake defatted through cold-pressing, Soxhlet extraction, and hexane	1:10	10[d]	1	--^	30 min at 8,000 x g[a,b]	5	7.4	6.25	--^
(Malomo and Aluko 2015a)	Hemp seed press-cake defatted by cold-pressing, then milled and sifted	1:20	8	2	37	60 min at 7,000 x g at 4°C[a] 60 min at 7,000 x g at 4°C[b]	5	7	--[c]	--^
(Raikos et al. 2015)	Press-cake of cold-pressed hemp seeds	1:20	10	2	25	30 min at 4,000 x g[a,b]	5	7	6.25	--^
(Ren et al. 2016)	Defatted hemp meal from company dried to moisture <2%	1:20	8.5	1	37	15 min at 8,000 x g at room temp[a,b]	4.5	7	--^	Yes
(Orio et al. 2017)	Ground hemp seeds defatted with hexane	1:7.5	10	1.5	Room temp	30 min at 8,000 x g at room temp[a,b]	5	--^	6.25	--^

[a] Centrifugation settings used after solubilization / [b] Centriguation settings used after precipitation
[c] Protein determined using Lowry method
[d] Solubilized twice at alkaline pH
[e] Stirred overnight at acidic pH at 4°C
^ Information not reported

Table 4 (continued).

			Solubilization				Precipitation	Neutralization		
Author(s) & Year	**Starting Material**	**Meal to Water Ratio (w/v)**	**pH**	**Time (hr)**	**Temp (°C)**	**Centrifugation**	**pH**	**pH**	**Conversion Factor**	**Dialysis**
(Hadnađev et al. 2018)	Hemp pellets defatted by cold-pressing and hexane	1:20	10	2	35	20 min at 6,000 x g 20 min at 7,500 x g	5[e]	7	5.7	Washed
(Wang et al. 2018)	Milled hemp seeds defatted with hexane	1:15	10	2	Room temp	20 min at 10,000 x g at 4°C[a] 15 min at 8,000 x g at 4°C[b]	4.5	7	6.25	--^
(Mamone et al. 2019)	Dehulled hempseed meal defatted at local oil extraction plant	1:20	10	4	35	30 min at 8,000 x g at 20°C[a] 10 min at 8,000 x g[b]	5	6.8	6.25	--^
(Shen et al. 2020a)	Dehulled and defatted hemp seeds	1:15	10	2	--^	20 min at 6,000 x g[a] 10 min at 6,000 x g[b]	5	7	6.25	--^
(Shen et al. 2020b)	Dehulled and defatted hemp seeds	1:15	10	2	--^	20 min at 6,000 x g[a] 10 min at 6,000 x g[b]	6	7	6.25	--^

[a] Centrifugation settings used after solubilization / [b] Centriguation settings used after precipitation

^ Information not reported

1.7.2 Salt-Assisted Extraction

Ammonium sulfate, sodium chloride, or potassium chloride can be used in salt-assisted protein extractions. This method is based off the principle of "salting-in" and "salting-out" proteins. At dilute concentrations of salt, commonly sodium chloride, the salt binds loosely to the proteins and increases the charge load, thus increasing protein solubility. This method works particularly well for protein sources with a large proportion of globulins, like hemp, as globulins are soluble in dilute salt solutions (Aluko 2017; Hadnađev et al. 2018). After solubilization, centrifugation is used to separate the protein-rich supernatant from the insoluble components (Sun and Arntfield 2010). Concentration of the protein fraction is commonly done by increasing the concentration of salt, commonly ammonium sulfate, resulting in the shielding of charges on the protein surface and consequently loss of solubility of the protein, which is then separated from the solubles by centrifugation. This purification process requires excessive amounts of water to remove the excess salt, creating a large waste stream. Accordingly, this process is not adopted industrially due to high water usage, low efficiency, high cost, and excessive waste stream. Conversely, membrane filtration, namely ultrafiltration coupled with diafiltration can be used to concentrate the proteins prior to drying (Boye et al. 2010a). While expensive, this process can be industrially feasible. **Table 5** summarizes the salt extraction conditions reported for hemp protein.

While salt-assisted protein extraction can produce an HPI with higher protein purity than pH extraction, it has not been as extensively studied (Hadnađev et al. 2018). However, the drawback of the salt-assisted extraction is that it has lower protein yields compared to pH extraction (Arntfield 2011; Hadnađev et al. 2018). Nevertheless, salt-extracted HPI demonstrated a relatively native structure compared to pH extracted HPI, demonstrated by more β-sheets and α-helices than random coil, and a higher enthalpy of denaturation (Tang et al. 2006; Hadnađev et al. 2018). Accordingly, salt-extracted HPI was more soluble than pH-extracted HPI between pH 3 – 5 and at pH 10, but less soluble between pH 6 – 9 (Hadnađev et al. 2018). While both HPIs had similar amino acid profiles, salt-extracted HPI had significantly more of the essential amino acids isoleucine and valine than pH-extracted HPI.

Table 5. Reported conditions used for salt-assisted extraction of hemp protein.

			Solubilization					
Author(s) & Year	**Starting Material**	**Meal to Water Ratio (w/v)**	**NaCl Concentration (M)**	**Time (hr)**	**Temp (°C)**	**Centrifugation**	**Filtration & Dialysis**	**Conversion Factor**
(Malomo and Aluko 2015b)	Defatted (through solvent-free mechanical pressing) hemp seed protein meal	1:10	0.5	1	24	60 min at 7,000 x g at 4°C[a,b]	Supernatant clarified w/ Whatman No 1 filter, dialyzed against water at 4 C for 5 days using 6 - 8 kDa MWCO dialysis tubing	--[c]
(Hadnađev et al. 2018)	Hemp pellets obtained by cold-pressing, defatted with hexane and milled	1:10	0.8	2	35	20 min at 6,000 x g[a] 20 min at 7,500 x g[b]	Supernatant was dialyzed against water (pH = 7.0) at 4°C for 72 hr using ultrafiltration membranes of cellulose (12000-14000 Da cutoff)	5.7

[a] Centrifugation settings used after solubilization / [b] Centrifugation settings used to collect precipitated protein after filtration/dialysis step
[c] Protein determined using Lowry method

While some differences between salt-extracted and pH-extracted HPIs have been identified, this area requires further exploration. As with pH extraction, salt extraction conditions have not been optimized nor adequately explored for industrial scale up. While some work has been done to explore protein functionality, such as solubility (Potin et al. 2019), the research findings are far from being complete and relevant to various applications. Thus, further research is needed to optimize the production of salt-extracted HPI for high yield and purity, and for desirable structural, functional, and nutritional properties.

1.7.3 Other Isolation Methods

Other protein isolation methods have been explored for hemp. Acid solubilization of defatted, milled hemp meal at pH 2 followed by precipitation at pH 5 produced a protein isolate with high purity (90% protein) but lower yield (10%) than alkaline solubilization (Teh et al. 2014). Compared to alkaline-extracted HPI, the acid-extracted HPI was lighter in color and more thermally stable, but contained a lower amount of all essential amino acids, an outcome that deemed this approach unfavorable.

Two other extraction methods were explored, enzyme assisted and alkaline solubilization coupled with membrane filtration. A cocktail of enzymes (cellulase, hemicellulase, xylanase, and phytase) were used at pH 5 to digest fiber and phytate in defatted hemp seed meal, followed by ultrafiltration/diafiltration to remove non-protein components (Malomo and Aluko 2015a). A protein concentrate (74% protein) was produced with higher protein solubility between pH 3 – 9 than HPI produced using alkaline solubilization and isoelectric precipitation. Another study explored the use of alkaline solubilization (pH 10 or 12) coupled with ultrafiltration/diafiltration to purify the protein instead of isoelectric precipitation (Potin et al. 2021). Solubilization at both pH values produced a protein isolate of similarly high purity (84 – 88% protein), but the isolate produced by solubilization at pH 12 had higher solubility (> 80%) between pH 7 – 9 though completely denatured. However, both of these methods of hemp protein extraction produced isolates of lower purities than that typically reported for alkaline extraction coupled with isoelectric precipitation and for salt extraction coupled with

membrane filtration. Therefore, alkaline solubilization and salt extraction methods show the most promise for producing an HPI with high purity and yield.

1.8 Limitations Associated with Hemp Protein Use

1.8.1 Color

One of the main limitations of hemp protein that must be considered in optimizing HPI extraction is the color of the isolate. In studies that used whole hemp seeds to extract protein following pH extraction, the resultant HPI had an unappealing darkish or greenish color (Girgih et al. 2014; Teh et al. 2014; Hadnađev et al. 2018). Hemp seed hulls contain phenolic compounds which, after solubilization at alkaline pH, contribute to undesirable color due to their oxidation during the process (Hadnađev et al. 2018). Shen et al. (2020a) suggested that the undesirable color of HPI could be resolved through thorough dehulling and separation. Currently, dehulling prior to commercial hemp seed oil extraction is not common, and dehulling is not always reported to be performed prior to protein extraction trials. Future research must further explore the effects of dehulling on HPI color as well as protein purity and yield.

1.8.2 Knowledge Gaps: Antinutritional Components, Nitrogen Conversion Factor, and Breeding for Hemp Protein Quality

Common in many plant protein sources, antinutritional components refer to compounds that decrease a nutrient's bioavailability or are toxic to the body (Farinon et al. 2020). These components can reduce digestibility through protein precipitation, inhibition of digestive enzymes, or through binding with other macro- or micronutrients, inhibiting absorption (Crescente et al. 2018). While only minimally reported, antinutritional components such as trypsin inhibitors, saponins, and phytic acid have been explored in hemp seed products (Farinon et al. 2020). The amounts of these antinutritional factors vary among different cultivars (Russo and Reggiani 2013, 2015). Phytic acid is the main antinutritional compound in hemp and is present in similar amounts in other oilseeds, legumes, and nuts (Pojić et al. 2014; Leonard et al. 2020).

However, it is difficult to determine whether antinutritional components are of major concern for hemp seeds, as their content varies in the different hemp seed parts

being evaluated. Pojić et al. (2014) milled hemp press cake and separated components based on particle size using sieves. The fractions with larger particle sizes were determined to contain the hulls, while the fractions with smaller particles sizes were determined to be mostly the protein-rich hemp heart. The authors reported higher amounts of phytic acid, trypsin inhibitor, and glucosinolates in the portions with smaller particle sizes. However, the hemp seeds were not dehulled prior to pressing; therefore, contamination of the hemp heart fraction by the papery testa and hull pieces likely occurred, as was evidenced by the visualized green color and by reported L* a* b* values. Therefore, it is not certain whether or not these antinutritional compounds reside in the hemp hearts, and thus would be a concern for hemp protein ingredients produced only from the hemp hearts (dehulled seeds). Overall, research on hemp antinutritional factors has focused on the amounts present in the seeds rather than the amounts left after processing for human consumption. Therefore, the presence of antinutritional factors must be further investigated in hemp products processed for human food use.

Another area of discrepancy in hemp research is the nitrogen conversion factor for determining protein content. Almost all studies on hemp protein use nitrogen conversion factor of 6.25 (**Tables 4** and **5**), even though this value can cause overestimation when the protein is high in amino acids with a relatively high % of nitrogen (Sriperm et al. 2011). The United States Department of Agriculture (USDA) reported a nitrogen conversion factor of 5.30 for hemp seeds, which presents a major discrepancy with what is currently being used in literature (USDA 2018). Therefore, future studies need to evaluate the appropriate nitrogen conversion factor to use for hemp in order to report more accurate protein contents.

Finally, breeding to improve hemp protein quality is unexplored. Hemp protein has limited nutritional quality due to its lysine deficiency and has functional limitations in comparison to soy protein. Research has demonstrated differences in protein content and amino acid composition among different cultivars (House et al. 2010; Russo and Reggiani 2015); however, previous breeding efforts have only focused on improving yields and agronomic characteristics (Fike 2016; Żuk-Gołaszewska and Gołaszewski 2020). Research examining differences in protein structural, functional, and nutritional

properties among hemp cultivars is needed to identify breeding strategies for improving hemp as a novel plant protein source.

1.9 Conclusions

With the increasing demand for plant proteins, hemp seeds show promise as a novel protein source that fits consumer trends. Hemp cultivation benefits the environment by 1) requiring minimal pesticide, herbicide, and fungicide usage; 2) improving the soil for future crops; and 3) providing raw materials for a variety of industrial uses, minimizing waste. As a protein ingredient, hemp seed protein is desirable because of its low allergenicity, high digestibility, and attractive amino acid profile. However, research on hemp protein is lagging behind other protein sources, as legal restrictions across the world stunted the growth of the hemp industry for decades. Removal of these restrictions and increased recognition of the nutritional benefits of hemp protein have paved the way for investigating its potential as a novel plant protein source. Currently, hemp seeds are used for their high edible oil content, while the remaining protein-rich press cake or meal has typically been underutilized. The protein from the defatted cake or meal can be extracted using pH or salt-assisted extraction to produce HPIs with high purity. However, the resulting HPIs have shown some functional limitations. More work is needed to optimize protein extraction conditions for improved yields, color, structure, function, and nutritional quality of HPI to fully utilize this novel protein. Additionally, variability in protein content and amino acid composition of hemp seeds from different cultivars indicate potential differences in protein structure, function, and nutritional quality. Such varietal differences can be leveraged to initiate targeted breeding to mitigate some of the limitations of hemp and enhances its viability as novel plant protein source.

Chapter 2: Optimization of Hemp Protein Extraction and Impact on Structural, Functional, and Nutritional Characteristics

2.1 Overview

Investigation into novel plant protein sources such as hemp (*Cannabis sativa* L.) is necessary to meet the growing consumer demand for protein. The aim of this study was to investigate the effects of two protein extraction methods (alkaline solubilization coupled with isoelectric precipitation and salt solubilization coupled with membrane filtration) on the structural, functional, and nutritional quality of hemp protein. Optimization of extraction parameters for both methods resulted in hemp protein isolates (HPI) with high purity (87 – 88% protein), favorable protein yield (>80% yield), and acceptable color. Structural and functional attributes in water and in 0.5 M NaCl of pH-extracted HPI (pH-HPI) and salt-extracted HPI (salt-HPI) were compared with commercial soy (cSPI) and pea protein isolates (cPPI). In general, the functionality of HPI at neutral pH was inferior to that of cSPI and cPPI, largely due to HPI's poor solubility. High surface hydrophobicity and a low surface charge explained the observed poor solubility of HPI. However, HPI demonstrated similar solubility at acidic pH in water and superior gel strength in 0.5 M NaCl to cSPI, and therefore demonstrated a potential to replace soy protein in certain food applications. Overall, pH-HPI demonstrated better overall functionality and had significantly higher digestibility ($P < 0.05$) than salt-HPI. Alkaline solubilization coupled with isoelectric precipitation was, therefore, determined to be the better extraction method for producing a HPI for food applications.

2.2 Introduction

The global protein ingredients market is predicted to grow from $42.5 billion in 2021 to $85.5 billion in 2028 (Grand View Research 2021). This growth is fueled by an increased interest in protein for its health-promoting properties. While animal-derived proteins make up a significant portion of the protein market, plant-derived proteins are gaining traction. More consumers are choosing a flexitarian, vegetarian, or vegan diet for a variety of reasons, including concerns about personal health, animal welfare, and the environment. Additionally, plant proteins can be produced in a cost-effective manner and can provide a viable alternative to traditional animal-derived proteins in various food applications (Ismail et al. 2020).

Currently, soybeans are the predominant source of plant protein ingredients (Grand View Research 2021). Soy protein has been well-established as a food ingredient for decades. However, soy is one of the "Big 9" allergens identified by the Food and Drug Administration (FDA) (Ismail et al. 2020). Pea protein is growing in popularity as a replacement of soy protein for having low allergenicity and acceptable functionality and nutritional quality (Boye et al. 2010a; Ismail et al. 2020; Grand View Research 2021). However, to meet the growing consumer demand for plant proteins, other novel sources must be explored.

Hemp (*Cannabis sativa* L.) is a promising plant protein source. The hemp plant has a deep rooting system, which can aerate the soil for other crops. Additionally, many of the nutrients contained in the plant are returned to the soil after harvest, enriching the soil (Aluko 2017). However, research on hemp as a food source has been hindered by legal restrictions due its similarities to marijuana, which contains appreciable amounts of Δ-9-tetrahydrocannabinol (THC), a psychoactive component (Callaway 2004; Small 2015; Aluko 2017). In the past two decades, legal restrictions on hemp cultivation and food use in North America were lifted, paving the way for the inclusion of hemp seeds and their products in food applications (Small 2015; Aluko 2017; Mattucci 2020).

Hemp seeds contain approximately 33-35% oil, which can be extracted using cold-pressing and sold for human consumption (House et al. 2010). The hemp seed meal remaining after oil extraction contains an appreciable amount of highly digestible protein (approximately 30-40% protein) with a favorable amino acid profile (Callaway 2004;

House et al. 2010). Milled into flour, the hemp meal has been sold directly to consumers as a dietary hemp protein powder (House et al. 2010). However, protein purification to produce a functional hemp protein isolate (HPI) could vastly increase hemp's use in different food applications.

Different protein extraction methods have been investigated to produce HPI. The most common method tested is alkaline extraction coupled with isoelectric precipitation, referred to as pH extraction. Typically for hemp, this method involves solubilization of the protein at pH 10 followed by precipitation at pH 5 to further purify the protein (Tang et al. 2006; Yin et al. 2008; Wang et al. 2008; Girgih et al. 2014; Malomo et al. 2014; Teh et al. 2014; Raikos et al. 2015; Orio et al. 2017; Hadnađev et al. 2018; Mamone et al. 2019; Shen et al. 2020a). While reported protein purity has been high (>80%), the protein yields have been modest, typically around 50% or less, with one exception of 73% yield reported by Tang et al. (2006) (Wang et al. 2018; Yin et al. 2008; Wang et al. 2008; Lu et al. 2010; Malomo et al. 2014; Teh et al. 2014; Ren et al. 2016; Orio et al. 2017; Hadnađev et al. 2018; Mamone et al. 2019; Shen et al. 2020a, b). Some work has been done investigating percent soluble protein at different solubilization pHs, but additional protein purification steps were not conducted and therefore overall protein yield could not be determined (Potin et al. 2019). An increase in protein yield was observed when using different precipitation pHs, but this difference was modest (Shen et al. 2020b). Therefore, more work is needed to determine the optimal extraction parameters to produce HPI using pH-assisted extraction.

Another protein extraction method, salt solubilization coupled with membrane filtration, has been investigated for the production of an HPI with high purity (Malomo and Aluko 2015b; Hadnađev et al. 2018). However, this protein extraction method has not been as thoroughly studied in comparison to pH extraction (Dapčević-Hadnađev et al. 2018, 2019; Hadnađev et al. 2018). Therefore, more work is needed to investigate optimal protein extraction conditions to produce HPI with high purity and yield following salt-assisted extraction.

One major drawback that has been reported when producing HPI is an offensive brown or greenish color. An unappealing color of HPI has been reported among studies using pH extraction (Girgih et al. 2014; Teh et al. 2014; Hadnađev et al. 2018). Hemp

hulls contain phenolic compounds, which can interact with the protein during extraction resulting in reduced protein solubility if not removed by dehulling and separation prior to extraction. These phenolic compounds become oxidized under harsh alkaline extraction conditions, contributing to the dark, greenish color of HPI. This color could decrease consumers' acceptance and limit the use of HPI in food applications. Recent work by Shen et al. (2020a) found that dehulling increased protein extraction yield and improved protein color. However, dehulling and separation prior to hemp protein extraction has been marginally investigated and should be further considered for the production of HPI with acceptable color and functionality.

Processing and extraction conditions affect the protein's structure, which impacts its functionality (Ismail et al. 2020). The impact of pH and salt extraction on protein structure and functionality has been studied (Dapčević-Hadnađev et al. 2018, 2019; Hadnađev et al. 2018). However, digestibility of the HPIs was not reported, and structural and functional characteristics were not compared to commonly used plant protein isolates, such as soy and pea protein isolates. Novel plant protein sources must demonstrate comparable or superior functional characteristics and nutritional quality to be adopted in the food industry.

The objective of this study, therefore, was to optimize protein extraction from hemp seed, following pH-assisted extraction and salt solubilization coupled with membrane filtration, to produce a protein isolate with acceptable color, purity, yield, structural and functional characteristics, as well as nutritional quality. This work will demonstrate how dehulled hemp seeds can be used to produce a protein ingredient with high protein purity and yield, and valuable attributes for food applications.

2.3 Materials and Methods

2.3.1 Materials

Surplus whole hemp seeds from the cultivar CFX-2 harvested in 2016 were kindly provided by Hemp Acres (Waconia, MN, USA). Soy protein isolate (cSPI, ProFam 974, 90.1% protein, 4.2% ash) and pea protein isolate (cPPI, ProFam 580, 79.5% protein, 5.6% ash) were kindly provided by Archer Daniels Midland (ADM) (Decatur, IL, USA).

All samples were stored in closed containers sealed with Parafilm at -20°C when not in use.

SnakeSkin™ dialysis tubing with a molecular weight cut-off of 3.5 kDa, Sartorius Vivaflow™ 200 Crossflow Cassettes for membrane filtration with a molecular weight cut-off of 3 kDa, and Sudan Red 7B were purchased from Thermo Fischer Scientific™ (Waltham, MA, USA). The enzyme kit for the extraction and quantification of total dietary fiber was purchased from Megazyme (NEOGEN Corporation, Lansing, MI, USA). Criterion™ TGX™ 4-20% precast gels (18 wells), 10X tris/glycine/sodium dodecyl sulfate (SDS) running buffer, Laemmli sample buffer, Precision Plus™ molecular weight marker, and Imperial™ Protein Stain were purchased from Bio-Rad Laboratories, Inc. (Hercules, CA, USA). Folded capillary tubes for zeta potential analysis were purchased from Malvern (Malvern, UK). Aluminum crucibles (40 µL, with pin) for use in DSC were purchased from Mettler-Toledo (Columbus, OH, USA). Electrophoresis grade sodium dodecyl sulfate (SDS), 2-mercaptoethanol (βME), Costar® 96-well black opaque plates, 8-anilino-1-napthalenesulfonic acid ammonium salt (ANS), and Celatom® (C8656) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Trypsin (T0303; from porcine pancreas Type IX-S, lyophilized powder, 13,000-20,000 BAEE units/mg protein), α-chymotrypsin (C4129; from bovine pancreas C4129 Type II, lyophilized powder, P40 units/mg protein), and protease (P5147; from *Streptomyces griseus* Type XIV, P3.5 units/mg solids) were used for protein digestibility assay and were purchased from Sigma-Aldrich. Casein (400601, high nitrogen, 80 mesh) for use as a reference in the protein digestibility assay was purchased from Dyets Inc. (Bethlehem, PA, USA). All other chemicals and reagents of analytical grade quality were purchased from Thermo Fischer Scientific or Sigma-Aldrich. Mazola pure corn oil was purchased from local grocery stores.

2.3.2 Production of Defatted Hemp Meal (DHM)

Hemp seeds were dehulled using an industrial-scale huller (Bühler MHSA huller, Bühler, Uzwil, Switzerland). Hull pieces were separated from hemp seeds using multiple sieves, a grain aerator, and a small-scale gravity separator table (Whippet V.80 Gravity Separator Table, Sutton, Steele & Steele, Inc., Dallas, TX, USA). Manual separation

using forceps was employed to remove residual hull pieces. Dehulled seeds were pressed using a hydraulic press (Carver Model C 8-ton manual bench top laboratory press with 2094 cage equipment, Carver Inc., Wabash, IN, USA) for 28 – 30 hours at ambient temperature with a maximum pressure of 16,000 psi. The pressed hemp cake was ground using a KitchenAid® (KitchenAid, Benton Harbor, MI, USA) coffee grinder. The ground cake was defatted using hexane in a ratio of 3:1 (hexane to hemp seeds) under constant agitation for one-hour, followed by another 30-minute cycle. Defatted cake was left overnight under the hood to evaporate off any residual hexane before being milled to 50-mesh using a cyclone sample mill (UDY Corp, Fort Collins, CO, USA). Defatted, milled hemp meal had less than 3.5% fat on wet basis as determined following the Mojonnier AOAC method 922.06. The protein content (~ 60% protein) of the defatted hemp meal (DHM) was determined by the Dumas AOAC method 990.03 using a nitrogen analyzer (LECO, St. Joseph, MI, USA) and a protein conversion factor of 5.30 (USDA 2018). Proximate composition of DHM can be found in **Table 21** in **Appendix A**.

2.3.3 pH Extraction of Hemp Protein

2.3.3.1 Preliminary Screening of pH Extraction Conditions

To determine optimal pH extraction conditions, several protein solubilization and precipitation pHs were tested. DHM was solubilized, in triplicate, in double distilled water (DDW) at 5% total solids for one hour at room temperature at either pH 7.0, 8.0, 9.0, 10.0, 11.0, or 12.0. The pH was checked and readjusted with 2 M NaOH after 30 minutes and right before centrifugation at 12,000 x g for 15 minutes. The supernatant was decanted, and the weight recorded. Protein content of the supernatant was determined following the Dumas method and was used to calculate the weight in grams of protein in the supernatant. The starting protein weight in grams in each solution was calculated by multiplying the protein content (%) of the DHM by the weight of the DHM powder. To determine the percent of soluble protein at each pH, **Eq. 4** was used (sample calculations shown in **Appendix B**). In the protein solubilization step, the ideal pH would be the one where most of the protein remains in solution.

$$\% \text{ soluble protein} = \frac{\text{Weight (g) of protein in supernatant}}{\text{Weight (g) of protein in starting solution}} \times 100 \qquad \textbf{Eq. 4}$$

To further purify the protein and separate it from other soluble components, the protein is precipitated at around its isoelectric point. Three precipitation pH levels were tested (pH 5.0, 5.5, and 6.0). To determine optimal precipitation pH, 5% total solids dispersions of DHM in DDW were prepared, in triplicate. Dispersions were adjusted to pH 10 and stirred for one hour at room temperature, followed by centrifugation at 12,000 x g for 15 minutes. Each supernatant was collected and adjusted to the precipitation pH using 2 M HCl, followed by centrifugation at 12,000 x g for 10 minutes. The supernatant was decanted and the weight recorded. The percent of soluble protein was determined using Dumas and **Eq. 4**. In the protein precipitation step, the ideal pH was considered to be the one where most of the protein would precipitate with minimal amount of protein remaining in solution.

2.3.3.2 Optimization of pH Extraction of Hemp Protein

Defatted hemp meal was used to produce HPI using alkaline solubilization coupled with isoelectric precipitation. In triplicate, DHM was fully dispersed in DDW at 5% total solids. The pH was adjusted to 10 or 11 using 2 M NaOH and stirred for one hour at room temperature, with pH readjustments performed after 30 minutes and after one hour of stirring. The dispersions were centrifuged at 12,000 x g for 15 minutes, and the supernatants were collected and neutralized. Then, each pellet was redispersed in water (5% total solids) and readjusted to pH 10 or 11 followed by stirring for another hour with pH adjustments after 30 minutes and after one hour of stirring. Each dispersion again was centrifuged at 12,000 x g for 15 minutes, and the supernatant was combined with the first supernatant and neutralized. The pellets were collected and lyophilized for mass balance determination. The pH of the combined supernatants was adjusted to pH 5 with 2 M HCl, followed by centrifugation at 12,000 x g for 10 minutes. The supernatant was collected and lyophilized for mass balance determination. The protein pellet was redispersed (1:4 w/w) in water and adjusted to pH 7.0 with 2 M NaOH. Once completely dispersed, the protein solution was dialyzed and lyophilized. The protein content of the

HPI, along with the residual pellet after protein solubilization and residual supernatant after protein precipitation, was determined following the Dumas method. Mass balance determination tracked the protein yield of the following fractions: HPI, the residual pellet after solubilization, and the residual supernatant after protein precipitation. The protein yield/lost/residue for each fraction was calculated (**Appendix C**). The ash content of each HPI was determined following the official AOAC 942.05 dry ashing method.

For protein characterization, 100 grams of DHM were used to produce one batch of pH-extracted HPI (pH-HPI) (85.8% protein, 2.0% ash) using the identified optimal extraction conditions. Samples were stored in closed containers sealed with Parafilm at -20°C when not in use.

2.3.4 Salt Extraction of Hemp Protein

2.3.4.1 Preliminary Screening of Salt Extraction Conditions

To determine optimal salt concentration for solubilization of hemp protein, four salt concentrations were tested. In triplicate, DHM was dispersed at 5% total solids in salt (NaCl) solutions at different concentrations 0.5 M, 0.75 M, 1 M, and 1.25 M and stirred for one hour in a water bath at 50°C. Dispersions were centrifuged at 12,000 x g for 20 minutes. The supernatants were decanted, and the weights recorded. The percent soluble protein was determined as outlined in **Section 2.3.3.1**. Once the optimal salt concentration was determined based on amount of protein solubilized, the extraction was repeated with protein solubilization carried out at ambient temperature to determine if heating was necessary. The percent soluble protein was determined using **Eq. 4**.

2.3.4.2 Optimization of Salt Extraction of Hemp Protein

DHM was used to produce HPI using salt solubilization coupled with membrane filtration. In triplicate, DHM was dispersed in 0.5 M or 0.75 M NaCl at 5% total solids and stirred for one hour in a water bath at 50°C. Dispersions were then centrifuged at 12,000 x g for 20 minutes, and the supernatants were collected. Each pellet was resolubilized in 0.5 M or 0.75 M NaCl at 5% total solids and stirred for another hour at 50°C. The dispersions were centrifuged at 12,000 x g for 20 minutes, and each supernatant was combined with the supernatant from the first solubilization. Each pellet

was saved and lyophilized for mass balance determination. The combined supernatants from each replicate were adjusted to pH 7 using 2 M NaOH or HCl and ultrafiltered using a lab scale Sartorius Vivaflow® 200 system with two membrane cassettes run side-by-side to increase efficiency. A peristaltic pump (Masterflex Easy Load Pump Head- Size 15, Masterflex Economy Drive Peristaltic Pump 230V, Sartorius) was used to push the protein solution across the membranes at a pressure of 2 – 2.5 bars. The membranes retained components that were 3 kDa or larger and recirculated them to the feed container, while components with molecular weights smaller than 3 kDa passed through as permeate and were discarded. The protein solution was concentrated down to ~25 mL and washed with six times the final volume using DDW. The retained protein solution was then dialyzed and lyophilized. The protein content of HPI and the discarded pellet was determined following the Dumas method. Mass balance determination tracked the protein yield of the following fractions: HPI and the residual pellet after solubilization. The protein yield/lost/residue for each fraction was calculated (**Appendix D**).

For protein characterization, 100 grams of DHM was used to produce one batch of salt-extracted hemp protein isolate (salt-HPI) following the identified optimal solubilization conditions, but with using a different membrane filtration unit. A pilot plant ultrafiltration/diafiltration plate-and-frame unit (DD20, Osmonics) was used with eight plates and a crossflow across a 3 kDa molecular-weight cut-off membrane (inlet pressure: 12-22 psi, outlet pressure: 25-35 psi). Filtration was determined to be complete when the permeate had ~0% total solids as measured by a refractometer. The resulting protein retentate was lyophilized to produce salt-HPI (86.7% protein, 5.1% ash). Samples were stored in closed containers sealed with Parafilm at -20°C when not in use.

2.3.5 Protein, Ash, and Fiber Content

The protein content of all protein isolates was determined using the Dumas method. A protein conversion factor of 6.25 was used for commercial soy protein (cSPI) and commercial pea protein isolates (cPPI), and a protein conversion factor of 5.30 was used for pH-HPI and salt-HPI. Ash content was determined using the AOAC dry ashing method (AOAC 942.05). Total dietary fiber of the DHM was measured using the Megazyme Total Dietary Fiber (TDF) Kit per the manufacturer's instructions based on

the AOAC Method 991.42 and AACC Method 32-07.01. An example calculation can be found in **Appendix E**.

2.3.6 Color Measurement

The color of all samples (cSPI, cPPI, pH-HPI, and salt-HPI) was measured in triplicate using a Chroma Meter CR-221 (Minolta Camera Co., Osaka, Japan) as outlined by Bu et al. (2022). Prior to sample analysis, the colorimeter was calibrated using a white CR-221 calibration plate (Minolta). Equivalent amounts of sample were weighed out to obtain a similar thickness of sample for all readings. The color measurements were recorded using the CIE (International Commission on Illumination) 1976 L* a* b* color system, where L* indicates lightness on a scale from 0 (black) to 100 (white), a* indicates redness (positive value) and greenness (negative value), and b* indicates yellowness (positive value) and blueness (negative value).

2.3.7 Protein Structural Characterization

2.3.7.1 Protein Profiling by Gel Electrophoresis

The protein profiling of cSPI, cPPI, pH-HPI, and salt-HPI using sodium dodecyl polyacrylamide gel electrophoresis (SDS-PAGE) was performed as outlined by Boyle et al. (2018). Samples were solubilized in DDW (2% w/v) for two hours before further dilution 1:4 with DDW. To analyze under non-reducing conditions, Laemmli buffer was added to a sample aliquot in a 1:1 ratio. To run under reducing conditions, a ratio of 0.95:1:0.05 of Laemmli buffer to sample to β-mercaptoethanol (βME) was used. Both samples under non-reducing and reducing conditions were heated in a boiling water bath for five minutes and allowed to cool to room temperature. Precision Plus™ MW protein standard (10 µL) and samples (5 µL, ~50 µg protein) were loaded onto a Criterion™ TGX™ 4-20% precast Tris-HCl gradient gel. The gel was electrophoresed at 200V, stained using Imperial Protein Stain™, destained using DDW, and imaged using Molecular Imager Gel Doc XR system (Bio-Rad Laboratories).

2.3.7.2 Thermal Denaturation by Differential Scanning Calorimetry (DSC)

Denaturation temperature and enthalpy of denaturation using a DSC instrument (DSC 1 STARe System, Mettler Toledo, Columbus, OH, USA) was determined for all samples using the method outlined by Bu et al. (2022), with modifications in the starting and final measurement temperature. Protein samples were dispersed in DDW (20% protein, w/v) and vortexed overnight at ambient temperature before analysis. An aliquot (20 µL) of each sample, in triplicate, was placed in an aluminum pan, sealed, and analyzed alongside an empty pan as a reference. The pans were kept at 25°C for five minutes before ramping up the temperature at a rate of 5°C per minute up to 115°C. Thermograms were recorded, and peaks were integrated to determine denaturation temperature (peak) and enthalpy of denaturation for each protein sample using the STARe Software version 11.00 (Mettler Toledo).

2.3.7.3 Protein Surface Properties

Surface hydrophobicity was determined for all samples using a spectrofluorometric method that utilizes 1-aniline-8-napthalene sulfonate (ANS) as outlined by Bu et al. (2022). In triplicate, samples were solubilized in 0.017M:0.165M citric acid:sodium phosphate buffer (0.05% protein, w/v) for two hours. Diluted protein solutions were prepared using the same buffer in concentrations ranging from 0.005 – 0.050% protein (w/v). Samples were vortexed and loaded (200 µL) into a 96-well black opaque well plate, along with six wells of 200 µL of the citric acid:sodium phosphate buffer as blanks. The relative fluorescence index (RFI) was measured using a microplate reader (BioTek Synergy HT, BioTek Instruments, Winooski, VT, USA). The RFI was measured at excitation and emission wavelengths of 400/30 (wavelength/bandwidth) and 460/40 nM and 40 gain. ANS probe solution (20 µL, 12.6 µg mL^{-1}) was added quickly to sample and blank wells and mixed using a multichannel pipettor. The plate and lid were covered with aluminum foil and allowed to sit for 15 minutes in the dark before measuring RFI again. Net RFI was calculated as described by Alizadeh-Pasdar and Li-Chan (2000). Net RFI was plotted against percent protein concentration and the slope (S_0) was used as an index of protein surface hydrophobicity (sample calculations and plot in **Figure 6** can be found in **Appendix F**).

Zeta potential was measured using a dynamic light scattering instrument, Malvern Nano Z-S Zetasizer, following the method outlined by Bu et al. (2022), with modifications in solubilization medium and number of measurements per sub-reading. Solutions were prepared, in triplicate, (0.1% protein, w/v) in DDW and solubilized for two hours. Before each sample was analyzed, the pH was adjusted to 7.0 using $\leq$1M HCl or NaOH. An aliquot (~1 mL) of each sample was inserted into a folded capillary cell and placed into the Zetasizer. After a 30-second equilibration period, the electrophoretic motility was measured by taking three sub-rep readings, each comprised of 20 measurements, with 30 seconds in between each sub-rep to allow for better stabilization of the readings. Malvern's Zetasizer software (version 7.13) was used to determine zeta potential using the Smoluchowski model. Zeta potential for each sample was also determined for samples solubilized in 0.5 M NaCl.

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

ATR-FTIR spectra for pH-HPI and salt-HPI were recorded using Fourier transform infrared spectrometer (Thermofisher Nicolett iS50 FTIR) following the method outlined by Bu et al. (2022). Protein samples were placed in a microcentrifuge tube with the lid open inside a desiccator for no less than 36 hours to dehydrate the sample and minimize instrument interference due to the presence of moisture. The protein powders were placed on diamond ATR and scanned from 400 to 4000 cm^{-1} by DLaTGS detector. The resulting ATR spectra were transformed to emission spectra using OMNIC® software, and the second derivative of the Amide I band (1600 cm^{-1} to 1700 cm^{-1}) was obtained by PeakFit v. 4.12 to determine the distribution of alpha-helix, beta-sheet, beta-turn, and random coil (**Appendix G, Figures 7** and **8**).

2.3.8 Protein Functional Characterization

2.3.8.1 Protein Solubility

The solubility of all protein samples was determined using the method outlined by Boyle et al. (2018), with modifications in the solubilization medium and acidic pH value. Protein solubility in DDW and in 0.5 M NaCl was measured. Solubilization at an acidic

pH of 3.2 instead of 3.4 was performed as it is still within the range used in industrial applications for acidic beverages but is further away from hemp protein's isoelectric point. In triplicate, protein dispersions (1% protein, w/v) in DDW and in 0.5 M NaCl were prepared and allowed to solubilize for two hours. After two hours, the pH was adjusted to pH 7.0 using 2 M HCl or NaOH, and 1-mL aliquots were placed into microcentrifuge tubes. Aliquots were assessed at room temperature and after heating at 80°C for 30 minutes. Both heated and non-heated samples were centrifuged at 15,682 x g for 10 minutes. Protein content of the supernatants and initial protein solutions were determined following the Dumas method. Protein solubility was expressed as shown in **Eq. 5** (sample calculations can be found in **Appendix H**).

$$\% \text{ protein solubility} = \frac{\% \text{ supernatant protein}}{\% \text{ initial protein}} \qquad \textbf{Eq. 5}$$

2.3.8.2 Gel Strength

The strength of heat-induced gels was measured as outlined by Boyle et al. (2018), with modifications in the solubilization medium and the texture analyzer probe speed. Protein solutions (15% protein, w/v) were prepared, in triplicate, either in DDW or 0.5 M NaCl, adjusted to pH 7.0 using 2 M NaOH or HCl, and solubilized for two hours. Aliquots (1 mL) were placed in microcentrifuge tubes and heated at 95°C ($\pm$ 2°C) in a water bath for 10 (cSPI, pH-HPI, salt-HPI) or 20 minutes (cPPI). Gels were allowed to cool to room temperature. Gel strength was determined using a TA-TX Plus Texture Analyzer (Stable Micro Systems LTD, Surrey, UK) with a 100 mm diameter probe, a 5 mm s^{-1} test speed, and a target distance of 0.5 mm from the plate. Gel strength was measured as the maximum force (in Newtons) that was needed to rupture the gel.

2.3.8.3 Water Holding Capacity

Water holding capacity (WHC) was measured as outlined by Boyle et. al (2018), with modifications in the solubilization medium. Protein solutions (15% protein, w/v) were prepared, in triplicate, in DDW or in 0.5 M NaCl, and solubilized for two hours. After solubilization, the pH was adjusted to 7.0 using 2 M NaOH or HCl. An aliquot (1

mL) was transferred to a microcentrifuge tube, and the weight of the solution was recorded (T_1). Samples were heated to 95°C (± 2°C) in a water bath for 10 (cSPI, pH-HPI, salt-HPI) or 20 minutes (cPPI), and gels were allowed to cool to room temperature before weighing (T_2). The samples were centrifuged at 1,000 x g for five minutes and inverted for 10 minutes to drain water expelled from gel. After 10 minutes, the weight of each gel was recorded (T_3). WHC was measured as the amount of water physically entrapped in the gel matrix using **Eq. 6** (sample calculations can be found in **Appendix I**).

$$\text{Water holding capacity} = 100 \times \left(\frac{T_3 - T_1}{T_2 - T_1}\right) \qquad \textbf{Eq. 6}$$

Where:

T_1 = weight of protein solution before heating

T_2 = weight of gel formed from protein solution + microcentrifuge tube after cooling

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

2.3.8.4 Emulsification Capacity

Emulsification capacity (EC) was determined using the method outlined by Boyle et al. (2018), with modifications in the solubilization medium. Protein solutions were prepared, in triplicate, in either DDW or 0.5 M NaCl at 1% protein (w/v) and solubilized for two hours. After solubilization, the pH was adjusted to 7.0 using 2 M NaOH or HCl. Corn oil dyed with 4 µg mL^{-1} of Sudan Red 7B was titrated at a steady flow rate of 2 mL per minute for the first three minutes and then increased to 6 mL per minute for the remainder of the titration, while mixing using a homogenizer (IKA® RW 20 Digital; IKA Works Inc., Wilmington, NC, USA) with a four-blade, 50 mm diameter shaft (IKA® R 1342) rotating at 860-870 rpm. The oil titration and simultaneous homogenization was continued until the emulsion formed was broken as indicated by a phase inversion. Emulsification capacity, expressed as g of oil emulsified per 1 g of protein, was calculated using **Eq. 7** (sample calculations can be found in **Appendix J**).

$$\text{Emulsification capacity} = \frac{\text{volume of oil titrated (mL) x density of oil (g/mL)}}{\text{weight of protein (g)}} \qquad \textbf{Eq. 7}$$

2.3.8.5 Emulsion Stability and Activity

Emulsification stability (ES) and activity index (EAI) were measured as outlined by Boyle et al. (2018), with modifications in the solubilization medium, volume of the sample solution, volume of corn oil, and the homogenizer used. Protein solutions (0.1% protein, w/v) were prepared, in triplicate, either in DDW or 0.5 M NaCl and solubilized for two hours before the pH was adjusted to 7.0 using ≤1 M NaOH or HCl. An aliquot (5 mL) was added to 1.67 mL of corn oil and homogenized at 18,000 rpm for one minute using an IKA T-25 ULTRA-TURRAX® high shear homogenizer (IKA Works, Inc., Wilmington, NC, USA). An aliquot (50 µL) of the homogenized emulsion was quickly added to a test tube with 0.1% SDS and vortexed for five seconds. Initial absorbance was measured at 500 nm using a UV/VIS spectrophotometer (Beckman Coulter DU 800, Brea, CA, USA). After 10 minutes, another aliquot (50 µL) of the emulsion was added to a test tube with 0.1% SDS, vortexed for five seconds, and the absorbance was measured again. The ES (min) and EAI ($m^2\ g^{-1}$) were calculated as described by Boyle et al. (2018) and Cameron et al. (1991), respectively, using **Eq. 8** and **9** (sample calculations can be found in **Appendix K**).

$$\text{ES (min)} = \frac{A_0}{A_0 - A_{10}} \text{ x 10 min} \qquad \textbf{Eq. 8}$$

$$\text{EAI} \left(\frac{m^2}{g}\right) = \frac{2T}{(1-\Phi)C} \qquad \textbf{Eq. 9}$$

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 the oil at 500 nm = $\frac{2.303A_0}{l}$

L = path length of the cuvette

2.3.8.6 Foaming Capacity and Stability

Foaming capacity and stability were measured following the method outlined by Boyle et al. (2018), with modifications in the solubilization medium. Protein solutions (0.5% protein, w/v) were prepared, in triplicate, either in DDW or in 0.5 M NaCl and solubilized for two hours before adjusting pH to 7.0 with 2 M NaOH or HCl. An aliquot (50 mL) was mixed at 800 rpm using a Sunbeam® hand mixer for 2 minutes and poured into a 250-mL squat form graduated cylinder. Foam volume and liquid volume measurements were recorded. After 30 minutes, the foam and liquid volumes were recorded again. Foaming capacity (mL foam g^{-1} protein) and foaming stability were calculated following **Eq. 10** and **11** (sample calculations can be found in **Appendix L**).

$$\text{Foaming capacity} = \frac{\text{initial solution volume - initial liquid volume}}{\text{grams of protein in solution}} \qquad \textbf{Eq. 10}$$

$$\text{Foaming stability} = \frac{\text{solution volume at 30 min - liquid volume at 30 min}}{\text{initial solution volume - initial liquid volume}} \times 100 \qquad \textbf{Eq. 11}$$

2.3.9 Amino Acid Composition and Nutritional Quality of Hemp Protein Isolates

2.3.9.1 Amino Acid Composition

Amino acid composition of the HPI samples was kindly analyzed by Jason Neufeld from the Department of Food and Human Nutritional Sciences at the University of Manitoba (Winnipeg, MB, Canada). Amino acids were hydrolyzed according to the AOAC official method 982.30, and cysteine and methionine were hydrolyzed using performic acid oxidation per the AOAC official method 994.12. Tryptophan was determined using alkaline hydrolysis as described by Nosworthy et al. (2017). Amino acid composition was used to calculate the amino acid score (AAS) using **Eq. 12** and the nitrogen conversion factor of each HPI sample (sample calculations can be found in **Appendix M** and **N**),

$$\text{AAS} = \frac{\text{first limiting amino acid content of test protein}}{\text{first limiting amino acid in reference amino acid pattern}} \qquad \textbf{Eq. 12}$$

where the reference amino acid is that which is required for preschool children ages 2 to 5 years as defined by FAO/WHO Expert Consultation (1991).

2.3.9.2 In Vitro Protein Digestibility

In vitro protein digestibility of the HPI samples was kindly measured by Adam Franczyk from the Department of Food and Human Nutritional Sciences at the University of Manitoba (Winnipeg, MB, Canada) using the pH drop method as outlined by Franczyk (2018), based on the method developed by Hsu et al. (1977) and modified by Tinus et al. (2012). The enzyme solution was prepared by weighing 1.6 mg trypsin, 3.1 mg chymotrypsin, and 1.3 mg peptidase for each mL of solution required. The enzyme solution was stirred and heated to 37°C (± 1°C) in a water bath for 10 minutes. The pH was adjusted to 8.0±0.05 using 0.1-1M NaOH or HCl and placed in an ice bath to cool to 0 – 4°C. In triplicate, protein samples were weighed (62.5 mg ± 3 mg of crude protein) into a beaker and 10 mL Milli-Q water added. Sample solutions were stirred and heated to 37°C in a water bath for one hour, or until there were no clumps present. The pH was also adjusted to 8.0 ±0.05 using 0.1-1M NaOH or HCl. Exact starting pH of each sample solution was recorded, and the enzyme solution (1 mL) was immediately added. After 10 minutes, the final pH of the sample solution was recorded. Casein was run as a control alongside the HPI samples to assure consistency of the results. *In vitro* protein digestibility was calculated using **Eq. 2** (**Section 1.6.2**, example calculations shown in **Appendix N**).

2.3.9.3 Protein Digestibility-Corrected Amino Acid Score (PDCAAS)

Protein digestibility-corrected amino acid score (PDCAAS) for the HPI samples was calculated using the amino acid score and *in vitro* protein digestibility measurements following **Eq. 12, 2,** and **13** (example calculations shown in **Appendix N**).

PDCAAS = AAS of first limiting amino acid x *in vitro* digestibility **Eq. 13**

2.3.10 Statistical Analysis

Analysis of variance (ANOVA) was performed using RStudio Version 1.3.1073 for Windows (RStudio, Boston, MA, USA). Tukey-Kramer multiple means comparison test was used to determine significant differences ($P \leq 0.05$) among the means ($n \geq 3$) of three or more samples. ANOVA tables can be found in **Appendix O (Tables 26 – 54)**. A student's unpaired t-test was used to determine significant differences ($P \leq 0.05$) between the means of two samples.

2.4. Results and Discussion

2.4.1 Optimization of pH Extraction of Hemp Protein

Hemp protein is soluble under alkaline conditions due to a relatively high net negative charge at pH levels way above the isoelectric point (pH 5 – 6 for hemp protein) (Tang et al. 2006; Malomo and Aluko 2015b; Hadnađev et al. 2018; Potin et al. 2019; Shen et al. 2020a). However, solubilization under alkaline conditions risks protein denaturation, and causes browning due to oxidation of polyphenols not removed during dehulling and separation (Damodaran 2017; Shen et al. 2020a). Therefore, in this study pH extraction conditions were investigated in terms of their impact on protein purity, yield, color and protein characteristics.

Hemp protein demonstrated low solubility between pH 7 – 9 (**Figure 2**). Significantly higher solubility at pH 10 and pH 11 was observed, with no significant differences in solubility at pH 11 and pH 12. The higher solubility at alkaline pH has been attributed to the solubilization of more edestin, which makes up 60 – 80% of the proteins in hemp (Park et al. 2012; Malomo and Aluko 2015b). Potin et al. (2019) observed a similar trend in protein solubility at pH 7 – 12. Due to higher solubility at both pH 10 and 11, production of HPI was performed at both solubilization pHs to evaluate differences in protein purity and yield (**Table 6**).

The isoelectric point of hemp protein has been reported to be between pH 5 – 6 (Tang et al. 2006; Hadnađev et al. 2018; Galves et al. 2019; Shen et al. 2020a, b). While differences in protein yield have been previously observed when precipitation pH varied between pH 5 and 6, with prior solubilization at pH 10 (Shen et al. 2020a,b), no such significant differences among the tested precipitation pHs were noted in this study.

Therefore, pH 5 was selected as the optimal precipitation pH to use for the production of HPI.

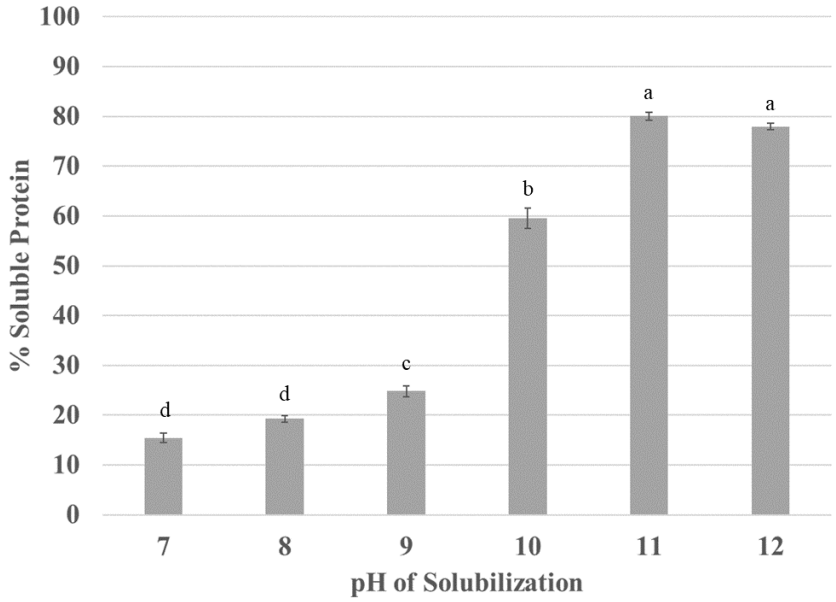


Figure 2. % Soluble protein at different solubilization pHs. Error bars represent standard error (n = 3). Different lowercase letters above the bars indicate significant differences among the samples according to the Tukey-Kramer multiple means comparison test ($P < 0.05$).

The significantly higher protein solubility at pH 11 than at pH 10 resulted in a significantly higher protein purity and yield for pH-HPI (**Table 6**). The difference in purity and yield can be explained by examining the protein lost in the discarded pellet and supernatant. The protein left in the discarded pellet represented the protein that was insoluble at the solubilization pH, while the residual protein in the discarded supernatant represented the protein that remained soluble at the precipitation pH. While there was a significant difference in the protein lost in the discarded supernatant, this difference was marginal. Solubilization at pH 10, on the other hand, resulted in a significantly higher protein residue in the discarded pellet as compared to solubilization at pH 11. Therefore, the higher protein purity and yield was attributed to a higher amount of protein being solubilized at pH 11 than at pH 10, complementing the screening results (**Figure 2**). The observed protein purity was comparable to previously reported values, while the protein

yield was markedly higher than reported yields (37.9 – 73.0%) (Wang et al. 2008, 2018; Yin et al. 2008; Malomo et al. 2014; Teh et al. 2014; Hadnađev et al. 2018; Shen et al. 2020a, b). The higher yield could be attributed to the double solubilization step, which allowed for more protein to be solubilized in an additional wash with a fresh alkaline solution. Interestingly, the protein yield for pH-HPI was much higher than that reported for soy protein (~60% protein yield), pea protein (~50 – 60% protein yield), and other oilseeds, such as camelina protein (36.8 – 38.4% protein yield) and sunflower protein (29.1 – 36.5% protein yield) following pH-assisted protein extraction (Kinsella 1979; Salgado et al. 2011; Boyle et al. 2018; Gao et al. 2020). These results demonstrated that alkaline extraction can be used successfully to produce an HPI with high protein purity and superior protein yield to that reported for other established and novel plant oilseed protein sources.

Table 6. Protein purity and yield as affected by the solubilization pH following alkaline solubilization coupled with isoelectric precipitation.

Solubilization pH	**HPI[1]**			**Discarded Pellet[2]**		**Discarded Supernatant[3]**	
	Protein Purity (%)	Protein Yield (%)	Ash (%)	Protein Purity (%)	Protein Residue (%)	Protein Purity (%)	Protein Lost (%)
10	84.8	64.2	1.75	45.2*	25.0*	30.2	7.39
11	88.1*	87.3*	2.37*	3.92	1.23	29.1	11.1*

[1] HPI – Hemp protein isolate. [2] Pellet discarded after alkaline solubilization. [3] Supernatant discarded after isoelectric precipitation. Protein purity (%) represents the amount of protein in the sample as determined by the Dumas method; Protein yield (%) represents the amount of protein extracted relative to the total amount of protein in the starting DHM; Protein residue (%) represents the amount of protein left in the discarded pellet relative to the total amount of protein in the starting DHM; Protein lost (%) represents the amount of protein lost to the discarded supernatant relative to the total amount of protein in the starting DHM. An asterisk (*) indicates a significant difference in each column as tested by the student's unpaired t-test ($P < 0.05$).

The high protein purity and yield observed in this study may also be attributed partially to the dehulling prior to defatting and protein extraction. Shen et al. (2020a) demonstrated a higher protein purity and yield for pH-extracted HPI from dehulled seeds (91.1% protein purity, 46.9% protein yield) than from non-dehulled seeds (82.2% protein

purity, 39.3% protein yield). Additionally, the high protein yield (73.0%) reported by Tang et al. (2006) was produced by pH-assisted extraction from dehulled hemp seeds. Hemp hulls contain phenolic compounds which, under alkaline conditions, can oxidize and promote protein polymerization, decreasing protein solubility (Ozdal et al. 2013). In addition to decreasing protein extraction yields, polymerization may have a negative impact on protein functionality. While there is not enough research to validate differences in functionality of proteins extracted from dehulled versus non-dehulled seeds, the reported higher protein extraction yields provided compelling data to support dehulling prior to protein extraction.

Difference in ash content between the two HPI samples, while significant, was likely due to slightly more salts produced with adjustment to a higher pH. The ash content of both HPIs was comparable to that of cSPI and cPPI (4.24% and 5.61%, respectively). Due to relatively high yield and purity, and low ash content, solubilization at pH 11 coupled with precipitation at pH 5 were selected as optimal pH extraction conditions. This study is the first to demonstrate successful production of HPI using solubilization at pH 11, rather than pH 10, coupled with precipitation at pH 5. The high protein yield and purity demonstrated feasibility of using these pH extraction parameters for industrial production of HPI.

2.4.2 Optimization of Salt Extraction of Hemp Protein

At low concentrations, salt enhances protein solubility by binding loosely to the protein and increasing electrostatic interactions with water. Subsequent centrifugation removes insoluble components (Smith 2017). This method demonstrates potential especially for plant proteins that contain mostly salt-soluble globulin-type proteins (Wolf 1970; Malomo and Aluko 2015b). Salt solubilization is less harsh than alkaline solubilization, preserving better the color and the protein native structure (Boyle et al. 2018). Once the protein is solubilized, it can be purified through the addition of more salt, which precipitates the protein, or through membrane filtration. One drawback of salt precipitation is that large amounts of water must be used to remove excess salt, which is expensive, wasteful, and thus not industrially feasible. In contrast, membrane filtration is a well-established industry purification process that has been shown to produce protein

isolates with high purity and good functionality (Boye et al. 2010a; Singhal et al. 2016). In this study, membrane filtration using ultrafiltration was used to purify proteins following salt solubilization.

When comparing protein solubility at different salt concentrations, hemp protein was significantly the least soluble at 0.5 M NaCl (**Figure 3**). While significantly more protein was solubilized at 1 M NaCl compared to 0.75 M, the difference was relatively small. Any salt added during extraction needs to be removed from the final protein isolate to avoid adverse effects on protein functionality (Tanger et al. 2022). The removal of salt requires large quantities of water, generating large amount of waste stream, thus limiting industrial feasibility. Accordingly, and considering the small difference in solubility, solubilization at 0.75 M instead of 1 M NaCl was further evaluated.

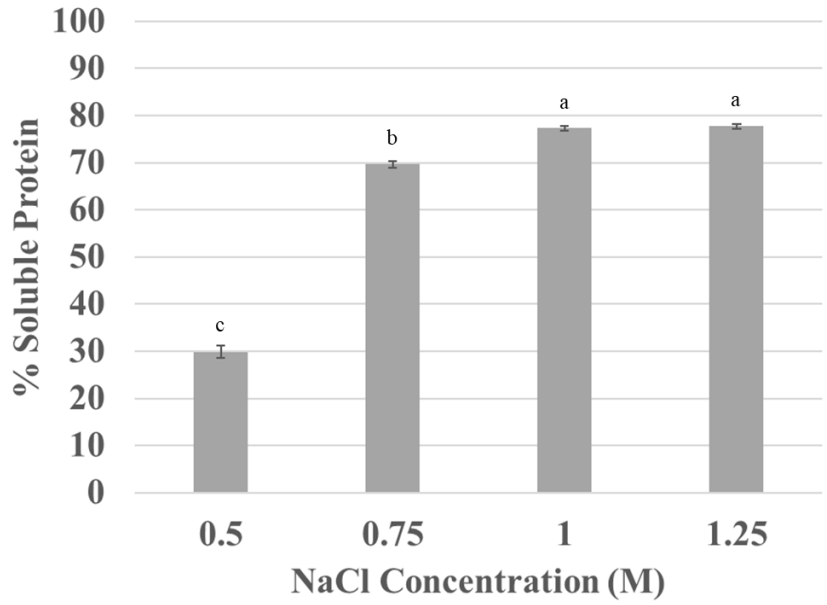


Figure 3. % Soluble protein at different salt (NaCl) concentrations when heated at 50°C. Error bars represent standard error (n = 3). Different lowercase letters above the bars indicate significant differences among the samples according to the Tukey-Kramer multiple means comparison test ($P < 0.05$).

Heating at 50°C during the solubilization step resulted in a 25% increase in soluble protein compared to solubilization at room temperature. This observation was in accordance to reported enhancement of camelina protein extraction when salt

solubilization was performed at 50°C (Boyle et al. 2018). Therefore, production of salt-HPI was carried out using solubilization in 0.5 M or 0.75 M NaCl at 50°C followed by ultrafiltration to determine the effect on protein purity and yield.

The significantly higher protein solubility at 0.75 M compared to 0.5 M NaCl resulted in a significantly higher protein purity and yield (**Table 7**). The protein content in the discarded pellet was significantly lower when solubilization was performed at 0.75 M compared to 0.5 M, explaining the observed higher protein yield. Similar to alkaline extraction coupled with isoelectric precipitation, salt solubilization produced an HPI (salt-HPI) with high protein purity and exceptionally high protein yield. The protein purity of salt-HPI is comparable to what has been reported for salt extracted pea protein (81 – 82%) and camelina protein (79.5 – 82.2%) (Tian et al. 1999; Karaca et al. 2011; Boyle et al. 2018). While Hadnađev et al. (2018) reported higher protein purity for their salt-extracted HPI, the protein purity was reported on dry basis and a higher nitrogen conversion factor (5.7) was used compared to the one (5.3) used in this study. The protein yield reported by Hadnađev et al. (2018), however, was half as that obtained in this study (40.2% compared with 81.6% protein yield). The higher yield in this study may be attributed to a second solubilization, which allowed for more protein to be solubilized with fresh salt solution, and a higher solubilization temperature used (50°C compared with 35°C). When comparing protein yield of salt-HPI to that of other salt extracted isolates, it was notably greater than that reported for pea protein (40% protein yield) and camelina protein (35.1 – 42.4% protein yield) (Tian et al. 1999; Boyle et al. 2018).

The high purity and yield in this study could be attributed to the use of membrane filtration for protein purification instead of salt precipitation. Following salt solubilization, Boyle et al. (2018) precipitated camelina protein using ammonium sulfite and used centrifugation to collect the precipitated proteins. However, some protein may have remained in solution and been discarded with the supernatant. In contrast, membrane filtration allows small molecular weight components, such as salts and small soluble sugars, to flow through the filters while retaining larger components, such as proteins. The protein yields reported with salt precipitation for camelina protein (35.1 – 42.4%) were much lower than observed in this study (81.6%) (Boyle et al. 2018). Additionally, reported protein purities were lower (79.5 – 82.2% protein) than observed

in this study (86.6% protein), as salt precipitation results in a higher residual salt content. These results demonstrated that following salt solubilization, membrane filtration is a more efficient purification process than salt precipitation.

Table 7. Purity and yield as effected by salt (NaCl) concentration following salt solubilization at 50°C coupled with ultrafiltration.

Salt Concentration (M)	**HPI[1]**			**Discarded Pellet[2]**	
	Protein Purity (%)	Protein Yield (%)	Ash (%)	Protein Purity (%)	Protein Residue (%)
0.5	82.7	50.3	8.38*	55.0*	40.9*
0.75	86.6*	81.6*	4.09	17.8	7.46

[1] HPI – hemp protein isolate. [2] Pellet discarded after salt solubilization. Protein purity (%) represents the amount of protein in the sample as determined by the Dumas method; Protein yield (%) represents the amount of protein extracted relative to the total amount of protein in the starting DHM; Protein residue (%) represents the amount of protein left in the discarded pellet relative to the total amount of protein in the starting DHM. An asterisk (*) indicates a significant difference in each column as tested by the student's two-sample unpaired t-test ($P < 0.05$).

The ash content was significantly higher in salt-HPI produced using 0.5 M NaCl than that produced using 0.75 M NaCl (**Table 7**), contrary to what was expected. This observation could be attributed to the effectiveness of the dialysis step. Due to the solubilization at higher salt concentration, more dialysis washes were performed, which resulted in a lower ash content. Given the higher yield and protein purity, solubilization at 0.75 M NaCl and at 50°C was determined as the optimal salt extraction conditions. These extraction parameters used slightly less salt (0.75 M compared with 0.8 M) and higher temperature (50°C compared with 35°C) than what has been used previously (Hadnađev et al. 2018), and resulted a markedly higher extraction yield. This work demonstrated that salt solubilization coupled with ultrafiltration could be an industrially feasible method for producing HPI.

2.4.3 Effect of Extraction Method on Color of HPI

The color of pH-HPI and salt-HPI was compared to that of cSPI and cPPI. Salt-HPI was the lightest and most neutral in color compared to the other isolates (**Table 8**). pH-HPI was significantly less light than cSPI and cPPI; however, the differences in lightness were small and might be negligible to the eye. The difference in lightness between salt-HPI and pH-HPI can be attributed to some browning that could have occurred due to the high alkalinity of the pH extraction. Salt-HPI had a higher lightness value than previously reported for HPI (Hadnađev et al. 2018; Galves et al. 2019; Shen et al. 2020a). pH-HPI had a higher lightness value than that reported for HPI produced from non-dehulled hemp, and a similar lightness value to the HPI produced from dehulled hemp (Shen et al. 2020a). The reported dark color of HPI was attributed to the oxidation of polyphenols, present in the hulls and seed coatings, during alkaline extraction (Girgih et al. 2014; Teh et al. 2014; Hadnađev et al. 2018). In this study, seeds were dehulled prior to defatting and protein extraction, providing further evidence that dehulling is a necessary step to avoid undesirable browning.

Table 8. Color (L* a* b*) of commercial soy protein reference (cSPI), commercial pea protein reference (cPPI), and pH-extracted and salt-extracted hemp protein isolates (pH-HPI and salt-HPI, respectively).

Protein Isolate	L*	a*	b*
cSPI	$86.2^{b\wedge}$	-0.2^{b}	$+14.8^{b}$
cPPI	86.6^{b}	$+0.2^{a}$	$+19.1^{a}$
pH-HPI	82.8^{c}	$+0.3^{a}$	$+11.5^{c}$
Salt-HPI	91.9^{a}	-0.1^{b}	$+5.22^{d}$

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

While statistically significant, the differences in redness/greenness among all four samples were minimal (**Table 8**). Both HPI samples were significantly less yellow than cSPI and cPPI, with salt-HPI demonstrating the least yellowness. Redness and yellowness for salt-HPI and pH-HPI were lower than that reported by Shen et al. (2020a)

for dehulled pH-HPI. Salt-HPI and pH-HPI produced in this study exhibited a light and neutral color, which is ideal for consumer acceptability in a variety of applications.

2.4.4 Effect of Extraction Method on Protein Structural Characteristics

2.4.4.1 Protein Profile

The protein profile of pH-HPI and salt-HPI was compared to that of cSPI and cPPI reference samples under reducing and non-reducing conditions using SDS-PAGE (**Figure 4**). cSPI and cPPI had a larger proportion of higher molecular weight (>50 kDa) globulin proteins compared to HPI samples. The main globulin proteins in soy are glycinin (11S; 320 kDa) and β-conglycinin (7S; 180-210 kDa), and in pea are legumin (11S; 320-380 kDa) and vicilin (7S; 175-180 kDa) (Boye et al. 2010a; Singh et al. 2015; Reinkensmeier et al. 2015). Additionally, cSPI and cPPI had a relatively smaller proportion of non-functional albumins (<20 kDa) compared to HPI samples (**Figure 4**, Lanes 2-3 and 6-7). The greater proportion of albumin proteins suggests that HPI might demonstrate limited functionality when compared to cSPI and cPPI.

All major hemp protein compounds were observed in both pH-HPI and salt-HPI. Under non-reducing conditions, an intense protein band around 50 kDa corresponded to the monomers of 11S edestin and to the subunits of 7S vicilin-like protein (**Figure 4**, Lanes 4-5). Another intense band around 13 kDa under non-reducing conditions corresponded to the 2S albumins. The band at 100 kDa under non-reducing conditions was attributed to protein aggregates linked by disulfide bonds (Wang et al. 2008; Hadnađev et al. 2018).

Under reducing conditions, two intense bands in both pH-HPI and salt-HPI at ~ 35 and ~ 20 kDa have been identified as the acidic and basic subunits of 11S edestin, respectively (**Figure 4**, Lanes 8-9). These subunits are linked by a disulfide bond as evident by their separation under reducing conditions. The presence of basic subunits of two different molecular weights (~18 and 20 kDa) has been previously reported (Wang et al. 2008; Potin et al. 2019). The fainter band still present at 50 kDa under reducing conditions corresponded to 7S vicilin-like protein (Potin et al. 2019). This protein is known to contain no disulfide linkages and to be much less abundant than 11S edestin (5% of total hemp proteins are 7S vicilin-like protein compared to 60 – 80% which are

11S edestin) (Sun et al. 2021). The two polypeptide chains under 10 kDa, observed under reducing conditions, indicated that these albumin chains are linked by disulfide linkages (Aluko 2017). The higher intensity of the albumin and edestin bands compared to that corresponding to vicilin-like protein indicated that they are present in relatively higher proportion.

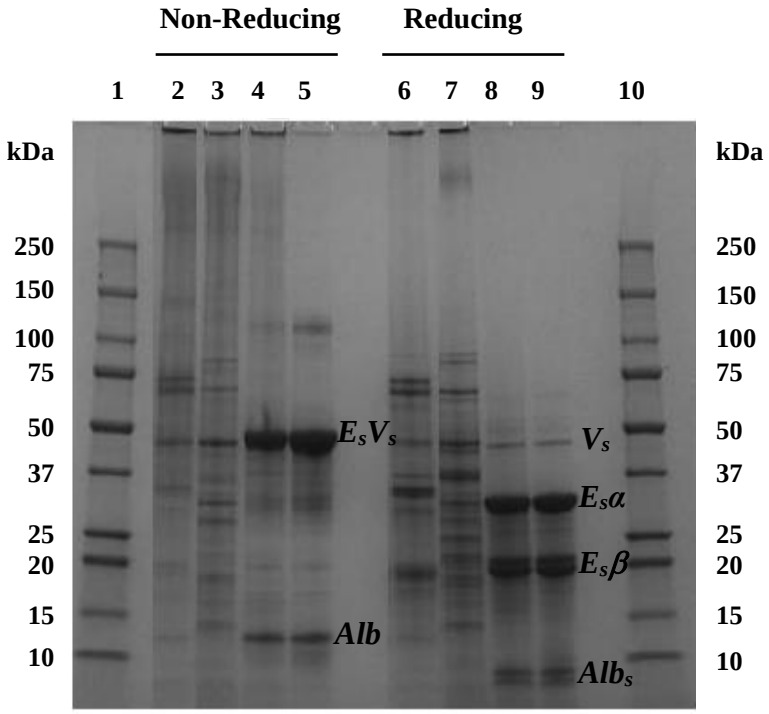


Figure 4. SDS-PAGE visualization of the protein profiles of hemp protein isolates (pH-HPI and salt-HPI) compared with commercially produced soy and pea protein isolates (cSPI and cPPI) under non-reducing (lanes 2-5) and reducing (lanes 6-9) conditions. Lane 1, 10: Molecular weight (MW) marker; Lane 2, 6: cSPI; Lane 3, 7: cPPI; Lane 4, 8: pH-HPI, Lane 5, 9: salt-HPI. E_s: subunits of 11S edestin; V_s: subunits of 7S vicilin-like protein; Alb: 2S albumins; $E_s\alpha$: acidic subunit cleaved from edestin; $E_s\beta$: basic subunit cleaved from edestin; Alb_s: albumin subunits.

Processing conditions can alter the inherent protein profile. Commercially produced protein isolates undergo heating for pasteurization and inactivation of antinutritional factors. Heating causes denaturation of the proteins, which may lead to polymerization via hydrophobic and disulfide linkages resulting in the formation of large insoluble aggregates (Akharume et al. 2021). Under non-reducing and reducing conditions, cSPI and cPPI had intense banding at the top of the gel, indicating presence of

protein polymers of molecular weights > 250 kDa (**Figure 4**, Lanes 2-3 and 6-7). The presence of these bands under non-reducing and reducing conditions suggested that irreversible protein aggregates were formed via covalent bonding. Additionally, intense streaking was evident for cSPI and cPPI under non-reducing conditions (**Figure 4**, Lanes 2-3). This streaking was somewhat lessened under reducing conditions, suggesting protein polymerization formed via covalent bonds attributed in part to disulfide linkages. These results indicated that cSPI and cPPI were most likely denatured.

Under non-reducing conditions, smearing in the upper part of the lane can be seen for pH-HPI and, to a lesser extent, for salt-HPI (**Figure 4**, Lanes 4-5). Under reducing conditions, smearing was no longer observed, indicating that polymerization in HPI occurred via disulfide linkages (**Figure 4**, Lanes 8-9). Higher extent of polymerization in pH-HPI compared to salt-HPI is attributed to the high alkalinity during protein extraction. High alkaline conditions promote denaturation and protein cross-linking via oxidation of the sulfhydryl groups (Damodaran 2017). The low degree of polymerization observed for salt-HPI suggested that it had undergone less protein denaturation than pH-HPI.

For the most part, the protein profiles of pH-HPI and salt-HPI were similar, suggesting that both protein extraction methods were successful in extracting similar protein components. The higher degree of polymerization noted for pH-HPI may have an impact on protein functionality.

2.4.4.2 Protein Denaturation

Proteins can become denatured by heat, extreme pH, shear, pressure, or organic solvents (Damodaran 2017). Employing these processing parameters during the production of a protein isolate can cause a loss of the protein's native structure, impacting functional and nutritional characteristics. Accordingly, the denaturation states of cSPI, cPPI, pH-HPI, and salt-HPI were evaluated using differential scanning calorimetry (DSC) (**Table 9**). Gradual heating of the protein samples during the DSC analysis produces thermograms showing endothermic peaks with denaturation temperature and enthalpy indices. The lack of endothermic peaks for cSPI and cPPI demonstrated that these protein samples were already denatured, which has been previously reported for commercial

protein isolates (Lee et al. 2003). This observation explained the degree of polymerization observed for these samples (**Figure 4**).

Endothermic peaks were seen for both HPI samples, indicating that these proteins had not been completely denatured during extraction (**Table 9**). Two peaks were observed for pH-HPI, corresponding to 11S edestin and the 7S vicilin-like protein. The denaturation temperature observed for 11S edestin was slightly lower than the reported range of 89 – 95°C (Tang et al. 2006; Wang et al. 2008; Hadnađev et al. 2018; Shen et al. 2020a, b). Differences in reported denaturation temperatures could be attributed to differences in moisture and salt content. The enthalpy of denaturation measured for pH-HPI was lower than the reported range of 5 – 11 J g^{-1} (Tang et al. 2006; Wang et al. 2008; Hadnađev et al. 2018; Shen et al. 2020a, b). However, previous studies did not report observation of a peak for 7S vicilin-like protein (Tang et al. 2006; Wang et al. 2008; Hadnađev et al. 2018; Shen et al. 2020a,b). Enthalpy of denaturation is measured by integrating the area of the endothermic peak. The combined enthalpy of denaturation for 11S edestin and 7S vicilin-like protein in pH-HPI fitted within the reported range and can be most explained by the differences in extraction conditions such as the pH used during solubilization and the final salt content.

One endothermic peak was observed for salt-HPI, which was attributed to 11S edestin (**Table 9**). The denaturation temperature was within the range (89 – 95°C) previously reported (Tang et al. 2006; Wang et al. 2008; Hadnađev et al. 2018; Shen et al. 2020a,b). However, the denaturation temperature was lower than that reported (96.2°C) for salt-extracted HPI (Hadnađev et al. 2018), which again could be attributed to differences in salt and moisture content. Additionally, the salt-HPI had a slightly lower enthalpy of denaturation than previously reported, which could have been similarly impacted by the residual salt content of the protein isolate. It is important to note that while the one endothermic peak of salt-HPI was attributed to 11S edestin, it is possible that this large peak encompasses both 11S edestin and 7S vicilin-like protein.

Based on observed enthalpy of denaturation, pH-HPI was determined to be more denatured than salt-HPI, supporting protein profiling observations (**Figure 4**). Differences in the denaturation state or degree of unfolding can impact the surface properties of the protein and in turn the functional properties of the two HPIs.

Table 9. Denaturation temperatures and enthalpy, surface hydrophobicity, surface charge, and secondary structure of commercial soy protein (cSPI), commercial pea protein (cPPI), and pH-extracted and salt-extracted hemp protein isolates (pH-HPI and salt-HPI, respectively).

Samples	Denaturation Temperature and Enthalpy				Surface Properties			Secondary Structure			
					Surface Hydrophobicity	Surface Charge					
	Denaturation Temperature	Enthalpy of Denaturation	Denaturation Temperature	Enthalpy of Denaturation		Dissolved in DDW	Dissolved in 0.5 M NaCl	α Helix	β Sheet	β Turn	Random Coil
	Td, °C	ΔH, J g-1	Td, °C	ΔH, J g-1	RFI	mV	mV	Relative Percentage			
	β-conglycinin (7S)		***Glycinin (11S)***								
cSPI	~	~	~	~	9968$^{b\wedge}$	-46.5$^{a\#}$	-7.1^{a}	N/A†	N/A	N/A	N/A
	Vicilin (7S)		***Legumin (11S)***								
cPPI	~	~	~	~	12656^{b}	-36.9$^{b\#}$	-7.0^{a}	N/A	N/A	N/A	N/A
	Vicilin-like Protein (7S)		***Edestin (11S)***								
pH-HPI	74.2	4.87	80.7	2.07	20062^{a}	-30.8$^{c\#}$	-6.3^{a}	17.5	39.9	25.8	16.8*
Salt-HPI	~	~	91.0*	19.2*	18035^{a}	-25.6$^{d\#}$	-6.9^{a}	24.5*	38.9	26.4	10.1

~ No peak of denaturation was observed; An asterisk (*) indicates a significant difference in each column as tested by the student's unpaired t-test ($P < 0.05$); $^{\wedge}$ Means ($n \geq 3$) in each column with different lowercase letters indicate significant differences among samples according to the Tukey-Kramer multiple means comparison test ($P < 0.05$); A number sign ($^{\#}$) indicates a significant difference between sample dissolved in DDW and in 0.5 M NaCl as tested by the student's unpaired t-test ($P < 0.05$); † N/A indicates that relative percentage of secondary structures were not determined, as inherent properties are different from different protein sources.

2.4.4.3 Protein Surface Properties

Both pH-HPI and salt-HPI had significantly higher surface hydrophobicity than cSPI and cPPI (**Table 9**). Surface hydrophobicity of pH-HPI and salt-HPI was almost double that of cSPI. This observation aligns with findings by Galves et al. (2019) who reported higher surface hydrophobicity for hemp protein compared to other oilseeds. The lower surface hydrophobicity of cSPI and cPPI compared to HPIs could be attributed to the formation of large polymers due to both hydrophobic interactions and disulfide interactions (**Figure 4**) among the denatured proteins. Additionally, hemp protein may have in general more hydrophobic residues on the surface compared to soy and pea protein. This high surface hydrophobicity could majorly impair protein solubility. High levels of hydrophobic residues on the surface may limit hemp protein's interactions with water and, subsequently, its functionality in aqueous systems.

Surface charge is another important property impacting protein functionality. The presence of charged residues on the protein's surface can increase protein-water interactions, improving overall functionality (Damodaran 2017). cSPI had a more negative surface charge than cPPI (**Table 9**), which agrees with the findings of Karaca et al. (2011). The surface charge of pH-HPI and salt-HPI were lower than that of cSPI and cPPI and were similar to the value reported by Galves et al. (2019). pH-HPI had a higher surface charge than salt-HPI. The higher ash (salt) content in salt-HPI compared to pH-HPI (**Tables 6** and **7**) may have caused partial shielding of the charges on the surface of the protein.

Surface charge of the proteins was also measured in 0.5 M NaCl solutions (**Table 9**). During preliminary testing with salt-HPI, poor solubility and sedimentation was observed. The predominant class of proteins in hemp, globulins, is salt-soluble, while the minor class, albumins, is water-soluble (Sun et al. 2021). Therefore, to fully investigate HPI's potential functionality in a food system, structural and functional testing was carried out in 0.5 M NaCl solution and in water. The salt concentration was selected based on preliminary testing. In 0.5 M NaCl, there was no significant difference in surface charge among the samples (**Table 9**). Salt at a concentration around 0.5 M can shield the charges on the surface of the protein (Damodaran 2017). Therefore, in 0.5 M

NaCl solution there was a significant decrease in measurable overall surface charge in all samples.

In the native state, proteins fold via intramolecular interactions to minimize free energy (Damodaran 2017). Generally, this state is achieved by orienting hydrophobic groups to the interior and hydrophilic groups to the exterior. However, inherent differences in the amino acid sequence affect how many hydrophobic residues remain on the surface. Plant proteins in general have high surface hydrophobicity compared to animal protein such as whey protein (Ismail et al. 2020). Overall, both HPIs had higher surface hydrophobicity and lower surface charge than cSPI and cPPI. These structural properties indicated that hemp protein will most likely interact less with water than soy and pea protein, and thus may have limited functionality.

2.4.4.4 Protein Secondary Structures

The impact of protein extraction method on the secondary structure of hemp protein was monitored using ATR-FTIR. FTIR allowed for the quantification of the relative abundance of α helix, β sheet, β turn, and random coil in pH-HPI and salt-HPI (sample spectrum **Figures 7** and **8** in **Appendix G**). While there was no significant difference in the relative abundance of β sheet and β turn between pH-HPI and salt-HPI, there were significant differences in the abundance of α helix and random coil (**Table 9**). Salt-HPI had a higher abundance of α helix than pH-HPI, while pH-HPI had a higher abundance of random coil than salt-HPI. Similarly, Hađnadev et al. (2018) noted a lower abundance of α-helix for pH-extracted HPI when compared with salt-extracted HPI. The abundance of α-helix decreases upon protein denaturation, while the abundance of unordered or random structures increases with higher alkalinity (Qi et al. 2004; Yu 2005; Mitra et al. 2017). Additionally, the ratio of β sheet to α helix was higher for pH-HPI than salt-HPI (2.28 and 1.58, respectively), reflecting the trend observed with protein denaturation (Yu 2005; Mitra et al. 2017). In general, pH-HPI demonstrated a loss of ordered secondary structure accompanied with relatively higher β-sheet, providing further evidence for its partial denaturation compared to salt-HPI. This change in structure may impact the functionality of pH-HPI as compared to salt-HPI.

2.4.5 Effect of Extraction Method on Protein Functional Characteristics

2.4.5.1 Protein Solubility

Solubility in water and in 0.5 M NaCl was measured at pH 3.2 and 7 at 1% protein under non-heated and heated (at 80°C) conditions to investigate their potential use in neutral and acidic beverages (**Table 10**). The concentration of protein was chosen based on preliminary testing demonstrating higher solubility at 1% protein for HPIs when compared with 2% and 5% protein concentration.

At pH 7 in DDW, cSPI had the highest solubility under heated and non-heated conditions (**Table 10**). This was attributed to cSPI having the lowest surface hydrophobicity and highest surface charge at pH 7, favoring protein-water interactions over protein-protein interactions. Soy protein maintained its solubility even when heated to temperatures around 70 – 80°C (Lee et al. 2003). Both pH-HPI and salt-HPI exhibited the lowest solubility at pH 7 under heated and non-heated conditions. HPI had overall lower surface charge and higher surface hydrophobicity than cSPI and cPPI, contributing to its lower solubility. Generally, a surface charge greater than $\pm$ 30 mV results in better protein solubility (Long and Labute 2010), which was seen for soy and pea but not for hemp protein. Low solubility of HPI at pH 7 regardless of extraction method has been previously reported (Malomo and Aluko 2015b; Hadnađev et al. 2018; Shen et al. 2020a, b), and is further explained by our observed surface properties.

At pH 3.2 in DDW, no statistically significant difference in solubility was observed among cSPI, pH-HPI, and salt-HPI (**Table 10**). The higher solubility of HPI at acidic pH can be explained by an increase in surface charge when compared to pH 7, which is closer to the isoelectric point for hemp protein (pH 5 – 6) (Tang et al. 2006; Hadnađev et al. 2018; Galves et al. 2019; Shen et al. 2020a,b). As a protein gets further from its isoelectric point, the surface charge increases. Above the isoelectric point, the protein will carry a net negative charge, while below the isoelectric point, the protein will carry a net positive charge (Damodaran 2017). A greater surface charge causes electrostatic repulsion between the protein molecules, encouraging hydration and increasing solubility. While zeta potential was only measured at pH 7 in this study, a greater surface charge has been reported for HPI at pH 3 compared to pH 7 (Shen et al.

2020a,b). Therefore, higher solubility at pH 3.2 can be explained by a higher net surface charge that could counteract the high surface hydrophobicity.

Protein solubility decreased for cSPI and cPPI at pH 7 under non-heated conditions when dissolved in 0.5 M NaCl, while protein solubility for pH-HPI and salt-HPI increased (**Table 10**). Commercial soy protein isolates have demonstrated low solubility in 0.5 M NaCl, especially when complete denaturation was confirmed (Lee et al. 2003). Different proteins precipitate out at different salt concentrations. While it appears that soy and pea were salted out, hemp protein was salted in at pH 7. The salt increased the charge load on the hemp protein, and in contrast shielded the charges on the surface of cSPI and cPPI. The impact of salt addition is dependent, however, on the pH, which dictates level of ionization of the amino acid residues on the surface of the protein. Mostly the globulins would have been salted in for hemp as they are the most abundant and are salt-soluble (Sun et al. 2021). At pH 3.2, the salt most likely shielded the positive charge on the surface of the HPI, explaining the observed decrease in the solubility of pH-HPI and salt-HPI in 0.5 M NaCl compared to water.

Overall, these results indicated that hemp protein could serve as a suitable alternative to soy protein in acidic beverages or could replace soy protein in high salt food applications where good protein solubility is desired at neutral pH. However, functionality of hemp protein in food systems at neutral pH may be limited, as good solubility is a precursor for other functional properties (Singh et al. 2008).

Table 10. Solubility, gel strength, and water holding capacity of commercial soy protein (cSPI), commercial pea protein (cPPI), and pH-extracted and salt-extracted hemp protein isolates (pH-HPI and salt-HPI, respectively).

Samples	Solubility in DDW (1% protein)				Solubility in 0.5 M NaCl (1% protein)				Gel Strength (15% protein)		Water Holding Capacity	
	pH 7		pH 3.2		pH 7		pH 3.2		DDW	0.5 M NaCl	DDW	0.5 M NaCl
	Non-heated	Heated at 80°C	Non-heated	Heated at 80°C	Non-heated	Heated at 80°C	Non-heated	Heated at 80°C	Strength (N)	Strength (N)	%	%
cSPI	67.8$^{a\wedge\#}$	82.5$^{a\#}$	52.9$^{a\#}$	65.2$^{a\#}$	26.6^{b}	28.6^{c}	16.6^{a}	18.8^{ab}	19.7$^{a\#}$	8.91^{c}	99.8^{a}	99.9^{a}
cPPI	41.8^{b}	60.1$^{b\#}$	16.2^{b}	28.0$^{b\#}$	24.4^{b}	34.1^{c}	13.4^{a}	14.5^{b}	2.07^{c}	N/A†	99.9^{a}	N/A†
pH-HPI	15.5^{c}	18.4^{c}	57.2$^{a\#}$	63.0$^{a\#}$	55.5$^{a\#}$	57.0$^{a\#}$	21.3^{a}	12.7^{b}	12.9^{b}	29.4$^{b\#}$	86.6$^{b\#}$	71.2^{c}
Salt-HPI	22.0^{c}	18.1^{c}	55.0$^{a\#}$	61.5$^{a\#}$	45.0$^{a\#}$	43.7$^{b\#}$	22.7^{a}	23.3^{a}	N/A†	36.4 $^{\Psi\,a}$	N/A†	81.4^{b}

$^{\wedge}$ Means ($n \geq 3$) in each column with different lowercase letters indicate significant differences among samples according to the Tukey-Kramer multiple means comparison test ($P < 0.05$); A number sign ($^{\#}$) indicates a significant difference between sample dissolved in DDW and in 0.5 M NaCl as tested by the student's unpaired t-test ($P < 0.05$); † No gels formed at 15% protein concentration under conditions specified in table; $^{\Psi}$ Salt-HPI gel formed in 0.5 M NaCl was not homogenous; bottom portion of gel was harder and upper portion of gel was softer.

2.4.5.2 Gel Strength and Water Holding Capacity

Gels were formed by heating above the protein's denaturation temperature. Different protein concentrations were tested to determine the least gelation concentration for HPI. pH-HPI formed a gel at 10% protein in water. However, in water salt-HPI would sediment out before heating, thus no gel network could be formed. Therefore, different concentrations of salt solution were tested for forming gels with salt-HPI. The least gelation concentration of salt-HPI was determined to be 12.5% protein in 0.5 M NaCl, as salt was needed to increase protein-water interactions and prevent immediate sedimentation. Gelation is an important functional property in meat, cheese, and bakery applications. Gelling properties are dependent on intrinsic factors, such as amino acid composition, protein profile, and protein size, and on extrinsic factors, such as temperature, pH, and ionic strength (Damodaran 2017).

cSPI produced the strongest gel when dissolved in water at 15% protein concentration (**Table 10**). Soy protein has been reported to have good gelation properties, with the 11S globulins being responsible for higher gel strength than 7S globulins (Kinsella 1979). cPPI has been reported to produce weaker gels than soy protein (Lam et al. 2018), which aligns with our results. pH-HPI produced a stronger gel than cPPI, while salt-HPI sedimented out before the gel network could form in water. This sedimentation may have occurred due to an imbalance of much stronger protein-protein interactions than protein-water interactions (Damodaran 2017), due to low surface charge and high surface hydrophobicity at pH 7. Generally, larger molecular weight proteins are needed for gel formation, as proteins smaller than 23 kDa generally have lower tendency to form a stable gel network (Damodaran 2017). However, if these smaller proteins contain sufficient sulfhydryl groups, polymerization can occur, leading to a strong gel network as is the case for whey protein (14 – 18 kDa) (Boland 2011; Damodaran 2017). pH-HPI's ability to form a moderately strong gel network may be due to the presence of disulfide linkages among the smaller molecular weight proteins observed by SDS-PAGE (**Figure 4**), promoting crosslinking via disulfide interchange. Additionally, HPI contains a similar proportion of β-sheets to what has been reported for soy protein (Zhu et al. 2020). β-sheet content has been positively correlated with gel strength (Guo et al. 2017), which may also contribute to pH-HPI's ability to form a strong gel.

When dissolved in 0.5 M NaCl, cSPI formed a weaker gel than in water, while cPPI did not form a gel (**Table 10**). This is in accordance with the observed reduced solubility in the salt solution. HPI gels were stronger in 0.5 M NaCl than in water, which agreed with the findings of Dapčević-Hadnađev et al. (2018). The improved gel strength of HPI may be desirable in high-salt comminuted meat products such as frankfurters, which contain salt concentrations around 0.4 M NaCl (Sun and Arntfield 2011). However, the gel produced by salt-HPI varied in consistency, with the gel being softer toward the top and harder toward the bottom. These results suggested that the addition of salt reduced, but did not eliminate, the sedimentation of salt-HPI. However, this phenomenon may not be an issue for texturization, as shear and mixing would provide continuous agitation (Zhang et al. 2019). Hemp protein has demonstrated the ability to cross-link, which could translate to good texturization potential as proteins unfold and aggregate during texturization (Zhang et al. 2019). The HPI gels produced demonstrated a more fibrous texture compared to soy and pea protein gels, which is desirable for texturization. Overall, pH-HPI compared to salt-HPI demonstrated promising gelation properties and texturization potential.

Water holding capacity (WHC) is another measure related to gelling ability and refers to the ability to physically entrap water within the protein gel matrix (Damodaran 2017). High WHC provides juiciness and tenderness for gel-type food applications, such as meat products or meat alternatives. WHC has yet to be reported for HPI. When solubilized in water, soy and pea protein isolates retained almost all the water (**Table 10**). WHC of cSPI remained unaffected when the gel was formed in 0.5 M NaCl, while the additional salt prevented cPPI gel formation. pH-HPI had high WHC when solubilized in water. However, solubilization in 0.5 M NaCl significantly reduced the WHC. The addition of salt can cause formation of a gel network with larger pores, resulting in decreased WHC (Dapčević-Hadnađev et al. 2018). Salt-HPI had significantly higher WHC than pH-HPI when the gel was prepared in 0.5 M NaCl. These results indicated that pH-HPI can provide good WHC in gel-type food applications, while salt-HPI may provide good WHC in the presence of higher salt concentrations.

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

When solubilized in water, cSPI had significantly the highest EC (**Table 11**), which agreed with the observations of Karaca et al. (2011) showing a higher EC for soy than pea protein. Neither HPI sample formed a visible emulsion when solubilized in water. These results are most likely due to poor protein-water interactions, and the resultant poor solubility at neutral pH (**Table 10**), rather than a lack of hydrophobic residues on the surface (**Table 9**), demonstrating a poor hydrophobic to hydrophilic balance. When solubilized in 0.5 M NaCl, both HPI samples produced emulsions with salt-HPI showing similar EC compared to cSPI, and pH-HPI showing similar EC to that of cPPI. The enhanced solubility of HPI at pH 7 in 0.5 M NaCl (**Table 10**) can explain the higher EC, as HPI was better dispersed and able to reach the oil-water interface instead of falling out of solution. When emulsions could be formed, salt-HPI demonstrated higher EC than pH-HPI, which aligns with the trend seen for salt- and pH-extracted camelina proteins (Boyle et al. 2018).

The emulsification properties of hemp protein have been previously evaluated by monitoring emulsion oil droplet size rather than by directly measuring EC (Malomo et al. 2014; Malomo and Aluko 2015b, a; Dapčević-Hadnađev et al. 2019). A smaller oil droplet size was related to good emulsification properties. At neutral pH, Malomo and Aluko (2015b) reported a smaller oil droplet size for pH-extracted HPI than hemp protein concentrate produced by solubilization with enzymes (cellulase, hemicellulase, xylanase, and phytase) followed by ultrafiltration, and commercial hemp protein concentrate. At acidic pH, Dapčević-Hadnađev et al. (2019) reported larger oil droplet sizes for pH-extracted HPI than salt-extracted HPI, which contributed to the authors' conclusions that salt-extracted HPI had superior emulsifying properties.

Table 11. Emulsification capacity, stability, and activity index and foaming capacity and stability of commercial soy protein (cSPI), commercial pea protein (cPPI), and pH-extracted and salt-extracted hemp protein isolates (pH-HPI and Salt-HPI, respectively).

Samples	Emulsification Capacity (1% protein)		Emulsification Stability		Emulsification Activity Index		Foaming Capacity		Foaming Stability	
	DDW	0.5 M NaCl	DDW	0.5 M NaCl	DDW	0.5 M NaCl	DDW	0.5 M NaCl	DDW	0.5 M NaCl
	g oil/ g protein	g oil/ g protein	min	min	m^2/g	m^2/g	mL foam/ g protein	mL foam/ g protein	%	%
cSPI	1194*$^{\#}$	832$^{a\wedge}$	11.5^{b}	12.3^{b}	144.8^{a}	268.4$^{a\#}$	144^{c}	413$^{b\#}$	74.1^{b}	74.8^{a}
cPPI	777$^{\#}$	707^{b}	12.5^{b}	11.5^{b}	185.5^{a}	273.3$^{a\#}$	292^{a}	717$^{a\#}$	13.7^{d}	56.9$^{a\#}$
pH-HPI	N/A†	740^{b}	N/A$^{\dagger\times}$	30.9^{a}	32.6$^{\wedge b}$	53.4$^{b\#}$	78.7^{d}	225$^{c\#}$	91.6$^{a\#}$	62.8^{a}
Salt-HPI	N/A†	831^{a}	16.7$^{\dagger a}$	26.1$^{a\#}$	147.2$^{\sim a\#}$	71.2^{b}	203^{b}	240^{c}	35.4$^{c\#}$	5.0^{b}

An asterisk (*) indicates a significant difference in each column as tested by the student's unpaired t-test ($P < 0.05$); † pH-HPI and salt-HPI did not form an emulsion when dissolved in DDW; $^{\wedge}$ Means ($n \geq 3$) in each column with different lowercase letters indicate significant differences among samples according to the Tukey-Kramer multiple means comparison test ($P < 0.05$); A number sign ($^{\#}$) indicates a significant difference between sample dissolved in DDW and in 0.5 M NaCl as tested by the student's unpaired t-test ($P < 0.05$); $^{\times}$ Measurements could not be made due to poor emulsion characteristics.

There was no significant difference in EAI and ES between cSPI and cPPI in water (**Table 11**). In water, salt-HPI had the highest ES (**Table 11**). However, it is important to note that both salt-HPI and pH-HPI did not form emulsions at 1% protein in water, which affected EAI readings. Because ES is calculated by comparing the difference in initial and final absorbance values, a low initial absorbance could cause a higher ES measurement, which is a systematic issue with this assay. The results of this study, however, aligned with the higher ES and EAI reported for pH-extracted HPI compared with SPI (Tang et al. 2006).

In 0.5 M NaCl, EAI of cSPI and cPPI increased, while no impact on ES was observed (**Table 11**). pH-HPI and salt-HPI had the highest ES, but EAI was low for both samples in 0.5 M NaCl. No significant difference in ES and EAI between salt-HPI and pH-HPI was observed, contrary to the findings reported by Dapčević-Hadnađev et al. (2019). The authors used higher protein concentrations (0.25 – 1.5% protein w/v) for emulsion preparation than used in this study (0.1% protein w/v). A higher protein concentration may be needed to better observe significant differences in emulsion properties, as the authors noted that pH-extracted HPI likely did not completely cover the surface of the oil droplets at low concentrations (0.25% protein). However, the effect of solubilization in salt solutions on HPI emulsions has not been previously reported. Results of this study, in general, highlighted the potential of improving emulsification properties of HPI upon the addition of salt.

Overall, hemp protein demonstrated inferior emulsification properties compared to soy and pea. Previous studies have noted hemp protein's inferior emulsification properties when compared to soy, and low solubility was determined to be the cause for hemp protein's underperformance as an emulsifier (Tang et al. 2006; Teh et al. 2014). When protein solubility is high (> 50%), surface hydrophobicity has a larger impact on emulsification properties, whereas when protein solubility is low (< 50%), solubility has a larger impact on emulsification properties (Li-Chan et al. 1984). Strategies to increase HPI solubility, such as solubilization in salt solution instead of water, could be used to improve emulsification properties.

2.4.5.4 Foaming Capacity (FC) and Stability (FS)

In water, cPPI had the highest FC, followed by salt-HPI. pH-HPI produced significantly less foam compared to all other samples. While foaming properties of salt-extracted HPI have not been reported, our findings align with a higher FC reported for salt-extracted camelina protein compared to pH-extracted camelina protein (Boyle et al. 2018). In 0.5 M NaCl, cPPI had the highest FC, but cSPI produced more foam than pH-HPI and salt-HPI. The improved solubility of HPI in the presence of salt (**Table 10**) may explain the significantly higher FC for pH-HPI in salt than in water, as hemp protein's FC has been positively correlated to its solubility (Malomo et al. 2014). These findings suggest that salt (NaCl) can be used to enhance foaming for pH-HPI in applications where foaming is desired. In applications at neutral pH where foaming is not desired, pH-HPI would be preferred instead of salt-HPI, and salt content in the formulation should be minimized.

In water, pH-HPI had the highest FS, while cPPI had the lowest FS. Malomo and Aluko (2015b) reported the highest FS at neutral pH for pH-extracted HPI compared to hemp protein meal, commercial hemp protein concentrate, and hemp protein concentrate produced using solubilization with the aid of enzymes (cellulase, hemicellulase, xylanase, and phytase) followed by membrane filtration (ultrafiltration). In 0.5 M NaCl, there was no significant difference in FS among cSPI, cPPI, and pH-HPI, while salt-HPI had significantly the lowest FS. The addition of salt was detrimental to FS of salt-HPI, suggesting that pH-HPI would be better for high salt applications in which stable foams are desired.

Overall, hemp protein demonstrated somewhat inferior foaming properties compared to soy and pea protein. While foaming properties are desirable in some applications such as cakes and bread, production of excess foam can create processing challenges when foaming is not a desirable property (Damodaran 2017). Hemp protein's inferior foaming properties compared to other plant proteins may actually be a positive attribute that limits unwanted foaming during processing of high protein formulations.

2.4.6 Amino Acid Composition and Nutritional Quality of HPIs

2.4.6.1 Amino Acid Composition of HPI Samples

The amino acid composition (**Table 12**) of hemp protein samples was used to evaluate hemp protein structural and functional characteristics. Amino acid composition can be used to determine a nitrogen conversion factor specific to the protein of interest. Typically, a conversion factor of 6.25 is used when a value specific to the protein is not known. This factor assumes 160 grams of nitrogen for every 1 kilogram of plant or animal protein (1000 g / 160 g = 6.25) (Sriperm et al. 2011). However, this factor would overestimate protein if a protein contained high amounts of amino acids with relatively high nitrogen percentages (Chang and Zhang 2017). Throughout this study, a conversion factor of 5.30 for hemp protein was used (USDA 2018). Calculations of the nitrogen conversion factor for pH-HPI and salt-HPI substantiated the use of 5.30 for hemp protein (**Table 24** and **25** in **Appendix M**).

Additionally, amino acid composition can help explain hemp protein functionality compared to other protein isolates. The HPIs contained a high amount of sulfur-containing amino acids. Sulfur-containing amino acids are involved in the formation of disulfide linkages, which can help in the formation of protein aggregates desirable for gelation or texturization (Tang et al. 2006; Damodaran 2017). The sulfur-containing amino acids might have contributed to the ability of HPI to produce strong gels when solubility in the medium was sufficient to prevent sedimentation.

The proportion of acidic and basic amino acids affects overall protein charge. The higher amount of acidic amino acids than basic amino acids explained the net negative charge of HPI at pH 7 (**Table 13**). Most food proteins have net negative charge at pH 7 and higher (Sathe et al. 2018). The proportion of acidic to basic amino acids is slightly lower than reported by Hadnađev et al. (2018) for salt-HPI (1.78) and pH-HPI (1.72); however, the authors reported similar solubility at pH 7 to our findings, suggesting that this discrepancy did not result in large functional differences.

Table 12. Amino acid content (g per 100 g protein, dry basis) of pH-extracted and salt-extracted hemp protein isolates (pH-HPI and salt-HPI, respectively).

Amino acids	pH-HPI	Salt-HPI
Alanine	3.87	3.71
Arginine	13.9	14.8
Aspartic acid	11.4	11.6
Cysteine	1.41	1.44
Glutamic acid	18.4	19.4
Glycine	3.70	3.65
Histidine	3.36	3.17
Isoleucine	4.34	4.22
Leucine	6.87	6.49
Lysine	3.41	3.21
Methionine	2.90	2.40
Phenylalanine	4.84	4.64
Proline	3.62	3.58
Serine	4.95	4.95
Threonine	3.28	3.17
Tryptophan	0.835	1.13
Tyrosine	3.70	3.57
Valine	5.14	4.93

Hydrophobicity of amino acids can also help explain overall protein behavior. Surprisingly, the percent of hydrophobic amino acids for the HPIs were lower than that of β-conglycinin and glycinin in soybean, which contain 41% and 39% hydrophobic amino acids, respectively (Damodaran 2017). Of the hydrophobic amino acids, phenylalanine, leucine, isoleucine, and valine are critical because of their degree of hydrophobicity compared to other hydrophobic residues (Mo et al. 2006). If they comprise more than 28% of the total amino acids, hydrophobic interactions will offset any electrostatic interactions, resulting in protein aggregation and minimal solubility across a wide pH

range. The percentages of these critical amino acids in pH-HPI and salt-HPI were below 28% and were similar to what is reported for the acidic subunit (20.1%) of soybean glycinin, which demonstrated good solubility across a wide pH range (Mo et al. 2006). Additionally, both HPIs had a similar proportion of all hydrophobic amino acids as the acidic subunit (36.6%) of glycinin. However, the acidic subunit had a much higher percentage of acidic amino acids (38.4%) and a much higher ratio of acidic to basic amino acids (2.76) than HPI, resulting in high solubility (> 80%) at pH 7. Therefore, it can be conjectured that hemp protein's solubility at neutral pH is largely hindered by a deficiency in charged residues, coupled with a large proportion of hydrophobic residues at the surface (**Table 9**).

Table 13. Percentage of key amino acids of pH-extracted and salt-extracted hemp protein isolates (pH-HPI and salt-HPI, respectively).

Sample	Sulfur-containing AA[1] (%)	Acidic AA[1] (%)	Basic AA[1] (%)	Ratio of Acidic to Basic AA[1]	Hydrophobic AA[1] (%)	Critical AA[1] (%)
pH-HPI	4.31	29.8	20.7	1.44	36.1	21.2
Salt-HPI	3.83	30.9	21.2	1.46	34.7	20.3

[1] Amino acids

2.4.6.2 Nutritional Quality of HPI Samples

One of the most fundamental roles of food proteins is providing nutritional value. Results confirmed that hemp protein contains the beneficial amino acids – arginine and the sulfur-rich methionine and cysteine – in higher amounts than has been reported for soy and pea (Callaway 2004; Hughes et al. 2011; Ma et al. 2017). Arginine may provide cardioprotective benefits, while methionine and cysteine are essential amino acids (House et al. 2010). Similar to other studies, lysine was determined to be the limiting amino acid in hemp and was used to calculate the amino acid score (**Table 14**).

pH-HPI had significantly higher *in vitro* digestibility than salt-HPI, which may be due to partial denaturation (**Table 9**). Partial denaturation can improve the digestibility of globular proteins, as unfolding can allow proteases better access to peptide bonds (Damodaran 2017). The higher *in vitro* digestibility resulted in a significantly higher

PDCAAS for pH-HPI than salt-HPI. While the PDCAAS of HPI has not been reported previously, the PDCAAS of pH-HPI and salt-HPI were within the range (0.48 – 0.61) reported by House et al. (2010) for whole hemp seeds, dehulled hemp seeds, and hemp seed meal. The PDCAAS of both pH-HPI and salt-HPI is superior to wheat gluten (0.25), suggesting that substitution of hemp protein for wheat gluten would result in an improvement in nutritional quality. The PDCAAS of hemp protein, however, is inferior to both soy protein (0.92 – 1) and pea protein (0.73 – 0.89) (Singh et al. 2008; House et al. 2010; Hughes et al. 2011; Ismail et al. 2020). However, hemp protein has similar digestibility to pea protein (82 – 85%) and soy protein (91 – 96%) (Han et al. 2007; Singh et al. 2008). As protein digestibility of both HPI is already quite high, an improvement in lysine content, potentially through breeding, could be a viable strategy to improve the overall nutritional value of hemp protein.

Table 14. Amino acid score, in vitro digestibility and protein digestibility-corrected amino acid score (PDCAAS) of pH-extracted and salt-extracted hemp protein isolates (pH-HPI and Salt-HPI, respectively).

Sample	Amino Acid Score[1]	*In vitro* Digestibility (%)[2]	PDCAAS
pH-HPI	0.642	90.9*	0.583*
Salt-HPI	0.618	87.0	0.537

[1] Calculated using the recommended amino acid scoring pattern for children (2 to 5 years) (FAO/WHO Expert Consultation, 1991); [2] Reported by Adam Franczyk, a graduate student in the Department of Food and Human Nutritional Sciences, University of Manitoba; An asterisk (*) indicates a significant difference in each column as tested by the student's two-sample unpaired t-test ($P < 0.05$).

2.5 Conclusions

Results demonstrated that both alkaline solubilization coupled with isoelectric precipitation and salt solubilization coupled with membrane filtration could be used to successfully produce HPI with high protein purities and yields desirable for industrial production. Higher protein yields than previously reported for HPI were achieved through the optimization of protein extraction parameters and the use of dehulling prior to defatting and protein extraction. Following optimization of protein extraction methods,

this work provided a comprehensive characterization of the effects on structure, function, and nutritional quality of HPI. Structural analysis demonstrated that pH-HPI was partially denatured compared to salt-HPI. However, partial denaturation of pH-HPI contributed to better functionality for certain applications compared to salt-HPI. Based on protein characterization results, alkaline solubilization coupled with isoelectric precipitation was determined to produce a more functional and nutritionally balanced HPI than salt extraction. While HPI demonstrated generally inferior functionality to cSPI and cPPI in water and dilute salt solutions, HPI showed potential to replace cSPI in certain applications. Further exploration into the functionality of pH-HPI within a food matrix should be explored. Based on HPI's poor solubility at neutral pH and lysine deficiency, future studies must focus on targeted breeding and protein modification as strategies to improve HPI for food applications. Nevertheless, this study demonstrated the potential of hemp protein to be a valuable novel plant protein ingredient.

Chapter 3: Characterization of Hemp Protein Isolates Extracted from Different Cultivars

3.1 Overview

Because hemp seeds have a greater market potential than hemp fiber, breeders are encouraged to identify breeding strategies to improve hemp as a food source. The goal of this work was to examine differences in hemp protein isolates (HPI, 87 – 89% protein) extracted from four industrial cultivars (CFX-2, Grandi, Joey, Picolo). The color, structure, function, and nutritional quality of the four HPI were characterized and compared to those of commercial soy (cSPI) and pea protein isolate (cPPI) references. All HPIs had an acceptable color in comparison to the references. Minimal differences in protein profile and structural properties were observed among the four HPI samples. Accordingly, minimal differences in protein functionality were observed. Three HPI samples produced stronger gels than cSPI at 15% protein, and all HPI samples had similar solubility to cSPI at acidic pH. There were no significant differences in *in vitro* digestibility among HPI cultivars, but differences in amino acid profile led to small significant differences in the protein digestibility corrected amino acid score (PDCAAS). Overall, given the minimal differences observed, more cultivars should be examined to screen for differences that could be used to breed for improved functional and nutritional properties of hemp protein for food applications.

3.2 Introduction

The global market for protein ingredients is projected to reach $85.5 billion in 2028, with food and beverage segments making up the largest share of the market. There is increased interest in consuming protein for its beneficial health properties. Specifically, the demand for plant-based sources of protein is increasing as they are perceived to be healthier than animal-based proteins. Plant proteins align with consumers' priorities related to dietary habits (following a flexitarian, vegetarian, or vegan diet), environmental sustainability, and animal welfare (Grand View Research 2021). Currently, soy, wheat, and pea are the major sources of plant protein ingredients. However, soy and wheat are "Big 9" allergens, while pea has limited functionality (Lam et al. 2018; FDA 2021). Therefore, it is important to identify novel sources of plant proteins to help meet the growing demand. One novel source that has been gaining traction is hemp oilseed.

Hemp (*Cannabis sativa* L.) has been cultivated for thousands of years for food, fiber, and medicinal use (Callaway 2004). One benefit of hemp is that almost all parts of the plant – stalk, leaves, flowers, and seeds – have an industrial use (Potin and Saurel 2020; Farinon et al. 2020). For example, the stalk can be used for paper and insulation, the leaves and flowers can be used in pharmaceuticals or supplements, and the seeds can be used for human food. However, legal restrictions throughout most of the 20th century stalled the hemp market in most western countries (Cherney and Small 2016; Fike 2016; Aluko 2017). Legal restriction were in place because hemp belongs to the same species that includes marijuana, which contains appreciable amounts of the psychotropic compound Δ-9-tetrahydrocannabinol (THC) (Small 2015). Breeding efforts have resulted in hemp plants consistently containing less than 0.3% THC of the weight of leaves and flower parts, too small of an amount to cause any narcotic effects (Callaway 2004; Cherney and Small 2016; Fike 2016; Johnson 2018). In the past two decades, legal restrictions on hemp have been lifted, allowing for renewed exploration into hemp as a crop (Farinon et al. 2020). In 2018, hulled hemp seeds, hemp seed protein powder, and hemp seed oil were granted "generally recognized as safe" (GRAS) status in the United States (FDA 2018).

Hemp seeds were originally a byproduct of the main purpose for hemp cultivation – fiber production (Fike 2016). Hemp stalks are made of two types of fiber – 1) long

phloem fibers that can be used in specialty paper and textiles and 2) short woody fibers that can be used in animal bedding and hemp-lime concrete (Cherney and Small 2016). Breeding efforts focused on producing tall plants with minimal branching to produce valuable long fibers (Small 2015; Cherney and Small 2016). However, the potential for growth of the oilseed market is greater than that of the fiber market, encouraging breeding for seed production or for dual-purpose fiber and seed production (Small 2015).

Desirable traits for seed production are shorter plants with more flowers that mature early for more efficient seed production (Small 2015; Fike 2016). Breeding for seed production has focused on increased yields, early flowering, suitability for a variety of agricultural conditions, and easy harvest and processing (Fike et al. 2020; Żuk-Gołaszewska and Gołaszewski 2020). While other desirable qualities have been identified, such as a larger seed size to aid with dehulling, and specific fatty acid or amino acid profiles for nutritional enhancement, breeding for these traits has been minimally explored (Fike 2016; Xu et al. 2020). Breeding efforts for soybeans, another oilseed, have been successful in altering the proportion of different protein components, impacting amino acid composition and protein functionality (Kim and Wicker 2005; Chen et al. 2020). Early work reported differences among hemp cultivars in seed components, which are worth exploring for food applications.

Hemp seeds from different cultivars have been reported to have varying crude oil content between 25.4 – 37.8% and a crude protein content between 19.5 – 35.6% (House et al. 2010; Russo and Reggiani 2015; Vonapartis et al. 2015; Galasso et al. 2016; Irakli et al. 2019; Lan et al. 2019; Schultz et al. 2020). Hemp seed oil is currently being extracted for its desirable ratio of omega-6 to omega-3 fatty acids, while the residual meal is being explored as a novel plant protein source (Callaway 2004; Aluko 2017). However, varietal differences in hemp protein profile, nutrition, and functionality have not been explored in relation to food use.

Hemp seed protein contains all of the essential amino acids and can be easily digested (House et al. 2010). However, hemp protein is deficient in lysine, an essential amino acid, causing it to be nutritionally inferior to soy and pea protein. Variabilities in amino acid profile among hemp cultivars have been identified (House et al. 2010; Russo

and Reggiani 2015). Therefore, future breeding efforts for food applications should include strategies to increase lysine content.

In addition to providing nutritional value, proteins are used for their functional properties in food applications. Research on hemp protein functionality, while not robust, has shown limitations in solubility, water binding, emulsification, and foaming properties (Tang et al. 2006; House et al. 2010; Potin and Saurel 2020). Water holding capacity and gelation specifically are largely unexplored. The functionality is heavily influenced by the protein structure, which has only been moderately explored for hemp protein. Differences in protein structure and functionality among hemp cultivars have yet to be explored. If differences exist in hemp protein among cultivars, then it would be possible to breed for improved protein functionality for food applications.

The objective of this study was to evaluate hemp protein isolates (HPIs) produced from four industrial cultivars for differences in color, protein extraction yields, and protein structural, functional, and nutritional properties. The cultivars explored in this study include cultivars bred for seed production (CFX-2, Grandi, Picolo) and for dual purpose seed and fiber production (Joey) (Lan et al. 2019). The information gathered from this work can be used to inform future hemp breeding programs focused on food applications.

3.3 Materials and Methods

3.3.1 Materials

Whole hemp seeds from the cultivars CFX-2, Grandi, Joey, and Picolo harvested in 2019 were kindly provided by Hemp Genetics International (North Saskatoon, SK, Canada). Soy protein isolate (cSPI, ProFam 974, 90.1% protein, 4.2% ash) and pea protein isolate (cPPI, ProFam 580, 79.5% protein, 5.6% ash) were kindly provided by Archer Daniels Midland (ADM) (Decatur, IL, USA). All seeds were stored in airtight containers at -20°C when not in use. All isolates were stored in closed containers sealed with Parafilm at -20°C when not in use.

SnakeSkin™ dialysis tubing with a molecular weight cut-off of 3.5 kDa, Sartorius Vivaflow™ 200 Crossflow Cassettes for membrane filtration with a molecular weight cut-off of 3 kDa, and Sudan Red 7B were purchased from Thermo Fischer Scientific™

(Waltham, MA, USA). Criterion™ TGX™ 4-20% precast gels (18 wells), 10X tris/glycine/sodium dodecyl sulfate (SDS) running buffer, Laemmli sample buffer, Precision Plus™ molecular weight marker, and Imperial™ Protein Stain were purchased from Bio-Rad Laboratories, Inc. (Hercules, CA, USA). Folded capillary tubes for zeta potential analysis were purchased from Malvern (Malvern, UK). Aluminum crucibles (40 µL, with pin) for use in DSC were purchased from Mettler-Toledo (Columbus, OH, USA). Electrophoresis grade sodium dodecyl sulfate (SDS), 2-mercaptoethanol (βME), Costar® 96-well black opaque plates, 8-anilino-1-napthalenesulfonic acid ammonium salt (ANS), and Celatom® (C8656) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Trypsin (T0303; from porcine pancreas Type IX-S, lyophilized powder, 13,000-20,000 BAEE units/mg protein), α-chymotrypsin (C4129; from bovine pancreas C4129 Type II, lyophilized powder, P40 units/mg protein), and protease (P5147; from *Streptomyces griseus* Type XIV, P3.5 units/mg solids) were used for protein digestibility assay and were purchased from Sigma-Aldrich. Casein (400601, high nitrogen, 80 mesh) for use as a reference in the protein digestibility assay was purchased from Dyets Inc. (Bethlehem, PA, USA). All other chemicals and reagents of analytical grade quality were purchased from Thermo Fischer Scientific or Sigma-Aldrich. Mazola pure corn oil was purchased from local grocery stores.

3.3.2 Production of Defatted Hemp Meal (DHM)

Hemp seeds from each cultivar were dehulled using a lab scale dehuller (Forsberg Model 7 F lab scale dehuller, Forsbergs Inc., Thief River Falls, MN, USA). Hull pieces were separated from hemp using a No. 8 U.S. standard stainless steel sieve, followed by two more cycles of dehulling at a higher speed and separation using a sieve. A small-scale gravity separator table (Whippet V.80 Gravity Separator Table, Sutton, Steele & Steele, Inc., Dallas, TX, USA) and manual separation using forceps were employed to remove residual hull pieces. Dehulled seeds were pressed, defatted, and milled as described in **Section 2.3.2**. Defatted, and milled hemp meal (DHM) from each cultivar had less than 3.5% fat on wet basis as determined following the Mojonnier AOAC method 922.06. The protein content (56.4 – 59.7% protein) of the DHM samples was

determined by the Dumas AOAC method 990.03 using a nitrogen analyzer (LECO, St. Joseph, MI, USA) and a protein conversion factor of 5.30 (USDA 2018).

3.3.3 Production of Hemp Protein Isolates (HPI)

HPI was produced from each cultivar following the identified optimized pH extraction (alkaline solubilization at pH 11 coupled with isoelectric precipitation at pH 5) outlined in **Section 2.3.3.2**. The protein content of HPI produced from each cultivar (87.8 – 89.0% protein) was determined by the Dumas AOAC method with a conversion factor of 5.30. Protein extraction yields ranged from 81.4 – 82.4%, similar to those obtained earlier (**Section 2.4.1**).

3.3.4 Protein and Ash Content

The protein contents of each sample (cSPI, cPPI, CFX-2 2019 HPI, Grandi HPI, Joey HPI, Picolo HPI) were determined using the Dumas method. A protein conversion factor of 6.25 was used for cSPI and cPPI, and a protein conversion factor of 5.30 was used for HPI. Ash content was determined using the AOAC dry ashing method (AOAC 942.05). Ash contents of HPI produced from the different cultivars ranged from 2.5 – 3.3%.

3.3.5 Color Measurement

The color of all samples was assessed, in triplicate, using a Chroma Meter CR-221 (Minolta Camera Co., Osaka, Japan), as described in **Section 2.3.6**.

3.3.6 Protein Structural Characterization

3.3.6.1 Protein Profiling by Gel Electrophoresis

Protein profiling of all samples using sodium dodecyl polyacrylamide gel electrophoresis (SDS-PAGE) was performed as outlined in **Section 2.3.7.1**. Briefly, samples (5µL; containing ~ 50 µg protein) and Precision Plus™ MW standard (10 µL) were loaded onto a Criterion™ TGX™ 4-20% precast Tris-HCl gradient gel. The gel was electrophoresed, stained/destained, and imaged.

3.3.6.2 Thermal Denaturation by Differential Scanning Calorimetry (DSC)

Denaturation temperature and enthalpy of denaturation using a DSC instrument (DSC 1 STARe System, Mettler Toledo, Columbus, OH, USA) was determined for all samples using the method described in **Section 2.3.7.2**. Thermograms were manually integrated to determine denaturation temperature and enthalpy of denaturation for each protein sample using the STARe Software version 11.00 (Mettler Toledo).

3.3.6.3 Protein Surface Properties

Surface hydrophobicity was determined for all samples following a spectrofluorometric method using 1-aniline-8-napthalene sulfonate (ANS) as outlined in **Section 2.3.7.3**. Zeta potential was measured using a dynamic light scattering instrument (Malvern Nano Z-S Zetasizer) as outlined in **Section 2.3.7.3**, with the exception that samples were only run in DDW (no analysis in 0.5 M NaCl). Zeta potential was determined by Malvern's Zetasizer software (version 7.13) using the Smoluchowski model.

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

ATR-FTIR spectra of all HPI samples were recorded using Fourier transform infrared spectrometer (Thermofisher Nicolett iS50 FTIR), following the method described in **Section 2.3.7.4**. ATR spectra were converted to transmission spectra using OMNIC® software. The second derivative of Amide I band (1600 cm^{-1} to 1700 cm^{-1}) was obtained by PeakFit v. 4.12 to determine the distribution of alpha-helix, beta-sheet, beta-turn, and random coil.

3.3.7 Protein Functional Characterization

3.3.7.1 Protein Solubility

Protein solubility at pH 7 and pH 3.2 at 1% protein concentration (w/v) was determined, in triplicate, as described in **Section 2.3.8.1** for all protein samples, with the exception that samples were only solubilized in DDW. Samples were assessed at room temperature and post thermal treatment (80°C for 30 minutes). Solubility was expressed

as the percentage of soluble protein (present in the supernatant after centrifugation) compared to the total protein content determined following the Dumas method.

3.3.7.2 Gel Strength

The strength of heat-induced gels was measured as described in **Section 2.3.8.2** at 15% protein (w/v, in DDW only), in triplicate, at pH 7. Gel strength was determined using a TA-TX Plus Texture Analyzer (Stable Micro Systems LTD, Surrey, UK) with a 100 mm diameter probe, a 5 mm s^{-1} test speed, and a target distance of 0.5 mm from the plate. Gel strength was measured as the maximum force (in Newtons) that was needed to rupture the gel.

3.3.7.3 Water Holding Capacity

Water holding capacity (WHC) of heat-induced gels was measured as outlined in **Section 2.3.8.3** at 15% protein (w/v, in DDW only), in triplicate, at pH 7. WHC was measured as the amount of water physically entrapped in the gel matrix.

3.3.7.4 Emulsification Capacity, Stability, and Activity

Emulsification capacity (EC) was determined, in triplicate, at 1% protein concentration (w/v, in DDW only), as described in **Section 2.3.8.4**. EC was expressed as g of oil emulsified per 1 g of protein. Emulsion stability (ES) and activity index (EAI) were measured as outlined in **Section 2.3.8.5** at 0.1% protein concentration (w/v, in DDW only). The ES (min) and EAI (m^2 g^{-1}) were calculated as described by Boyle et al. (2018) and Cameron et al. (1991), respectively.

3.3.7.5 Foaming Capacity and Stability

Foaming capacity (FC) and stability (FS) were measured as described in **2.3.8.6**. Protein solutions (0.5% w/v, in DDW only) were prepared, in triplicate, at pH 7. FC was expressed as mL foam per 1 gram of protein, and FS was calculated as mL foam after mixing divided by mL of foam after 30 minutes (expressed as a percent).

3.3.8 Amino Acid Composition and Nutritional Quality of HPI

3.3.8.1 Amino Acid Composition

Amino acid composition of each HPI sample was kindly analyzed by Jason Neufeld from the Department of Food and Human Nutritional Sciences at the University of Manitoba (Winnipeg, MB, Canada), as described in **Section 2.3.9.1**. Amino acid composition was used to calculate the amino acid score (AAS) using the reference amino acid pattern required for preschool children ages 2 to 5 years as defined by FAO/WHO Expert Consultation (1991).

3.3.8.2 In Vitro Protein Digestibility

In vitro protein digestibility of each HPI sample was kindly measured by Adam Franczyk from the Department of Food and Human Nutritional Sciences at the University of Manitoba (Winnipeg, MB, Canada), as described in **Section 2.3.9.2**. Casein was run alongside hemp samples as a control to confirm consistency of results compared with previous analyses.

3.3.8.3 Protein Digestibility-Corrected Amino Acid Score (PDCAAS)

Protein digestibility-corrected amino acid score (PDCAAS) was calculated for each HPI sample, as described in **Section 2.3.9.3**, using the amino acid score and *in vitro* protein digestibility.

3.3.9 Statistical Analysis

Analysis of variance (ANOVA) was performed using RStudio Version 1.3.1073 for Windows (RStudio, Boston, MA, USA). Tukey-Kramer multiple means comparison test was used to determine significant differences ($P \leq 0.05$) among the means ($n \geq 3$) of three or more samples. ANOVA tables can be found in **Appendix O (Tables 55 – 75)**. A student's unpaired t-test was used to determine significant differences ($P \leq 0.05$) between the means of two samples.

3.4 Results and Discussion

3.4.1 Effect of Cultivar on Color of HPI

HPI produced from the four different cultivars had acceptable color, with minor differences across samples (**Table 15**). While all HPI samples were less light than cSPI and cPPI, the difference was marginal and may be negligible to the eye. Similar to previous observations (**Section 2.4.3**), lightness values were comparable to that reported by Shen et al. (2020a) for HPI produced from dehulled hemp seeds. All four HPI samples were less yellow than that of HPI reported by Shen et al. (2020a).

The differences in color among HPI samples from the four cultivars may have been due to inherent variability in pigmentation among the cultivars. Differences may also reflect natural variation among batches of HPI due slight differences in the proportion of hull pieces remaining in the DHM samples. These findings demonstrated that a light-colored protein isolate could be produced from all four hemp cultivars. Overall, these results demonstrated that HPIs from different cultivars had similar light, bland colors desirable for a variety of food applications.

Table 15. Color (L* a* b*) of commercial soy protein (cSPI), commercial pea protein (cPPI), and hemp protein isolates (HPI) extracted from four different industrial hemp cultivars (CFX-2 2019, Grandi, Joey, Picolo) using alkaline solubilization coupled with isoelectric precipitation.

Protein Isolate	L*	a*	b*
cSPI	$86.2^{a\wedge}$	-0.2^{e}	$+14.8^{b}$
cPPI	86.6^{a}	$+0.2^{d}$	$+19.1^{a}$
CFX-2 2019 HPI	79.8^{d}	$+1.2^{b}$	$+13.6^{d}$
Grandi HPI	78.4^{e}	$+1.5^{a}$	$+14.8^{b}$
Joey HPI	81.0^{c}	$+1.3^{b}$	$+14.5^{c}$
Picolo HPI	82.6^{b}	$+1.0^{c}$	$+13.0^{e}$

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

3.4.2 Effect of Cultivar on Protein Structural Characteristics

3.4.2.1 Protein Profile

The protein profiles of HPI from different cultivars were compared under reducing and non-reducing conditions using SDS-PAGE (**Figure 5**). The protein profile of all HPI samples was similar to that observed (**Figure 4)** and discussed in **Section 2.4.4.1.**

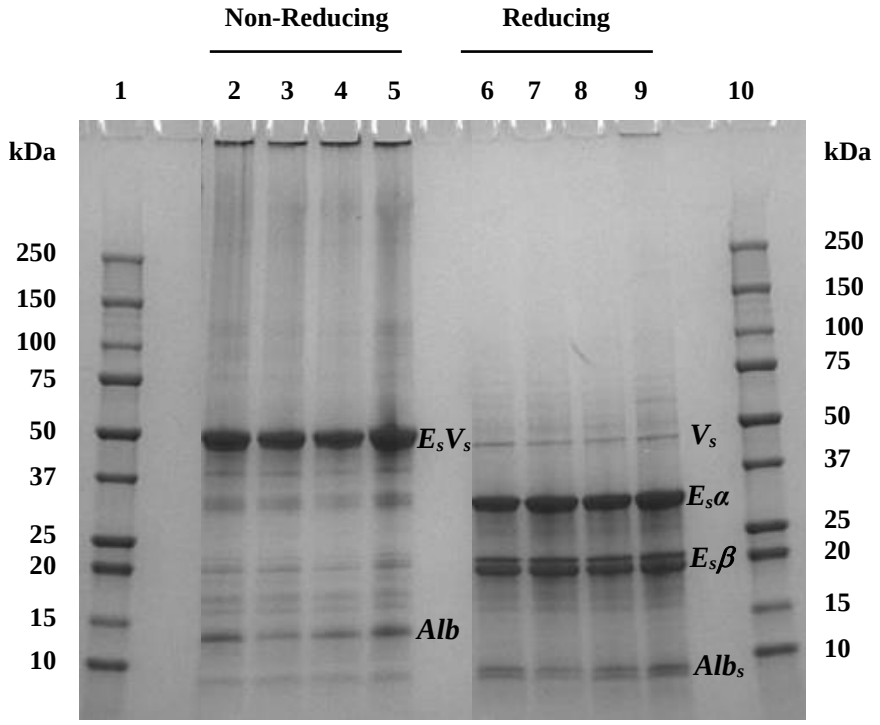


Figure 5. SDS-PAGE visualization of the protein profiles of hemp protein isolates from different cultivars (CFX-2 2019 HPI, Grandi HPI, Joey HPI, Picolo HPI) under non-reducing (lanes 2-5) and reducing (lanes 6-9) conditions. Lane 1, 10: Molecular weight (MW) marker; Lane 2, 6: CFX-2 2019 HPI, Lane 3, 7: Grandi HPI; Lane 4, 8: Joey HPI; Lane 5, 9: Picolo HPI. E_s: subunits of 11S edestin; V_s: subunits of 7S vicilin-like protein; Alb: 2S albumins; $E_s\alpha$: acidic subunit cleaved from edestin; $E_s\beta$: basic subunit cleaved from edestin; Alb_s: albumin subunits.

For all HPI samples, smearing in the upper part of the lanes was observed under non-reducing conditions (**Figure 5**, Lanes 2-5). Under reducing conditions (**Figure 5**, Lanes 6-9), this smearing almost completely disappeared, which indicated the presence of protein polymers linked via disulfide bonds. This observation was expected, as all HPI cultivars were extracted using solubilization at a high alkaline pH, which promoted

polymerization via disulfide linkages. The smearing due to the presence of disulfide linked polymers was similar to that seen previously for pH-HPI (**Figure 4**).

All protein bands were similar in intensity across all cultivars (**Figure 5**, Lanes 2-5 and 6-9), which indicated minimal qualitative differences in the distribution of protein subunits in the four cultivars. This observation was similar to that reported for soy and sunflower proteins. Zhu et al. (2020) observed minimal differences in protein profile among SPI samples from 12 cultivars. Similarly, Raymond et al. (1991) noted comparable protein profiles among nine sunflower cultivars. Regardless of such similarity in protein profile, differences in the abundance of each hemp protein component may still exist. Kim and Wicker (2005) noted similar visual SDS-PAGE results for two soybean cultivars, but abundance of each protein subunit differed when, as assessed by densitometry measurement. These differences translated to pronounced differences in functional properties. The cultivar with a higher ratio of glycinin (11S) to β-conglycinin (7S) produced a tofu with higher hardness, chewiness, and gumminess measurements. The higher proportion of sulfur-containing amino acids in glycinin was cited as the reason for this difference.

SDS-PAGE confirmed the presence of all major subunits in these four hemp cultivars in visually similar proportions. Further characterization of structural and functional properties of HPI samples might elucidate any potential differences in protein distribution among cultivars.

3.4.2.2 Protein Denaturation

Two endothermic peaks were observed for each HPI sample, which were attributed to 11S edestin and 7S vicilin-like protein (**Table 16**). The temperature of denaturation for 11S edestin and 7S vicilin-like protein were similar to that measured for pH-HPI (**Table 9**). Among HPI samples from different cultivars, there were significant, yet marginal, differences in denaturation temperatures for 7S vicilin-like protein and 11S edestin.

Minimal differences in enthalpy of denaturation for 11S edestin and 7S vicilin-like protein among all HPI samples were observed. The enthalpies of denaturation for HPIs from different cultivars were higher than measured for pH-HPI (**Table 9**), possibly

due to differences in growing year or storage conditions prior to being received. Picolo HPI had the highest enthalpy of denaturation for edestin and vicilin-like protein among HPI samples. These results suggested that Picolo HPI had slightly higher thermal stability than the other cultivars. Chen et al. (2020) reported statistically significant differences in denaturation properties of SPI from different soy cultivars. However, differences were also marginal (92.6 – 96.5°C for denaturation temperature, 0.98 – 2.21 J g^{-1} for enthalpy of denaturation) (Chen et al. 2020).

3.4.2.3 Protein Surface Properties

All HPI samples had lower surface charge and higher surface hydrophobicity than cSPI and cPPI (**Table 16**). Among HPI samples, significant differences in surface charge were observed between CFX-2 2019 and Picolo HPI. However, differences in surface charge were minimal, where all samples had a surface charge less than the $\pm$ 30 mV generally needed for protein repulsion and favored protein-water interactions (Long and Labute 2010). In contrast, significant differences among surface charge of SPI from different cultivars were reported (Chen et al. 2020). At pH 8, SPI from two cultivars had a more negative surface charge than -30 mV (-38.9 – -37.4 mV), while SPI from the other three cultivars had a less negative surface charge (-29.1 – -21.9 mV). While minimally observed in our study, significant differences in surface charge might exist among other hemp cultivars.

No significant differences in surface hydrophobicity among HPI from different cultivars were observed (**Table 16**). In contrast, significant differences in surface hydrophobicity of SPI extracted from different cultivars have been reported (Chen et al. 2020; Zhu et al. 2020). These reported varietal differences could suggest that variability in hemp cultivars other than those in this study may exist. A drastic improvement in surface hydrophobicity would be needed, as all HPI samples had a much higher surface hydrophobicity than cSPI and cPPI (**Table 9**). Both surface charge and hydrophobicity are important indications of protein-water interactions necessary for good functionality. Minor difference is surface properties of the HPI from the four cultivars might not result in impactful differences in functional properties.

Table 16. Denaturation temperatures and enthalpy, surface hydrophobicity, and surface charge of commercial soy protein (cSPI), commercial pea protein (cPPI), and hemp protein isolates (HPI) from different cultivars (CFX-2 2019, Grandi, Joey, Picolo).

Samples	Denaturation Temperature and Enthalpy				Surface Properties		Secondary Structure			
	Denaturation Temperature	Enthalpy of Denaturation	Denaturation Temperature	Enthalpy of Denaturation	Surface Charge	Surface Hydrophobicity	α Helix	β Sheet	β Turn	Random Coil
	Td, °C	ΔH, J g-1	Td, °C	ΔH, J g-1	mV	RFI	Relative Percentage			
	β-conglycinin (7S)		***Glycinin (11S)***							
cSPI	~		~		-46.5^{a}	9968^{b}	N/A†	N/A	N/A	N/A
	Vicilin (7S)		***Legumin (11S)***							
cPPI	~		~		-36.9^{b}	12656^{b}	N/A	N/A	N/A	N/A
	Vicilin-like Protein (7S)		***Edestin (11S)***							
CFX-2 2019 HPI	77.5$^{b\wedge}$	5.76^{b}	81.0^{a}	2.63^{b}	-27.5^{d}	20009^{a}	19.5^{a}	39.5^{b}	26.5^{a}	14.4^{a}
Grandi HPI	78.4^{a}	5.99^{b}	81.0^{a}	2.86^{b}	-28.8^{cd}	22544^{a}	18.0^{a}	41.6^{ab}	25.4^{a}	15.0^{a}
Joey HPI	76.4^{c}	6.44^{b}	79.2^{b}	3.13^{ab}	-28.4^{cd}	21310^{a}	19.9^{a}	43.1^{a}	24.9^{a}	12.1^{a}
Picolo HPI	78.8^{a}	8.11^{a}	81.2^{a}	3.93^{a}	-29.9^{c}	21356^{a}	17.9^{a}	40.6^{ab}	27.1^{a}	14.4^{a}

~ No peak of denaturation was observed; $^{\wedge}$ Means ($n \geq 3$) in each column with different lowercase letters indicate significant differences among samples according to the Tukey-Kramer multiple means comparison test ($P < 0.05$) ; † N/A indicates that relative percentage of secondary structures were not determined, as inherent properties are different from different protein sources.

3.4.2.4 Protein Secondary Structure

ATR-FTIR was used to monitor differences in the secondary structure of HPI from different cultivars. FTIR allowed for the quantification of the relative abundance of α helix, β sheet, β turn, and random coil in HPI samples from all cultivars (**Table 16**). No significant difference in abundance of α-helices, β-turns, and random coils was observed. The abundance of β-sheets in CFX-2 2019 HPI and Joey HPI were significantly different, but this difference did not translate to noteworthy differences in thermal stability between these two samples. Overall, minimal differences in secondary structure among the four cultivars explored in this study were observed.

3.4.3 Effect of Cultivar on Protein Functional Characteristics

3.4.3.1 Protein Solubility

Solubility in water at 1% protein concentration was measured at pH 3.2 and 7 under non-heated and heated (at 80°C) conditions to investigate the effect of cultivar on HPI's potential to be used in neutral and acidic beverages (**Table 17**). At neutral pH, all HPI samples had poor solubility, regardless of heating conditions. These results reinforced the poor solubility of HPI in water observed earlier (**Table 10**), and previously reported (Tang et al. 2006; Hadnađev et al. 2018; Shen et al. 2020a,b). The significant, yet minor, differences in surface charge between HPI samples (**Table 16**) did not translate to differences in solubility at neutral pH. In contrast, Chen et al. (2020) observed lower solubility for SPI from a cultivar that also had significantly the lowest surface charge. However, the variability in surface charge at pH 8 (-38.9 – -21.9 mV) was much larger in that study than what was observed in this study at pH 7 (-29.9 – -27.5 mV). The soybean cultivars were bred for differences in protein content and amino acid profile, which may have resulted in greater differences in protein properties of interest for food applications. The poor solubility observed for all HPI samples at pH 7 might negatively impact other functional properties.

Table 17. Solubility, gel strength, water holding capacity, emulsification capacity, stability, and activity index, and foaming capacity and stability of commercial soy protein (cSPI), commercial pea protein (cPPI), and hemp protein isolates (HPI) from four industrial cultivars (CFX-2 2019, Grandi, Joey, Picolo).

Samples	Solubility (1% protein)				Gel Strength (15% protein)	Water Holding Capacity (15% protein)	Emulsification Capacity (1% protein)	Emulsification Stability	Emulsification Activity Index	Foaming Capacity	Foaming Stability
	pH 7		pH 3.2								
	Non-heated	Heated at 80°C	Non-heated	Heated at 80°C	Strength (N)	%	mL oil/ g protein	min	m^2/g	mL foam/ g protein	%
cSPI	$67.8^{a\wedge}$	82.5^{a}	52.9^{ab}	65.2^{ab}	19.7^{c}	99.8^{a}	1194*	11.5^{c}	144.8^{b}	144^{b}	74.1^{b}
cPPI	41.8^{b}	60.1^{b}	16.2^{c}	28.0^{c}	2.1^{d}	99.9^{a}	777	12.5^{c}	185.5^{a}	292^{a}	13.7^{c}
CFX-2 2019 HPI	7.5^{c}	11.2^{c}	55.9^{ab}	62.0^{ab}	19.6^{c}	90.3^{b}	N/A†	42.9^{ab}	38.8^{c}	93.3^{d}	92.0^{a}
Grandi HPI	9.2^{c}	9.5^{c}	49.2^{b}	53.8^{b}	23.9^{a}	92.7^{b}	N/A	29.2^{bc}	45.8^{c}	128^{bc}	84.5^{ab}
Joey HPI	8.9^{c}	10.3^{c}	66.8^{a}	71.0^{a}	21.7^{b}	91.0^{b}	N/A	51.7^{a}	37.5^{c}	117^{c}	76.1^{ab}
Picolo HPI	9.7^{c}	10.7^{c}	66.6^{a}	71.6^{a}	23.2^{ab}	93.8^{ab}	N/A	49.7^{a}	44.5^{c}	116^{c}	87.3^{ab}

^ Means ($n \geq 3$) in each column with different lowercase letters indicate significant differences among samples according to the Tukey-Kramer multiple means comparison test ($P < 0.05$). An asterisk (*) indicates a significant difference in each column as tested by the student's unpaired t-test ($P < 0.05$); † N/A indicates that HPI samples did not form an emulsion when dissolved in DDW.

At pH 3.2, on the other hand, some differences in solubility among the four HPI samples were observed (**Table 17**). Grandi HPI had significantly lower solubility than Joey and Picolo HPI. The difference in solubility could not be explained by the noted structural properties (**Table 16**), but probably could be attributed to differences in amino acid composition. Joey and Picolo HPIs had good solubility (>70%) similar to what has been reported for pH-extracted HPI at pH 3 (71.2%) measured at 1% total solids (Dapčević-Hadnađev et al. 2019). All four HPI samples demonstrated similar solubility to cSPI, which was similar to the trend observed for both pH-HPI and salt-HPI (**Table 10**). This observation confirms the potential of HPI for use in acidic beverage applications.

3.4.3.2 Gel Strength and Water Holding Capacity

HPI from three different cultivars (Grandi, Joey, Picolo) produced significantly stronger gels at 15% protein than cSPI (**Table 17**). There was no significant difference in gel strength between the other HPI cultivar (CFX-2 2019) and cSPI. The lower gel strength of CFX-2 2019 HPI compared to the other cultivars could be in part due to the lower abundance of β-sheets (**Table 16**). A higher β-sheet content has been correlated with higher gel strength (Guo et al. 2017). Since all HPI samples produced gels that had similar or superior gel strength to cSPI, HPI might be a suitable substitute for cSPI in gel-type food applications.

Minimal differences in WHC among HPI samples was observed (**Table 17**). CFX-2 2019, Grandi, and Joey HPIs had significantly lower WHC than cSPI and cPPI, although the difference was minimal. WHC of Picolo HPI was not significantly different from that of cSPI and cPPI. Overall, the acceptable WHC of all four HPI samples, along with a high gel strength, suggested that HPI should be explored for gel-type food applications.

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

When EC was measured at 1% protein, none of the HPI samples formed a visible emulsion (**Table 17**). Results were similar to those noted for both pH- and salt- HPI

samples (**Table 11**)**.** Results can be explained by the extremely poor solubility of all HPI samples at pH 7, which prevented HPI from reaching the oil-water interface.

Some differences in ES among all HPI samples were observed, although marginal. In contrast, Zhu et al. (2020) reported differences in ES among SPI from different cultivars. ES for all HPI samples were within the range reported for ES for pH-extracted HPI (14 – 100 min) (Tang et al. 2006; Yin et al. 2008). Based on the wide range in ES reported for HPI, it is difficult to determine whether differences in ES among cultivar would be impactful.

No significant differences in EAI were observed for all HPI samples (**Table 17**). All EAI values were significantly lower than cSPI and cPPI. Similarly, Tang et al. (2006) reported a lower EAI for HPI than SPI. EAI of all HPI samples were similar to that reported for pH-extracted HPI by Yin et al. (2008). Our findings supported the poor emulsifying properties of HPI at pH 7, regardless of cultivar.

3.4.3.4 Foaming Capacity (FC) and Stability (FS)

Minimal differences in FC among HPI samples were noted (**Table 17**). Joey and Picolo HPIs had significantly higher FC than CFX-2 2019 HPI, although differences were marginal. All HPI samples had significantly lower FC than cPPI, and all HPI samples except for Grandi HPI, which had significantly lower FC than cSPI (**Table 17**). FC for all HPI samples was slightly higher than that observed earlier for pH-HPI in water (**Table 11**), which could be attributed to differences in harvest year or growing conditions.

No significant differences in FS among all HPI samples was observed. Similarly, Aluko and McIntosh (2001) reported no differences in FC or FS for pH-extracted canola protein isolates from different cultivars. Overall, HPI samples from all four cultivars demonstrated similar foaming properties, which were somewhat inferior to cSPI and cPPI.

3.4.4 Amino Acid Composition and Nutritional Quality of HPI from Different Cultivars

3.4.4.1 Amino Acid Composition of HPI from Different Cultivars

The amino acid composition of all HPI samples from the four cultivars (**Table 18**) was determined. The amino acid composition was used to calculate the nitrogen conversion factor for each HPI sample (**Appendix M**) and to evaluate the proportion of key amino acids (**Table 19**) for structural and functional consideration.

Table 18. Amino acid content (g per 100 g protein, dry basis) of hemp protein isolates (HPI) from four cultivars (CFX-2 2019, Grandi, Joey, Picolo).

Amino acids	CFX-2 2019 HPI	Grandi HPI	Joey HPI	Picolo HPI
Alanine	3.93	4.02	3.96	4.00
Arginine	14.0	13.8	14.0	13.8
Aspartic acid	11.5	11.8	11.5	11.5
Cysteine	1.23	1.15	1.26	1.21
Glutamic acid	18.5	18.1	18.2	18.2
Glycine	3.60	3.73	3.56	3.78
Histidine	2.93	2.91	3.09	2.94
Isoleucine	4.43	4.42	4.41	4.40
Leucine	6.90	7.04	6.97	7.05
Lysine	3.55	3.43	3.53	3.43
Methionine	2.59	2.29	2.57	2.54
Phenylalanine	4.88	4.95	4.90	4.90
Proline	3.63	3.66	3.64	3.68
Serine	4.97	4.97	4.96	5.02
Threonine	3.39	3.46	3.38	3.43
Tryptophan	1.20	1.25	1.30	1.18
Tyrosine	3.59	3.72	3.51	3.62
Valine	5.20	5.31	5.20	5.26

Table 19. Percentage of key amino acids of hemp protein isolates (HPI) from four cultivars (CFX-2 2019, Grandi, Joey, Picolo).

Sample	Sulfur-containing AA[1] (%)	Acidic AA[1] (%)	Basic AA[1] (%)	Ratio of Acidic to Basic AA[1]	Hydrophobic AA[1] (%)	Critical AA[1] (%)
CFX-2 2019 HPI	3.82	30.0	20.5	1.46	36.4	21.4
Grandi HPI	3.44	29.9	20.2	1.48	36.7	21.7
Joey HPI	3.83	29.7	20.7	1.44	36.5	21.5
Picolo	3.76	29.7	20.2	1.47	36.6	21.6

[1] Amino acids

While the proportion of sulfur-containing amino acids were lower for all cultivars (**Table 19**) than determined for pH-HPI (**Table 13**), gelling properties were not negatively impacted. Percentage of acidic, basic, hydrophobic, and critical amino acids, as well as the ratio of acidic to basic amino acids, were similar among all HPI samples and pH-HPI from **(Table 13)**. In contrast, Zhu et al. (2020) reported significant differences in percentage of acidic, basic, and hydrophobic amino acids, and in the ratio of acidic to basic amino acids among SPI from 12 different cultivars. House et al. (2010) reported variability in the amino acid composition of hemp proteins found in whole hemp seeds, dehulled hemp seeds, and hemp seed meal from four different cultivars. Amino acid composition can vary depending on cultivar, growing year, environmental conditions, and processing. More cultivars need to be studied to establish differences in amino acid composition that would translate to noteworthy structural and functional differences.

3.4.4.2 Nutritional Quality of HPI from Different Cultivars

Lysine was the limiting amino acid for all HPI samples and was used to determine the amino acid score (**Table 20**). The amino acid scores of all four HPI samples were higher than determined for pH-HPI (**Table 14**), which was likely due to differences in growing year or conditions. House et al. (2010) observed a range in amino acid score of 0.54 – 0.69 for dehulled hemp seeds from different cultivars. Differences in lysine

content between cultivars could be leveraged to breed hemp cultivars for improved lysine content.

No statistically significant difference in *in vitro* digestibility was observed among HPI from different cultivars (**Table 20**). Minimal differences in PDCAAS were observed. However, a significant difference in PDCAAS between CFX-2 2019 HPI and Picolo HPI was noted. In contrast, Zarkadas et al. (2007) found no significant differences in PDCAAS among 14 soybean cultivars. However, the authors used the same true protein digestibility value (from the US Federal Register) for all cultivars. Any difference in PDCAAS among cultivars would be worth exploring to improve the nutritional quality of HPI through breeding.

Table 20. Amino acid score, in vitro digestibility and protein digestibility-corrected amino acid score (PDCAAS) of hemp protein isolates (HPI) from four cultivars (CFX-2 2019, Grandi, Joey, Picolo).

Sample	Amino Acid Score[1]	*In vitro* Digestibility (%)[2]	PDCAAS
CFX-2 2019 HPI	0.706	90.8	0.641[a^]
Grandi HPI	0.684	91.8	0.630[ab]
Joey HPI	0.692	90.4	0.625[ab]
Picolo HPI	0.684	90.5	0.619[b]

[1] Calculated using the recommended amino acid scoring pattern for children (2 to 5 years) (FAO/WHO Expert Consultation, 1991); [2] Reported by Adam Franczyk, a graduate student in the Department of Food and Human Nutritional Sciences, University of Manitoba; No significant differences observed among *in vitro* digestibility according to the Tukey-Kramer multiple means comparison test ($P < 0.05$); [^] Means (n = 3) in each column with different lowercase letters indicate significant differences among samples according to the Tukey-Kramer multiple means comparison test ($P < 0.05$).

3.5 Conclusions

Results demonstrated minimal structural differences among HPI extracted from the four cultivars, which contributed to only slight, mostly not significant, differences in functionality and nutritional quality. This study is the first to examine the impact of cultivar on structural, functional, and nutritional properties of HPI. Results of such work can be used to inform future breeding efforts focused on food applications. However,

further work should focus on screening more hemp cultivars to identify differences that would lead to desirable improvements in functionality and nutritional quality. This study provided a comprehensive analysis of HPI properties of interest for food applications, and confirmed that production of HPI with acceptable color, regardless of cultivar, was possible. Results confirmed that HPI from all cultivars had some similar or superior functional properties to cSPI for certain food applications. Overall, this work demonstrated that HPI should continue to be investigated as a novel plant protein source.

Chapter 4: Overall Conclusions, Implications, and Recommendations

Protein extraction methods were optimized for dehulled hemp seeds, and structural, functional, and nutritional properties of the resultant HPI produced from different cultivars were characterized. Extraction parameters for two industry feasible methods, alkaline solubilization coupled with isoelectric precipitation and salt solubilization coupled with membrane filtration, were tested. When extraction conditions were optimized, both methods produced HPI with high protein purity, yield, and acceptable color. Protein profiling of salt-HPI and pH-HPI revealed visual similarities in the proportion of hemp protein subunits, however, pH-HPI exhibited more protein polymerization. Polymerization, a lower enthalpy of denaturation, and a higher ratio of β-sheet to α-helix confirmed the partially denatured state of pH-HPI compared to salt-HPI. Both HPIs had higher surface hydrophobicity and lower surface charge than cSPI and cPPI, which hindered their solubility and emulsification properties in water under neutral conditions. However, pH-HPI produced a stronger gel than cPPI. Additionally, salt-HPI had higher foaming capacity than cSPI. Under acidic conditions, both HPIs had similar solubility to cSPI, likely due to increased surface charge further away from hemp protein's isoelectric point. A dilute salt solution (0.5 M NaCl) was needed to enhance solubilization of salt-HPI in order to form a gel. Salt improved some HPI functionality, such as gel strength and solubility at neutral pH, while negatively impacting others, such as water holding capacity and foaming stability. In terms of nutritional quality, pH-HPI had a significantly higher PDCAAS than salt-HPI due to higher amino acid score and *in vitro* digestibility. In general, pH-HPI was determined to have better functional and nutritional characteristics than salt-HPI; therefore, pH-assisted protein extraction was preferred over salt-assisted extraction.

The optimized alkaline solubilization with isoelectric precipitation protocol was used to extract HPI from four industrial hemp cultivars grown in Canada and the northern United States. Minimal differences in protein profile and structural characteristics were observed among the four HPI samples. Accordingly, minimal differences in protein functionality and nutritional quality were noted. HPI from all cultivars had similar solubility to cSPI at acidic pH, likely due to higher surface charge at acidic pH than

neutral. Three HPI cultivars had superior gel strength to cSPI at neutral pH, which has been attributed to disulfide interchange as indicated by protein polymerization, observed via SDS-PAGE, and by the relatively higher proportion of β-sheets. More hemp cultivars should be screened for differences that could be used to improve HPI through breeding.

Novel plant protein sources must demonstrate similar or superior functionality to current plant protein sources to be adopted in the industry. This work indicated that HPI could be used as a replacement for soy or pea protein for certain food applications. Compared to soy protein, hemp protein has more desirable market traits (non-GMO, low allergenicity), and can deliver comparable functionality for some applications (solubility at acidic pH, gel strength in water and in salt, emulsification capacity in salt). Compared to pea protein, hemp protein has better functionality for certain applications (solubility at acidic pH, gel strength in water and in salt, foaming stability in water). The observed high gel strength and acceptable water holding capacity suggested that HPI has potential for plant-based meat analogue application. Similar solubility at acidic pH, on the other hand, suggested that HPI can potentially replace cSPI in acidic beverage applications. Additionally, HPI can be produced with higher protein yields than soy and pea, making HPI production industrially feasible and desirable.

In light of these findings, more work is needed to evaluate HPI's use in various food applications. Currently, hemp seed oil is being extracted from whole hemp seeds, resulting in a residual, protein-rich meal that is high in polyphenols. The presence of polyphenols during protein extraction can negatively impact protein functionality due to oxidation and resulting protein polymerization; therefore, functionality of HPI produced from non-dehulled seeds compared with dehulled seeds should be further explored. A proven improvement in protein functionality, coupled with the acceptable color of HPI when produced from dehulled seeds, would validate the necessity of the dehulling step prior to defatting and protein extraction. Additionally, further work to determine the potential use of HPI should include flavor characterization. Sensory acceptability is essential for the adoption of such a protein ingredient in food applications.

Finally, because of the observed functionality limitations, especially solubility, as well as lysine deficiency, strategies to improve HPI's functionality and nutritional quality should be explored. Protein modification (e.g., enzymatic hydrolysis, glycation, cold plasma,

transglutaminase treatment), and continued screening of hemp cultivars to develop breeding strategies could potentially improve functionality. Screening of more hemp cultivars for improved lysine content or blending of HPI with protein sources high in lysine (peas, chickpeas) could be further explored to improve nutritional quality. Overall, this work demonstrated that hemp protein extraction is industrially feasible and should continue to be explored as a promising functional and nutritional plant protein ingredient.

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Appendix A: Proximate Analysis of Defatted Hemp Meal (DHM)

Table 21. Proximate analysis of defatted hemp meal (DHM) prepared from dehulled CFX-2 2016 hemp seeds.

Component	% on Wet Basis
Fat	2.33
Protein	59.8
Ash	12.5
Moisture	6.84
Carbohydrates[1]	18.5
Total Dietary Fiber	9.06
Other Carbohydrates[1]	9.44

[1] Determined by subtraction

Appendix B: Sample Calculations for Determining % Soluble Protein in Extraction Screening Methods

Calculating % soluble protein for pH and salt extraction screening methods:

$$\% \text{ soluble protein} = \frac{\text{mass (g) of supernatant x \% protein in supernatant}}{\text{mass (g) of dry sample x \% protein in sample}} \times 100 \qquad \textbf{Eq. 4}$$

Example calculation of % soluble protein for pH extraction screening testing solubilization at pH 10 for one replicate:

$$\% \text{ soluble protein} = \frac{47.3668 \text{ g x } 1.945\%}{2.5005 \text{ g x } 58\%} \times 100 = 63.52\%$$

Appendix C: Sample Calculations for Determining Protein Yields Using Mass Balance of pH Extraction

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

$$\%\ \text{protein yield in pH-HPI} = \frac{\text{pH-HPI mass (g) x pH-HPI protein purity (\%)}}{\text{DHM mass (g) x DHM protein purity (\%)}} \times 100 \qquad \textbf{Eq. 14}$$

$$\%\ \text{protein residue in pellet} = \frac{\text{pellet mass (g) x pellet protein purity (\%)}}{\text{DHM mass (g) x DHM protein purity (\%)}} \times 100 \qquad \textbf{Eq. 15}$$

$$\%\ \text{protein lost in supernatant} = \frac{\text{supernatant mass (g) x supernatant protein purity (\%)}}{\text{DHM mass (g)x DHM protein purity (\%)}} \times 100 \qquad \textbf{Eq. 16}$$

Example calculation of each fraction for one replicate of optimized pH extraction (solubilization at pH 11):

$$\%\ \text{protein yield in pH-HPI} = \frac{2.8503\ \text{g} \times 87.92\%}{5.0008\ \text{g} \times 58.20\%} \times 100 = 86.10\%$$

$$\%\ \text{protein residue in pellet} = \frac{0.9165\ \text{g} \times 3.51\%}{5.0008\ \text{g} \times 58.20\%} \times 100 = 1.11\%$$

$$\%\ \text{protein lost in supernatant} = \frac{1.1563\ \text{g} \times 29.63\%}{5.0008\ \text{g} \times 58.20\%} \times 100 = 11.77\%$$

Table 22. Mass balance for hemp protein optimized pH extraction (solubilization at pH 11).

Fraction	Mass (g)	Protein Purity (%)	Mass of Protein (g)	Protein Yield (%)
DHM	5.0008	58.20	2.9105	
pH-HPI	2.8503	87.92	2.5059	86.10
Pellet	0.9165	3.51	0.0322	1.11
Supernatant	1.1563	29.63	0.3426	11.77
Sum of Fractions	3.7668		2.8806	
Recovered Material (%)	98.45			98.98

Appendix D: Samples Calculations for Determining Protein Yields Using Mass Balance of Salt Extraction

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

$$\%\ \text{protein yield in salt-HPI} = \frac{\text{salt-HPI mass (g) x salt-HPI protein purity (\%)}}{\text{DHM mass (g) x DHM protein purity (\%)}} \times 100 \quad \textbf{Eq. 17}$$

$$\%\ \text{protein residue in pellet} = \frac{\text{pellet mass (g) x pellet protein purity (\%)}}{\text{DHM mass (g) x DHM protein purity}} \times 100 \quad \textbf{Eq. 18}$$

Example calculation of each fraction for one replicate of optimized salt extraction (solubilization using 0.75 M NaCl):

$$\%\ \text{protein yield in salt-HPI} = \frac{2.7288\ \text{g} \times 87.43\%}{5.0010\ \text{g} \times 58\%} \times 100 = 82.25\%$$

$$\%\ \text{protein residue in pellet} = \frac{1.2657\ \text{g} \times 17.58\%}{5.0010\ \text{g} \times 58\%} \times 100 = 7.67\%$$

Table 23. Mass balance for hemp protein optimized salt extraction (solubilization using 0.75 M).

Fraction	Mass (g)	Protein Purity (%)	Mass of Protein (g)	Protein Yield (%)
DHM	5.0010	58.0	2.9006	
Salt-HPI	2.7288	87.43	2.3858	82.25
Pellet	1.2657	17.58	0.2225	7.67
Sum of Fractions	3.9945		2.6082	
Recovered Material (%)	79.87			89.92

Appendix E: Sample Calculations for Determining Total Dietary Fiber

Calculating total dietary fiber (TDF) in DHM:

$$\% \text{ TDF} = \frac{\frac{R_1+R_2}{2} - p - A - B}{\frac{m_1+m_2}{2}} \times 100 \qquad \textbf{Eq. 19}$$

Where:

R_1 = residual weight (g) from m_1

R_2 = residual weight (g) from m_2

m_1 = sample weight (g) 1

m_2 = sample weight (g) 2

A = ash weight (g) from R_1

p = protein weight (g) from R_2

$$B = \text{blank} = \frac{BR_1 + BR_2}{2} - BP - BA \qquad \textbf{Eq. 20}$$

Where:

BR = blank residue

BP = blank protein from BR_1

BA = blank ash from BR_2

Example calculation for TDF of DHM:

$$\% \text{ TDF} = \frac{\frac{0.3557 \text{ g} + 0.3488 \text{ g}}{2} - 0.1604 \text{ g} - 0.0966 \text{ g} - 0.0046 \text{ g}}{\frac{1.0003 \text{ g} + 1.0004 \text{ g}}{2}} \times 100 = 9.06\%$$

$$\text{Blank} = \frac{0.0132 \text{ g} + 0.0102 \text{ g}}{2} - 0.0071 \text{ g} - 0 \text{ g} = 0.0046 \text{ g}$$

Residues were corrected for ash and protein using the dry ashing method (AOAC 942.05) and the Kjeldahl method (AOAC 981.10). A protein conversion factor of 5.30 was used for hemp per the USDA.

Appendix F: Sample Calculation for Determining Surface Hydrophobicity Index

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

$$\text{Net RFI} = \text{RFI}_{final} - \text{RFI}_{initial} \quad \textbf{Eq. 21}$$

$$\text{RFI}_{initial} = \text{Sample}_{initial} - \text{Blank}_{initial} \quad \textbf{Eq. 22}$$

$$\text{RFI}_{final} = \text{Sample}_{final} - \text{Blank}_{final} \quad \textbf{Eq. 23}$$

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 salt-HPI at 0.05% protein:

$\text{RFI}_{initial} = 9 - 16.5 = -7.5$

$\text{RFI}_{final} = 854 - 20.2 = 833.8$

Net RFI = 833.8 - -7.5 = 841.3

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 6**.

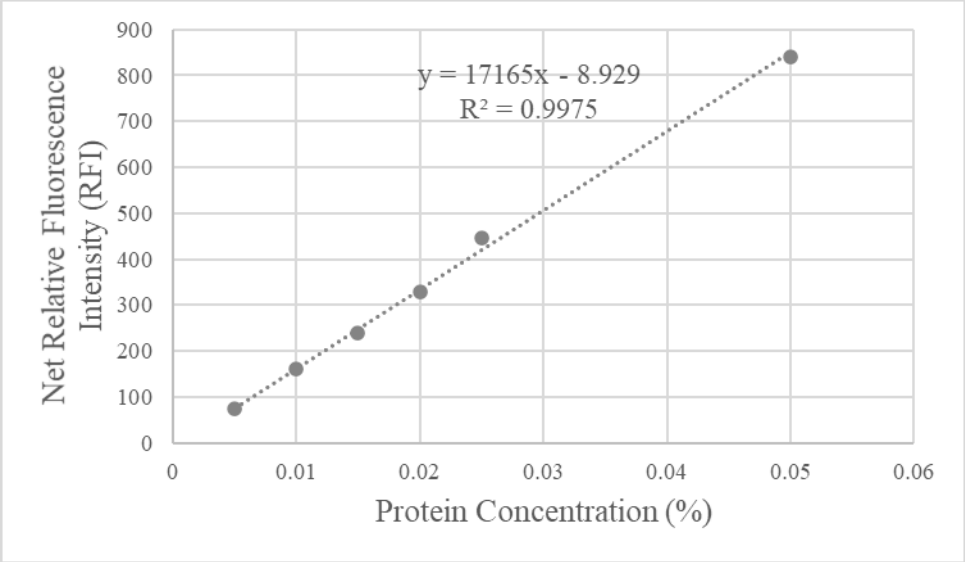

Figure 6. Net Relative Fluorescence Intensity (RFI) plotted against protein concentration (%) for salt-HPI to determine surface hydrophobicity index.

The slope of the trendline in **Figure 6** is the surface hydrophobicity index 17165, r^2 = 0.9975.

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

Appendix G: Sample Spectrum for Determining Protein Secondary Structure

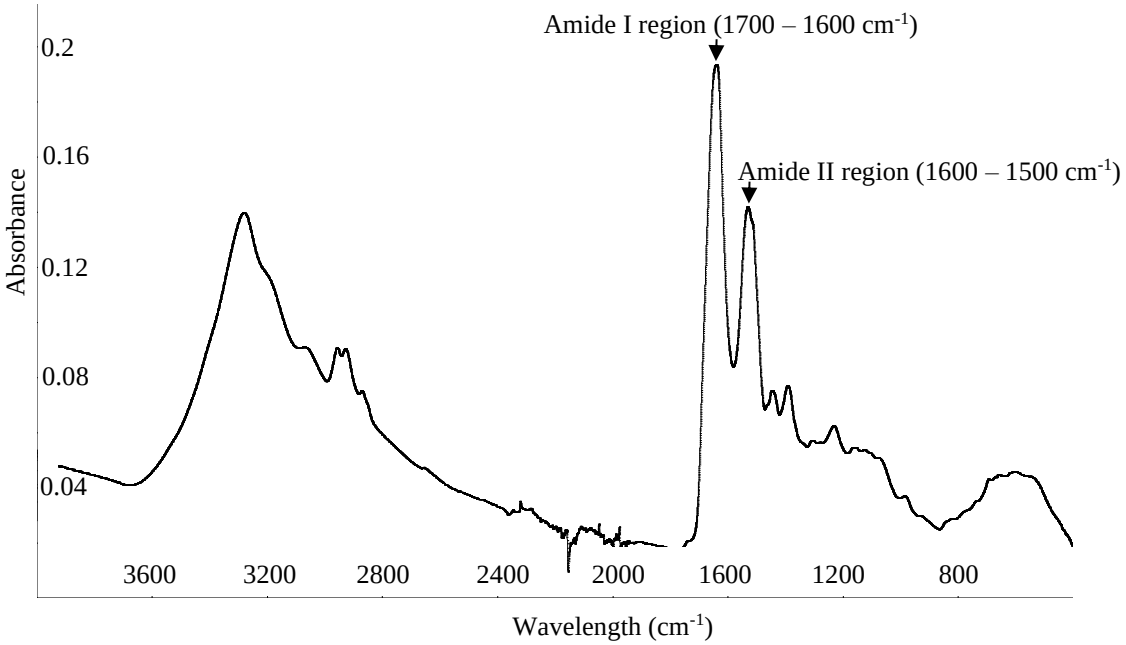

Figure 7. Original FTIR-ATR spectrum of salt-HPI.

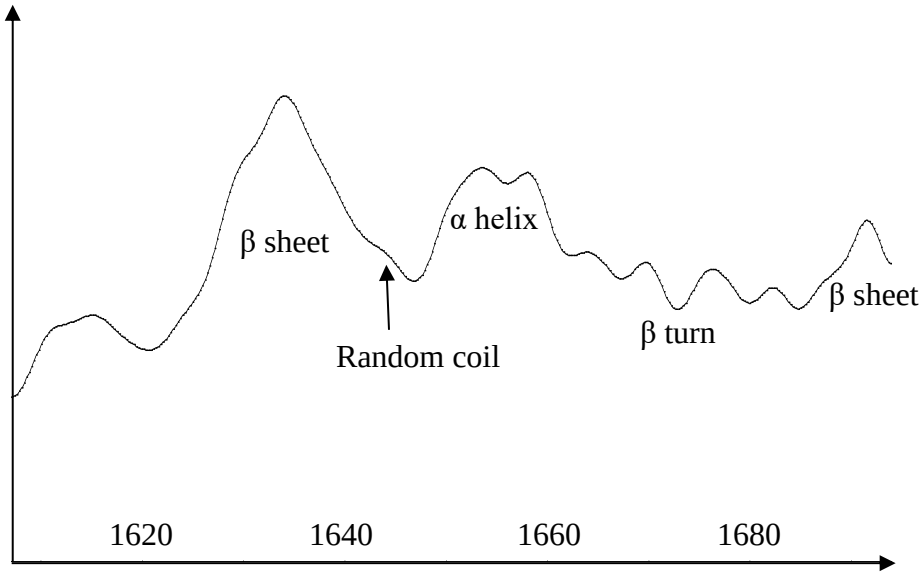

Figure 8. Second-derivative spectrum of salt-HPI.

The original FTIR-ATR spectrum of salt-HPI was shown in **Figure 7**. Second derivative of the Amide I band (1600 cm-1 – 1700 cm-1) **(Figure 8)** was obtained by PeakFit v. 4.12 to identify alpha-helix, beta-sheet, beta-turn, and random coil, according to the range of 1648 – 1660, 1612 – 1641 and 1684 – 1694, 1662 – 1684, and 1640 – 1650, respectively.

Appendix H: Sample Calculation for Determining Protein Solubility

Calculating protein solubility for pH-HPI at pH 3.2 non-heated in water:

$$\% \text{ protein solubility} = \frac{\% \text{ supernatant protein}}{\% \text{ initial protein}} \times 100 \qquad \textbf{Eq. 5}$$

$$\% \text{ protein solubility} = \frac{0.673\%}{1.08\%} \times 100 = 62.31\%$$

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

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

Calculating water holding capacity (WHC) for salt-HPI in 0.5 M NaCl:

$$WHC = 100 \times \left(\frac{T_3 - T_1}{T_2 - T_1}\right) \qquad \textbf{Eq. 6}$$

$$WHC = 100 \times \left(\frac{1.7075\ g - 1.0492\ g}{1.8350\ g - 1.0492\ g}\right) = 83.77\%$$

Where:

T_1 = weight (g) of protein solution before heating

T_2 = weight (g) of protein solution + microcentrifuge tube after cooling

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

Appendix J: Sample Calculation for Determining Emulsification Capacity

Calculating emulsification capacity (EC) for pH-HPI in 0.5 M NaCl:

$$EC = \frac{\text{volume of oil titrated (mL) x density of oil } (\frac{g}{mL})}{\text{mass of protein (g)}} \qquad \textbf{Eq. 7}$$

$$EC = \frac{40 \text{ mL x } 0.93 \frac{g}{mL}}{0.05 \text{ g}} = 744 \frac{\text{g protein}}{\text{g oil}}$$

Where:

0.93 g/mL = density of corn oil

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

Appendix K: Sample Calculation for Determining Emulsion Stability and Emulsification Activity Index

Calculating emulsion stability (ES) for salt-HPI in 0.5 M NaCl:

$$ES\ (min) = \frac{A_0}{A_0 - A_{10}} \times 10\ min \qquad \textbf{Eq. 8}$$

$$ES\ (min) = \frac{0.1179}{0.1179 - 0.0702} \times 10\ min = 24.72\ minutes$$

Where:

A_0 = absorbance at 0 min

A_{10} = absorbance at 10 min

Calculating emulsification activity index (EAI) for salt-HPI in 0.5 M NaCl:

$$EAI\left(\frac{m^2}{g}\right) = \frac{2T}{(1-\Phi)C} \qquad \textbf{Eq. 9}$$

$$EAI\left(\frac{m^2}{g}\right) = \frac{2 \times \frac{2.303 \times 0.1179}{0.01\ m}}{(1 - 0.25) \times 1\ g/m^3} = 72.44\ \frac{m^2}{g}$$

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

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

L = path length of the cuvette

= 10 mm = 0.01 m

Appendix L: Sample Calculation for Determining Foaming Capacity and Stability

Calculating foaming capacity (FC) of pH-HPI in DDW:

$$FC = \frac{\text{initial solution volume - initial liquid volume}}{\text{grams of protein in solution}} \qquad \textbf{Eq. 10}$$

$$FC = \frac{\text{initial solution volume - initial liquid volume}}{\text{grams of protein in solution}}$$

$$= \frac{63 \text{ mL} - 44 \text{ mL}}{0.25 \text{ g}} = 76 \text{ mL foam per g protein}$$

Calculating foaming stability (FS) of pH-HPI in DDW:

$$FS = \frac{\text{solution volume at 30 min - liquid volume at 30 min}}{\text{initial solution volume - initial liquid volume}} \times 100 \qquad \textbf{Eq. 11}$$

$$FS = \frac{\text{solution volume at 30 min - liquid volume at 30 min}}{\text{initial solution volume - initial liquid volume}} \times 100$$

$$= \frac{62 \text{ mL} - 44 \text{ mL}}{63 \text{ mL} - 44 \text{ mL}} \times 100 = 95\%$$

Appendix M: Sample Calculation for Determining Nitrogen Conversion Factor

Table 24. Amino acid composition and % nitrogen for pH-HPI used to calculate nitrogen conversion factor.

AA[1]	AA[1] Mol[2] Weight	AAA[3] Weight	% AA Dry Weight of Sample^	% N[4] in AA[1]	% N[4] in AAA[3]	% AAA[3] in Dry Sample	% N[4] in AAA[3] in Dry Sample
Alanine	89.09	71.09	3.29	15.72	19.70	2.62	0.52
Arginine	174.2	156.2	11.81	32.16	35.87	10.59	3.80
Aspartic acid	133.1	115.1	9.69	10.52	12.17	8.38	1.02
Cysteine	121.06	103.06	1.20	11.56	13.58	1.02	0.14
Glutamic acid	147.13	129.13	15.66	9.52	10.85	13.74	1.49
Glycine	75.05	57.05	3.14	18.66	24.55	2.39	0.59
Histidine	155.16	137.16	2.86	27.08	30.63	2.53	0.77
Isoleucine	131.17	113.17	3.68	10.68	12.38	3.18	0.39
Leucine	131.17	113.17	5.84	10.68	12.38	5.03	0.62
Lysine	146.19	128.19	2.90	19.16	21.85	2.54	0.55
Methionine	149.21	131.21	2.46	9.39	10.68	2.17	0.23
Phenylalanine	165.19	147.19	4.11	8.48	9.52	3.66	0.35
Proline	115.13	97.13	3.08	12.17	14.43	2.60	0.37
Serine	105.09	87.09	4.21	13.33	16.09	3.49	0.56
Threonine	119.12	101.12	2.79	11.76	13.85	2.37	0.33
Tryptophan	204.23	186.23	0.71	13.72	15.05	0.65	0.10
Tyrosine	181.19	163.19	3.15	7.73	8.58	2.83	0.24
Valine	117.15	99.15	4.36	11.96	14.13	3.69	0.52

[1] AA – amino acid, [2] Mol – molecular, [3] AAA – anhydrous amino acid, [4] N – nitrogen
^ % dry weight of amino acid profile was determined as described in **Section 2.3.9.1** and corrected for moisture content of sample

Calculating values presented in **Table 24,** showing example calculations for alanine:

$$\text{AAA weight} = \text{AA molecular weight - molecular weight of } H_2O = 89.09\,\frac{g}{mol} - 18\,\frac{g}{mol} = 71.09\,\frac{g}{mol}$$

$$\%\text{ N in AAA} = \frac{\text{AA molecular weight}}{\text{AAA weight}} \text{ x \% N in AA} = \frac{89.09\text{ g/mol}}{71.09\text{ g/mol}} \text{ x } 15.72\% = 19.70\%$$

$$\%\text{ AAA in dry sample} = \frac{\text{anhydrous AA weight}}{\text{AA molecular weight}} \text{ x \% AA dry weight in sample}$$

$$= \frac{71.09\text{ g/mol}}{89.09\text{ g/mol}} \text{ x } 3.29\% = 2.62\%$$

$$\%\text{ N in AAA in dry sample} = \frac{\%\text{ N in AAA}}{100} \text{ x \% AAA in dry sample} = \frac{19.70\%}{100} \text{ x } 2.62\% = 0.52\%$$

Example calculation for determining nitrogen conversion factor for pH-HPI using values from **Table 24**:

$$\text{Amide \%N (w/w)} = \text{measured \% ammonia (w/w)} \text{ x } \frac{14}{17} = 1.11\% \text{ x } \frac{14}{17} = 0.91$$

$$k_A \text{ (N-factor maximum upper limit)} = \frac{\text{total \% AAA in dry sample}}{\%\text{ N in AAA + amide N\% (w/w)}}$$

$$= \frac{73.5}{(12.59 + 0.91)} = 5.4$$

$$k_P \text{ (N-factor minimum lower limit)} = \frac{\text{total \% AAA in dry sample}}{\%\text{ N in dry sample determined by Dumas method}}$$

$$= \frac{73.5}{16.76} = 4.4$$

$$\text{k1 (N-factor plausible upper limit)} = \frac{k_A + k_P}{2} + \frac{k_A - k_P}{4} = \frac{5.4 + 4.4}{2} + \frac{5.4 - 4.4}{4} = 5.2$$

$$\text{k2 (N-factor plausible lower limit)} = \frac{k_A + k_P}{2} - \frac{k_A - k_P}{4} = \frac{5.4 + 4.4}{2} - \frac{5.4 - 4.4}{4} = 4.6$$

$$\text{Estimated N-factor} = \frac{k_A + k_P}{2} = \frac{5.2 + 4.6}{2} = 4.9$$

Table 25. Summary of calculated upper and lower limits and estimated nitrogen conversion factor for all HPI samples.

Sample	k_A	k_P	k1	k2	Estimated N factor
pH-HPI	5.4	4.4	5.2	4.6	4.9
Salt-HPI	5.4	4.5	5.2	4.7	4.9
CFX-2 2019 HPI	5.4	4.6	5.2	4.8	5.0
Grandi HPI	5.5	4.7	5.3	4.9	5.1
Joey HPI	5.4	4.6	5.2	4.8	5.0
Picolo HPI	5.5	4.7	5.3	4.9	5.1

N factors for HPI samples were calculated using The National Renewable Energy Laboratory's (NREL) Nitrogen-to-Protein Factor Calculator, available at https://www.nrel.gov/bioenergy/assets/docs/calculation_sheet_n_factor.xls.

Appendix N: Sample Calculation for Determining Amino Acid Score, *In Vitro* Protein Digestibility, and Protein Digestibility-Corrected Amino Acid Score (PDCAAS)

Calculating amino acid score (AAS) for pH-HPI, where the first limiting amino acid is lysine:

$$\text{AAS} = \frac{\text{first limiting amino acid content of test protein}}{\text{first limiting amino acid in reference amino acid pattern}} \qquad \textbf{Eq. 12}$$

$$\text{AAS} = \frac{37.21 \text{ mg lysine / g protein}}{58 \text{ mg lysine / g protein}} = 0.642$$

where the reference amino acid is that which is required for preschool children ages 2 to 5 years as defined by FAO/WHO Expert Consultation (1991).

Calculating *in vitro* protein digestibility for pH-HPI:

$$\textit{In vitro} \text{ protein digestibility} = 65.66 + (18.10 \times \Delta pH_{10\ min}) \qquad \textbf{Eq. 2}$$

$$\textit{In vitro} \text{ protein digestibility} = 65.66 + (18.10 \times (7.99 - 6.59)) = 90.9\%$$

where $\Delta pH_{10\ min}$ is the change in pH measured initially and the pH measured after 10 minutes of mixing with the enzyme cocktail.

Calculating protein digestibility-corrected amino acid score (PDCAAS) for pH-HPI:

PDCAAS = AAS of first limiting amino acid x *in vitro* digestibility **Eq. 13**

PDCAAS = 0.642 x 0.909 = 0.583

Appendix O: ANOVA Tables

Table 26. Analysis of variance on the effect of solubilization pH on % soluble hemp protein.

Sample Analysis	Source of Variation	DF	SS	MS	F	P
HPI solubilized at pH 7, 8, 9, 10, 11, 12	Between Groups	5	13379.0	2675.79	684.48	2.656×10^{-14}
	Residual	12	46.9	3.91		
	Total	17	13425.9			

Table 27. Analysis of variance on the effect of salt (NaCl) concentration (M) on % soluble hemp protein.

Sample Analysis	Source of Variation	DF	SS	MS	F	P
HPI solubilized in 0.5 M, 0.75 M, 1 M, 1.25 M NaCl	Between Groups	3	4686.5	1562.17	784.67	3.234×10^{-10}
	Residual	8	15.9	1.99		
	Total	11	4702.4			

Table 28. Analysis of variance on the effect of protein sample on color value indicating lightness (L*).

Sample Analysis	Source of Variation	DF	SS	MS	F	P
cSPI, cPPI, pH-HPI, Salt-HPI	Between Groups	3	129.246	43.082	633.17	7.601×10^{-10}
	Residual	8	0.544	0.068		
	Total	11	129.79			

Table 29. Analysis of variance on the effect of protein sample on color value indicating redness and greenness (a*).

Sample Analysis	Source of Variation	DF	SS	MS	F	P
cSPI, cPPI, pH-HPI, Salt-HPI	Between Groups	3	0.55216	0.184053	31.552	8.789×10^{-5}
	Residual	8	0.04667	0.005833		
	Total	11	0.59883			

Table 30. Analysis of variance on the effect of protein sample on color value indicating yellowness and blueness (b*).

Sample Analysis	Source of Variation	DF	SS	MS	F	P
cSPI, cPPI, pH-HPI, Salt-HPI	Between Groups	3	306.599	102.2	12722	4.746×10^{-15}
	Residual	8	0.064	0.008		
	Total	11	306.663			

Table 31. Analysis of variance on the effect of protein sample on surface charge (mV) measured with sample dissolved in water.

Sample Analysis	Source of Variation	DF	SS	MS	F	P
cSPI, cPPI, pH-HPI, Salt-HPI	Between Groups	3	2181.01	727.00	280.15	$< 2.2 \times 10^{-16}$
	Residual	32	83.04	2.59		
	Total	35	2264.05			

Table 32. Analysis of variance on the effect of protein sample on surface charge (mV) measured with sample dissolved in 0.5 M sodium chloride solution.

Sample Analysis	Source of Variation	DF	SS	MS	F	P
cSPI, cPPI, pH-HPI, Salt-HPI	Between Groups	3	3.666	1.2220	0.2889	0.833
	Residual	32	135.341	4.2294		
	Total	35	139.007			

Table 33. Analysis of variance on the effect of protein sample on surface hydrophobicity.

Sample Analysis	Source of Variation	DF	SS	MS	F	P
cSPI, cPPI, pH-HPI, Salt-HPI	Between Groups	3	196581400	65527133	25	0.0002041
	Residual	8	20969077	2621135		
	Total	11	217550477			

Table 34. Analysis of variance on the effect of protein sample on protein solubility non-heated at pH 7 in DDW.

Sample Analysis	Source of Variation	DF	SS	MS	F	P
cSPI, cPPI, pH-HPI, Salt-HPI	Between Groups	3	4981.9	1660.63	32.962	7.488×10^{-5}
	Residual	8	403.0	50.38		
	Total	11	5384.9			

Table 35. Analysis of variance on the effect of protein sample on protein solubility heated at pH 7 in DDW.

Sample Analysis	Source of Variation	DF	SS	MS	F	P
cSPI, cPPI, pH-HPI, Salt-HPI	Between Groups	3	9191.1	3063.71	355.35	7.552×10^{-9}
	Residual	8	69.0	8.62		
	Total	11	9260.1			

Table 36. Analysis of variance on the effect of protein sample on protein solubility non-heated at pH 3.2 in DDW.

Sample Analysis	Source of Variation	DF	SS	MS	F	P
cSPI, cPPI, pH-HPI, Salt-HPI	Between Groups	3	3429.9	1143.31	33.442	7.101×10^{-5}
	Residual	8	273.5	34.19		
	Total	11	3703.4			

Table 37. Analysis of variance on the effect of protein sample on protein solubility heated at pH 3.2 in DDW.

Sample Analysis	Source of Variation	DF	SS	MS	F	P
cSPI, cPPI, pH-HPI, Salt-HPI	Between Groups	3	2825.48	941.83	26.385	0.0001681
	Residual	8	285.56	35.69		
	Total	11	3111.04			

Table 38. Analysis of variance on the effect of protein sample on protein solubility non-heated at pH 7 in 0.5 M NaCl.

Sample Analysis	Source of Variation	DF	SS	MS	F	P
cSPI, cPPI, pH-HPI, Salt-HPI	Between Groups	3	2011.69	670.56	26.861	0.0001577
	Residual	8	199.71	24.96		
	Total	11	2211.4			

Table 39. Analysis of variance on the effect of protein sample on protein solubility heated at pH 7 in 0.5 M NaCl.

Sample Analysis	Source of Variation	DF	SS	MS	F	P
cSPI, cPPI, pH-HPI, Salt-HPI	Between Groups	3	1393.07	464.36	43.57	2.659×10^{-5}
	Residual	8	85.26	10.66		
	Total	11	1478.33			

Table 40. Analysis of variance on the effect of protein sample on protein solubility non-heated at pH 3.2 in 0.5 M NaCl.

Sample Analysis	Source of Variation	DF	SS	MS	F	P
cSPI, cPPI, pH-HPI, Salt-HPI	Between Groups	3	164.29	54.762	2.9274	0.09976
	Residual	8	149.66	18.707		
	Total	11	313.95			

Table 41. Analysis of variance on the effect of protein sample on protein solubility heated at pH 3.2 in 0.5 M NaCl.

Sample Analysis	Source of Variation	DF	SS	MS	F	P
cSPI, cPPI, pH-HPI, Salt-HPI	Between Groups	3	178.220	59.407	7.1777	0.01531
	Residual	7	57.936	8.277		
	Total	10	236.156			

Table 42. Analysis of variance on the effect of protein sample dissolved in DDW on gel strength.

Sample Analysis	Source of Variation	DF	SS	MS	F	P
cSPI, cPPI, pH-HPI, Salt-HPI	Between Groups	3	1016.14	338.71	2612.8	$< 2.2 \times 10^{-16}$
	Residual	14	1.81	0.13		
	Total	17	1017.95			

Table 43. Analysis of variance on the effect of protein sample dissolved in 0.5 M NaCl on gel strength.

Sample Analysis	Source of Variation	DF	SS	MS	F	P
cSPI, cPPI, pH-HPI, Salt-HPI	Between Groups	3	4254.9	1418.31	452.92	6.238×10^{-15}
	Residual	15	47.0	3.13		
	Total	18	4301.9			

Table 44. Analysis of variance on the effect of protein sample dissolved in DDW on water holding capacity.

Sample Analysis	Source of Variation	DF	SS	MS	F	P
cSPI, cPPI, pH-HPI, Salt-HPI	Between Groups	3	22927.2	7642.4	1049.6	9.399×10^{-16}
	Residual	13	94.7	7.3		
	Total	16	23021.9			

Table 45. Analysis of variance on the effect of protein sample dissolved in 0.5 M NaCl on water holding capacity.

Sample Analysis	Source of Variation	DF	SS	MS	F	P
cSPI, cPPI, pH-HPI, Salt-HPI	Between Groups	3	24541.9	8180.6	3856.6	$< 2.2 \times 10^{-16}$
	Residual	12	25.5	2.1		
	Total	15	24567.4			

Table 46. Analysis of variance on the effect of protein sample dissolved in 0.5 M NaCl on emulsification capacity measured at 1% protein.

Sample Analysis	Source of Variation	DF	SS	MS	F	P
cSPI, cPPI, pH-HPI, Salt-HPI	Between Groups	3	46976	15658.5	44.458	8.943×10^{-7}
	Residual	12	4226	352.2		
	Total	15	51202			

Table 47. Analysis of variance on the effect of protein sample dissolved in DDW on emulsification stability.

Sample Analysis	Source of Variation	DF	SS	MS	F	P
cSPI, cPPI, pH-HPI, Salt-HPI	Between Groups	3	758.35	252.784	147.92	7.759×10^{-11}
	Residual	14	23.93	1.709		
	Total	17	782.28			

Table 48. Analysis of variance on the effect of protein sample dissolved in 0.5 M NaCl on emulsification stability.

Sample Analysis	Source of Variation	DF	SS	MS	F	P
cSPI, cPPI, pH-HPI, Salt-HPI	Between Groups	3	1272.25	424.08	77.656	3.97×10^{-10}
	Residual	17	92.84	5.46		
	Total	20	1365.09			

Table 49. Analysis of variance on the effect of protein sample dissolved in DDW on emulsification activity index.

Sample Analysis	Source of Variation	DF	SS	MS	F	P
cSPI, cPPI, pH-HPI, Salt-HPI	Between Groups	3	61282	20427.3	34.502	1.013×10^{-6}
	Residual	14	8289	592.1		
	Total	17	69571			

Table 50. Analysis of variance on the effect of protein sample dissolved in 0.5 M NaCl on emulsification activity index.

Sample Analysis	Source of Variation	DF	SS	MS	F	P
cSPI, cPPI, pH-HPI, Salt-HPI	Between Groups	3	201086	67029	233.21	5.246×10^{-14}
	Residual	17	4886	287		
	Total	20	205972			

Table 51. Analysis of variance on the effect of protein sample dissolved in DDW on foaming capacity.

Sample Analysis	Source of Variation	DF	SS	MS	F	P
cSPI, cPPI, pH-HPI, Salt-HPI	Between Groups	3	60014	20004.5	177.41	5.774×10^{-7}
	Residual	7	789	112.8		
	Total	10	60803			

Table 52. Analysis of variance on the effect of protein sample dissolved in 0.5 M NaCl on foaming capacity.

Sample Analysis	Source of Variation	DF	SS	MS	F	P
cSPI, cPPI, pH-HPI, Salt-HPI	Between Groups	3	443369	147790	1405.6	4.311×10^{-10}
	Residual	7	736	105		
	Total	10	444105			

Table 53. Analysis of variance on the effect of protein sample dissolved in DDW on foaming stability.

Sample Analysis	Source of Variation	DF	SS	MS	F	P
cSPI, cPPI, pH-HPI, Salt-HPI	Between Groups	3	9610.9	3203.6	92.311	5.425×10^{-6}
	Residual	7	242.9	34.7		
	Total	10	9853.8			

Table 54. Analysis of variance on the effect of protein sample dissolved in 0.5 M NaCl on foaming stability.

Sample Analysis	Source of Variation	DF	SS	MS	F	P
cSPI, cPPI, pH-HPI, Salt-HPI	Between Groups	3	6361.3	2120.44	45.832	5.715×10^{-5}
	Residual	7	323.9	46.27		
	Total	10	6685.2			

Table 55. Analysis of variance on the effect of protein sample on color value indicating lightness (L*).

Sample Analysis	Source of Variation	DF	SS	MS	F	P
cSPI, cPPI, CFX-2 2019 HPI, Grandi HPI, Joey HPI, Picolo HPI	Between Groups	5	168.183	33.637	1209.2	8.834×10^{-16}
	Residual	12	0.334	0.028		
	Total	17	168.517			

Table 56. Analysis of variance on the effect of protein sample on color value indicating redness and greenness (a*).

Sample Analysis	Source of Variation	DF	SS	MS	F	P
cSPI, cPPI, CFX-2 2019 HPI, Grandi HPI, Joey HPI, Picolo HPI	Between Groups	5	6.7968	1.35935	855.54	7.002×10^{-15}
	Residual	12	0.0191	0.00159		
	Total	17	6.8159			

Table 57. Analysis of variance on the effect of protein sample on color value indicating yellowness and blueness (b*).

Sample Analysis	Source of Variation	DF	SS	MS	F	P
cSPI, cPPI, CFX-2 2019 HPI, Grandi HPI, Joey HPI, Picolo HPI	Between Groups	5	68.145	13.6291	1283.7	6.176×10^{-16}
	Residual	12	0.127	0.0106		
	Total	17	68.272			

Table 58. Analysis of variance on the effect of protein sample on denaturation temperature of 7S vicilin-like protein measured using differential scanning calorimetry (DSC).

Sample Analysis	Source of Variation	DF	SS	MS	F	P
CFX-2 2019 HPI, Grandi HPI, Joey HPI, Picolo HPI	Between Groups	3	9.6403	3.2134	74.688	3.427×10^{-6}
	Residual	8	0.3442	0.0430		
	Total	11	9.9845			

Table 59. Analysis of variance on the effect of protein sample on enthalpy of denaturation of 7S vicilin-like protein measured using differential scanning calorimetry (DSC).

Sample Analysis	Source of Variation	DF	SS	MS	F	P
CFX-2 2019 HPI, Grandi HPI, Joey HPI, Picolo HPI	Between Groups	3	10.1237	3.3746	8.8359	0.006414
	Residual	8	3.0553	0.3819		
	Total	11	13.179			

Table 60. Analysis of variance on the effect of protein sample on denaturation temperature of 11S edestin measured using differential scanning calorimetry (DSC).

Sample Analysis	Source of Variation	DF	SS	MS	F	P
CFX-2 2019 HPI, Grandi HPI, Joey HPI, Picolo HPI	Between Groups	3	8.4725	2.82416	45.661	2.23×10^{-5}
	Residual	8	0.4948	0.06185		
	Total	11	8.9673			

Table 61. Analysis of variance on the effect of protein sample on enthalpy of denaturation of 11S edestin measured using differential scanning calorimetry (DSC).

Sample Analysis	Source of Variation	DF	SS	MS	F	P
CFX-2 2019 HPI, Grandi HPI, Joey HPI, Picolo HPI	Between Groups	3	2.9088	0.96959	6.5124	0.01535
	Residual	8	1.1911	0.14888		
	Total	11	4.0999			

Table 62. Analysis of variance on the effect of protein sample on surface charge (mV).

Sample Analysis	Source of Variation	DF	SS	MS	F	P
cSPI, cPPI, CFX-2 2019 HPI, Grandi HPI, Joey HPI, Picolo HPI	Between Groups	5	2489.64	497.93	198.55	$< 2.2 \times 10^{-16}$
	Residual	48	120.38	2.51		
	Total	53	2610.02			

Table 63. Analysis of variance on the effect of protein sample on surface hydrophobicity.

Sample Analysis	Source of Variation	DF	SS	MS	F	P
cSPI, cPPI, CFX-2 2019 HPI, Grandi HPI, Joey HPI, Picolo HPI	Between Groups	5	419930120	83986024	39.566	4.758×10^{-7}
	Residual	12	25472453	2122704		
	Total	17	445402573			

Table 64. Analysis of variance on the effect of protein sample on protein solubility non-heated at pH 7 in DDW.

Sample Analysis	Source of Variation	DF	SS	MS	F	P
cSPI, cPPI, CFX-2 2019 HPI, Grandi HPI, Joey HPI, Picolo HPI	Between Groups	5	9478.1	1895.62	62.394	3.608×10^{-8}
	Residual	12	364.6	30.38		
	Total	17	9842.7			

Table 65. Analysis of variance on the effect of protein sample on protein solubility heated at pH 7 in DDW.

Sample Analysis	Source of Variation	DF	SS	MS	F	P
cSPI, cPPI, CFX-2 2019 HPI, Grandi HPI, Joey HPI, Picolo HPI	Between Groups	5	15580.8	3116.16	841	7.757×10^{-15}
	Residual	12	44.5	3.71		
	Total	17	15625.3			

Table 66. Analysis of variance on the effect of protein sample on protein solubility non-heated at pH 3.2 in DDW.

Sample Analysis	Source of Variation	DF	SS	MS	F	P
cSPI, cPPI, CFX-2 2019 HPI, Grandi HPI, Joey HPI, Picolo HPI	Between Groups	5	5212.6	1042.54	27.235	3.714×10^{-6}
	Residual	12	459.3	38.28		
	Total	17	5671.9			

Table 67. Analysis of variance on the effect of protein sample on protein solubility heated at pH 3.2 in DDW.

Sample Analysis	Source of Variation	DF	SS	MS	F	P
cSPI, cPPI, CFX-2 2019 HPI, Grandi HPI, Joey HPI, Picolo HPI	Between Groups	5	4027.9	805.57	26.46	4.34×10^{-6}
	Residual	12	365.3	30.44		
	Total	17	4393.2			

Table 68. Analysis of variance on the effect of protein sample dissolved in DDW on gel strength.

Sample Analysis	Source of Variation	DF	SS	MS	F	P
cSPI, cPPI, CFX-2 2019 HPI, Grandi HPI, Joey HPI, Picolo HPI	Between Groups	5	1665.63	333.13	445.72	$< 2.2 \times 10^{-16}$
	Residual	22	16.44	0.75		
	Total	27	1682.07			

Table 69. Analysis of variance on the effect of protein sample dissolved in DDW on water holding capacity.

Sample Analysis	Source of Variation	DF	SS	MS	F	P
cSPI, cPPI, CFX-2 2019 HPI, Grandi HPI, Joey HPI, Picolo HPI	Between Groups	5	426.83	85.366	7.8064	0.000236
	Residual	22	240.58	10.935		
	Total	27	667.41			

Table 70. Analysis of variance on the effect of protein sample dissolved in DDW on emulsification stability.

Sample Analysis	Source of Variation	DF	SS	MS	F	P
cSPI, cPPI, CFX-2 2019 HPI, Grandi HPI, Joey HPI, Picolo HPI	Between Groups	5	6005.8	1201.16	15.758	1.092×10^{-5}
	Residual	16	1219.6	76.23		
	Total	21	7225.4			

Table 71. Analysis of variance on the effect of protein sample dissolved in DDW on emulsification activity index.

Sample Analysis	Source of Variation	DF	SS	MS	F	P
cSPI, cPPI, CFX-2 2019 HPI, Grandi HPI, Joey HPI, Picolo HPI	Between Groups	5	80526	16105.2	132.23	1.99×10^{-12}
	Residual	16	1949	121.8		
	Total	21	82475			

Table 72. Analysis of variance on the effect of protein sample dissolved in DDW on foaming capacity.

Sample Analysis	Source of Variation	DF	SS	MS	F	P
cSPI, cPPI, CFX-2 2019 HPI, Grandi HPI, Joey HPI, Picolo HPI	Between Groups	5	56491	11298.1	284.18	3.058×10^{-11}
	Residual	11	437	39.8		
	Total	16	56928			

Table 73. Analysis of variance on the effect of protein sample dissolved in DDW on foaming stability.

Sample Analysis	Source of Variation	DF	SS	MS	F	P
cSPI, cPPI, CFX-2 2019 HPI, Grandi HPI, Joey HPI, Picolo HPI	Between Groups	5	9112.2	1822.44	54.217	2.23×10^{-7}
	Residual	11	369.8	33.61		
	Total	16	9482			

Table 74. Analysis of variance on the effect of protein sample on in vitro protein digestibility.

Sample Analysis	Source of Variation	DF	SS	MS	F	P
CFX-2 2019 HPI, Grandi HPI, Joey HPI, Picolo HPI	Between Groups	3	4.0333	1.3444	0.9915	0.4444
	Residual	8	10.8472	1.3559		
	Total	11	14.8805			

Table 75. Analysis of variance on the effect of protein sample on PDCAAS.

Sample Analysis	Source of Variation	DF	SS	MS	F	P
CFX-2 2019 HPI, Grandi HPI, Joey HPI, Picolo HPI	Between Groups	3	7.9615	2.65383	4.0444	0.05061
	Residual	8	5.2494	0.65618		
	Total	11	13.2109			