FUNCTIONALITY AND NUTRITIONAL VALUE OF FABA BEAN PROTEIN ISOLATES: COMPARISON TO MAJOR LEGUME PROTEINS IN THE MARKET

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ABSTRACT

The present research compared the functional and nutritional values of protein isolates of faba bean, yellow pea, and soy to investigate the utilization potential of Canadian faba beans as value-added ingredients in food formulations. In the first study, protein isolates of faba bean (FPI) cultivars Fabelle, Malik, and Snowbird, yellow pea (PPI) CDC Amarillo, and soybean (SPI) AAC 26-15 were prepared by alkaline extraction and isoelectric precipitation (AE-IP) and evaluated for their physicochemical and functional properties. The reduced water usage from the 1:10 flour:solvent ratio to 1:8 maintained the protein yield, whereas the ratio of 1:6 significantly lowered protein extractability. The faba bean seeds were rich in protein (29.34-34.35%), higher than that of pea (23.10%), while being equivalently low in lipids (1.11-1.35%). In summary of the quality attributes, with little impact of protein composition in terms of the legumin:vicilin (L/V) ratio, the functionalities (protein solubility, water and oil holding capacity, foaming and emulsifying properties) of FPI were mostly similar to those of PPI while being comparable or higher to those of SPI. The cultivar Snowbird had a few attributes generally different from those of Fabelle and Malik. In the second study, legume flours and isolates prepared in Study 1 were compared for their nutritional values. The faba bean samples were largely comparable to pea and soy for the content of total phenolic compounds, condensed tannins, and phytic acid. Meanwhile, they were less concentrated with the raffinose family oligosaccharides (RFO) and contained additional vicine and convicine at varying levels. However, since methionine and cysteine were more limiting in the faba bean samples, they had lower protein quality (*in vitro* protein digestibility corrected amino acid score; IV-PDCAAS) than pea and soy, especially for isolates (Snowbird < Fabelle < Malik) due to the loss of albumins during AE-IP. Overall, the findings suggested that FPI could replace PPI or SPI in formulating products that require additional or improved functionality, whereas when used as nutritional ingredients, a complementary blend with cereals would be beneficial. Future studies should investigate the modification of wet extraction processes to minimize nutritional losses.

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ΔA	Change in absorbance
A_0	Absorbance of the diluted emulsion at time zero
A_{10}	Absorbance of the diluted emulsion after 10 min
AA	Amino acid
AAFC	Agriculture and Agri-Food Canada
AAS	Amino acid score
Abs	Absorbance
AE-IP	Alkaline extraction-isoelectric precipitation
ANF	Antinutritional factor
ANOVA	One-way analysis of variance
AOAC	Association of Official Analytical Chemists
BAEE	Nα-Benzoyl-L-arginine ethyl ester
β	Correction factor
c	Weight of protein per volume
CDC	Crop Development Centre
CE	Catechin equivalent
CIGI	Canadian International Grains Institute
CPI	Chickpea protein isolate
CS	Creaming stability
CT	Condensed tannins
d.b.	Dry basis
DF	Diafiltration
EAA	Essential amino acid
EAI	Emulsifying activity index
EC	Emulsion capacity
ES	Emulsion stability
ESI	Emulsifying stability index
ε	Permittivity
η	Viscosity
FAO	Food and Agriculture Organization of the United Nations
FC	Foaming capacity

FE	Foam expansion
FF	Faba bean flour
F_{max}	Maximum force
FPC	Faba bean protein concentrate
FPI	Faba bean protein isolate
FS	Foam stability
$f(\kappa\alpha)$	Smoluchowski approximation
g	Gravitational force
GAE	Gallic acid equivalent
γ	Surface and interfacial tension
G6PD	Glucose-6-phosphate dehydrogenase
HPLC	High performance liquid chromatography
ISO	International Organization for Standardization
IVPD	*In vitro* protein digestibility
IV-PDCAAS	*In vitro* protein digestibility corrected amino acid score
LAA	Limiting amino acid
LPI	Lentil protein isolate
L/V	Legumin:vicilin
MP	Micellar precipitation
N	Dilution factor
%N	%Nitrogen
OHC	Oil holding capacity
O/W	Oil-in-water
PA	Phytic acid
PDCAAS	Protein digestibility corrected amino acid score
PER	Protein efficiency ratio
PF	Pea flour
pH	Acidity in logarithmic scale
ΔpH	Change in pH
φ	Volume fraction
pI	Isoelectric point
PPC	Pea protein concentrate
PPI	Pea protein isolate

PVDF	Polyvinylidene fluoride
r	Pearson correlation
R	Radius
RDI	Reasonable Daily Intake
RFO	Raffinose family oligosaccharide
rpm	Revolutions per minute
SAA	Sulfur-containing amino acid
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SE	Salt extraction
SF	Soy flour
SPI	Soybean protein isolate
SPSS	Statistical Product and Service Solutions
t	Time interval
TFD	True fecal digestibility
TPC	Total phenolic compounds
U_E	Electrophoretic mobility
UF	Ultrafiltration
UPLC	Ultra performance liquid chromatography
V-C	Vicine and convicine
V_{f0}	foam volume at time zero
V_{f30}	foam volume after 30 min
V_{li}	Volume of the protein solution
v/v	Volume to volume
WFP	World Food Program
WHC	Water holding capacity
WHO	World Health Organization
W/O	Water-in-oil
w/v	Weight to volume
w/w	Weight to weight
ζ	Zeta potential

1. INTRODUCTION

1.1. Overview

Food proteins are an essential source of nutrition to supply energy and maintain growth and development of the human body. Presently, the dietary protein market has seen a significant rise in plant-based alternatives, predominantly prepared from cereals and legumes. The evolving consumer preferences for non-meat diets are encouraged by the health-promoting benefits of plant consumption and concerns over the environment and animal welfare (Lynch et al., 2018). Legumes (e.g., pea, chickpea, lentil, faba bean, and soybean) are good sources of protein with adequate amounts of essential amino acids (Boye et al., 2010b). Since legumes are relatively high in lysine while limiting in sulfur-containing amino acids (SAA) methionine and cysteine, they are often consumed together with cereals by many people worldwide to meet their daily requirements for protein (Nosworthy et al., 2017b). The protein content of legumes is generally higher than that of cereal crops, as the root nodules fix atmospheric nitrogen and provide for protein synthesis (Biswas & Gresshoff, 2014). Legumes are abundant in dietary fiber, minerals, and vitamins required for human health, of which folate, iron, magnesium, potassium, and zinc are richer in content than those of cereals (Singh, 2017). Studies have shown that the consumption of legumes may contribute to potential health benefits relevant to the prevention of cardiovascular diseases, type-2 diabetes, hypertension, osteoporosis, gastrointestinal disorders, adrenal disease, and cancer (Boye et al., 2010b; Hu, 2003).

Legumes can be processed into flours, protein concentrates and isolates with varying composition and functionality. Incorporating legume protein ingredients provides foods with favorable quality attributes and influences their sensory and mechanical characteristics. Examples of legume protein functionality include solubility, water and oil holding capacity, and foaming and emulsifying properties (Boye et al., 2010a). The major storage proteins in legumes are globulins, which are further categorized into legumins (11S) and vicilins (7S) (Shevkani et al., 2019). These two protein types differ in size, structure, and amino acid composition, hence varying functionality (Koyoro & Powers, 1987). For instance, pure vicilin solutions prepared from pea showed better emulsifying and gelation properties than pure

legumin solutions in the work of Barac et al. (2010). It has been hypothesized that the legumin:vicilin (L/V) ratio, which varies among species and cultivars, has a significant impact on the functionality of legume proteins.

Faba bean (*Vicia faba* L.), also known as fava bean, broad bean, and horse bean, is a cold season annual crop that is widely grown and consumed in the mid-Eastern region of the world, while the use as livestock feed is more extensive in North America (Bilalis et al., 2003; Vioque et al., 2012). Over the past two decades, the global production of dry faba beans increased from 3.6 to 5.4 million tons with China, Ethiopia, and the United Kingdom being the top producers (FAO, 2021). While being lower than that of the leading countries, there has been a significant increase in faba bean production in Canada, especially from the prairie provinces (McGill et al., 2016). Presently, the Canadian-grown faba bean primarily targets the domestic feedstock market, while a smaller portion is being exported as food (Khazaei et al., 2021). The ethnic food market accounts for the majority of domestic faba bean consumption with only limited growth (McGill et al., 2016). According to Bhatty (1974), dry faba bean seeds sourced from Canada had approximately 26.4-37.4% of crude protein, higher than that of pea, lentil, chickpea, and beans, 6.4-8.4% of crude fiber, and very low levels of lipid (~1.8%). The high protein content and favorable processing characteristics (e.g., low oil content) of faba beans offer opportunities to develop economically valuable ingredient fractions for expanding their utilization (Tyler et al., 1981).

Currently, value-added applications of faba bean fractions (e.g., protein isolates) are limited, primarily due to several reasons. Firstly, there are antinutritional factors (ANF) such as tannin, vicine, and convicine in faba beans, and some others generally found in pulse crops (e.g., phytic acid). The consumption of such compounds negatively affects the digestion and assimilation of nutrients (Gulewicz et al., 2014). On the other hand, a gap of scientific knowledge is present in relation to new faba bean cultivars grown in Canada, as the plant has been considered a minor crop until recently (Khazaei et al., 2021). Advanced utilization of Canadian faba beans requires an updated understanding of the recent market classes to fully benefit from emerging opportunities. A comprehensive investigation into the major seed components and their quality attributes, both functional and nutritional, with direct comparison to major legume proteins in the current market, is critical.

The overarching goal of this research was to evaluate the functional and nutritional values of protein isolates sourced from three faba bean cultivars currently grown in Canada (Fabelle, Malik, and Snowbird), and to compare the quality attributes with those of yellow pea

(CDC Amarillo) and soybean (AAC 26-15), the two dominant sources of legume protein in the market. The faba bean cultivars were selected to reflect the current market classes, with Fabelle being medium seeded, regular tannin, and low vicine and convicine, Malik being large seeded and regular tannin, and Snowbird being small/medium seeded and low tannin (CFIA, 2021; Saskatchewan Pulse Growers, 2021). In Study 1, the physicochemical properties (proximate composition, L/V ratio, surface charge, and interfacial tension) and functionality (protein solubility, water and oil holding capacity, foaming capacity and stability, and emulsifying activity and stability index) of the legume protein isolates were evaluated. The effect of reducing water usage in the alkaline extraction-isoelectric precipitation (AE-IP) method on the protein yield, and the impact of protein composition (L/V ratio) on the quality attributes, were emphasized for faba bean only. In the second study, flours and isolates prepared in Study 1 were examined for ANF, including total phenolic compounds, condensed tannins, phytic acid, vicine, convicine and oligosaccharides. The protein quality of the samples was assessed by evaluating the amino acid composition, amino acid scores, *in vitro* protein digestibility (IVPD) and *in vitro* protein digestibility corrected amino acid scores (IV-PDCAAS). The findings from both studies provide insight into the utilization potential of Canadian faba beans as value-added ingredients in food product formulations and a clear picture of how they compared to pea and soy.

1.2. Objectives

The primary objective was to compare the quality attributes of protein isolates prepared from Canadian faba bean to those from yellow pea and soybean, with specific objectives being:

1) Evaluating the physicochemical and functional properties of protein isolates prepared from faba bean, yellow pea, and soybean in Study 1 while investigating

a) the effect of reducing water usage during AE-IP on the protein yield (of the faba bean cultivar Fabelle only), and

b) the impact of protein composition (L/V ratio) on the quality attributes of protein isolates (of faba bean only), and

2) Evaluating the nutritional value (levels of ANF and protein quality) of flours and protein isolates prepared from faba bean, yellow pea, and soybean in Study 2

1.3. Hypotheses

Addressing the objectives of this research, the following hypotheses were tested:

1) Protein isolates prepared from faba bean cultivars, Fabelle, Malik, and Snowbird, are comparable in physicochemical and functional properties to those of yellow pea (CDC Amarillo) and soy (AAC 26-15), and

a) the reduced water usage from 1:10 flour:solvent ratio (w:v) to 1:8 and 1:6 during AE will lower the extractability and the yield of protein when recovered by IP, and

b) the L/V ratio of faba bean protein isolates has a relationship with their physicochemical and functional attributes, and

2) Flours and protein isolates prepared from the faba bean cultivars, Fabelle, Malik, and Snowbird, are comparable in nutritional value (levels of ANF and protein quality) to those from yellow pea (Amarillo) and soy (AAC 26-15)

2. LITERATURE REVIEW

2.1. Pulses – Overview

Pulses are members of the legume family harvested for their dry edible seeds and have been cultivated and consumed globally as an important source of nutrition for at least 10,000 years (FAO, 2015; Mudryj et al., 2014). Pulses are rich in protein, of which the content is almost double that of cereal crops (Boye et al., 2010a; Curran, 2012; Tiwari & Singh, 2012). Due to the relatively high content of lysine, pulses are consumed in combination with cereal grains by populations around the world to meet their protein needs, particularly in regions where the consumption of animal proteins is limited due to religious beliefs, ethical concerns, or the unavailability of animal products (Liener, 1962). Concerns related to common food allergens, including soy, gluten, and nuts in other plant materials, also lead to the inclusion of pulses in people's diets. In addition, pulses offer an abundance of fiber, vitamins, minerals, and antioxidants and provide health benefits relevant to preventing chronic diseases and cancer (Hu, 2003; Roy et al., 2010). Moreover, the cultivation of pulses is considered environmentally sustainable as the plants fix atmospheric nitrogen via their rhizobia bacteria, thus reducing the release of greenhouse gases from synthetic fertilizer manufacture (Biswas & Gresshoff, 2014).

Pulses' highly nutritious and sustainable profile has encouraged consumption and sparked interest in replacing traditional animal proteins with fractionated pulse alternatives in food products, especially in North America. The functionality of pulse proteins, including solubility, water and oil-holding capacity, emulsifying and foaming properties, allows pulses to be formulated in a variety of products with varying functional and sensory qualities. Despite being applied in food products, expanding the utilization of pulses could be challenged by ANF, compounds that are naturally generated in plants that reduce the bioavailability of nutrients in the human body upon consumption (Fekadu Gemede, 2014). Pulses contain a series of ANF, including phenolics, tannins, phytates, lectins, saponins, and enzyme inhibitors (e.g., trypsin inhibitors, chymotrypsin inhibitors, and α-amylase inhibitors). Due to their presence, the protein digestibility of pulses is generally lower than that of cereals, limiting crop utilization

(Tiwari & Singh, 2012). The utilization of pulse ingredients in formulated foods faces obstacles also from the off-flavor compounds, the unfamiliarity of new proteins by the formulators, limited availability of new protein sources, and the relatively high production cost to extract and isolate protein ingredients (Green, 2019).

Faba bean (*Vicia faba* L.) is a cold season annual crop cultivated in many regions around the world for its use in food, feed, and agronomic practices (Köpke & Nemecek, 2010). Also known as fava bean, broad bean, and horse bean, faba bean is widely grown and consumed as an inexpensive grain in the mid-Eastern region, while in Europe, it is primarily cultivated for livestock feed (Bilalis et al., 2003; Wei, 2019). Compared to other pulses, faba bean is a strong nitrogen fixer and has been increasingly used as a cover crop to improve soil quality in recent years (Etemadi et al., 2018). Due to the high protein content of faba bean seeds and the additional crop rotation advantage, the global production of faba bean has been increasing at a steady rate over the past two decades (FAO, 2021). In North America, Canada is emerging as a faba bean producer, with most agronomic activities occurring in Alberta, Saskatchewan, and Manitoba (McGill et al., 2016). The cultivation of faba bean in the Canadian prairies is ideal as the crop is well adapted to wet and cold environments (Link et al., 2010). In 2020, the insured faba bean seeded area in Saskatchewan was 52,387 acres (~21,200 hectares), which was a dramatic increase from 26,803 acres (~10,847 hectares) in 2018 (Friesen, 2021).

Canada produces a variety of faba beans differing in seed size, tannin, vicine, and convicine contents, of which the large, tannin (e.g., Malik) and small, low tannin (Snowbird) cultivars are preferred for food and feed use, respectively (McGill et al., 2016). According to the 2021 production data in the Canadian prairies, the cultivar Snowbird takes up 83% of the total documented acres, while Malik and other tannin cultivars account for less than 8% (Friesen, 2021). The low vicine and convicine cultivar (e.g., Fabelle) is fairly new and has attracted attention as the consumption of faba beans with such composition reduces the risk of favism. As of now, the animal feed is the primary domestic market for Canadian faba bean supply (Khazaei et al., 2021) while a large portion goes abroad as exports for food use; the domestic niche ethnic food market has a limited growth (McGill et al., 2016). Since faba bean is high in protein and fiber and low in lipids, opportunities exist in developing ingredient fractions useful in plant protein-based food product formulations. In response to the rapidly growing demand for plant-based proteins, the Canadian production of faba bean is expected to increase over the next ten years, with the majority going into the fractionated ingredient market and very little into the traditional ones for food and feed (Khazaei et al., 2021).

2.2. Protein composition (11S, 7S)

Pulse seed proteins are composed of dilute salt-soluble globulins, water-soluble albumins, alcohol-soluble prolamins, and dilute acid/base-soluble glutelins (Osborne, 1909). Globulins represent the majority of the pulse proteins (70-80%), and albumins come in second (10-20%), while prolamins and glutelins account for <5% (Shevkani et al., 2019). Similar to most pulses, the major storage proteins in faba bean are globulins (69.5-78.1%) (El Fiel et al., 2002). Based on the sedimentation coefficient, globulins are further characterized into legumin (11S, ~300-400 kDa) and vicilin (7S, ~180 kDa). The 11S legumin is a hexamer comprised of six acidic-basic (α-β) monomers (~60 kDa of each) linked by disulfide bonds (González-Pérez & Arellano, 2009). Generally, the 11S globulins contain high amounts of sulfur-containing cysteine and methionine. On the other hand, the 7S vicilin type proteins are trimeric molecules consisting of three monomers (~50-60 kDa of each) held together by hydrophobic interactions (González-Pérez & Arellano, 2009). Consequently, this 7S protein fraction is low in cysteine. The convicilin (~220-290 kDa), another type of the 7S globulins, is present in pulse seeds in lesser amounts. It has a homologous core structure to that of vicilin and is distinguished by the presence of a highly charged hydrophilic N-terminal extension (Barac et al., 2015b). Compared with vicilin, convicilin has a very distinct amino acid profile, of which the residues of SAA are present in its primary structure, and it also lacks carbohydrate side chains (Barac et al., 2015b). There have been 29 different legume species from 4 genera (*Pisum, Lens, Vicia* and *Latyruus spp*.) containing convicilin gene sequences (Saenz de Miera et al., 2008). As reported by Nikolić et al. (2012), the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) showed that faba bean seeds have a protein composition similar to that of pea with major bands for convicilin (~74 kDa), vicilin (~50 kDa), α-legumin (~36 kDa) and β-legumin (~24 kDa).

The functionality of pulse proteins depends on the protein composition, which differs among species and cultivars. Currently, studies on the impact of protein composition mainly focus on the ratio of 11S:7S (Tay et al., 2006). As an example, a high vicilin content is often linked to high protein solubility due to its polar amino acid profile and low molecular weight (Barac et al., 2015a; Kimura et al., 2008; Koyoro & Powers, 1987). Vicilin is more abundant in negatively charged amino acids, including glutamic acid and aspartic acid, contributing to a much more hydrophilic profile than the legumin fraction (Koyoro & Powers, 1987; Lam et al., 2017; Rubio et al., 2013). The carbohydrate moieties on vicilin extension regions were also

reported to promote hydration under neutral and weakly alkaline conditions (Kimura et al., 2008). Pure vicilin solutions sourced from pea showed better emulsifying and gelation properties than pure legumin solutions in the work of Barac et al. (2010), while Koyoro and Powers (1987) also found that foams of green pea legumin showed lower foaming stability than that of the respective vicilin fraction. Legumin is less surface-active than vicilin due to its rigid structure based on disulfide linkages, which hinders conformational change at the interfacial space. The high molecular weight and low charge profile of legumin also lead to low solubility in solution, limiting diffusion. It was suggested that 7S proteins, when analyzed as pure protein fractions, tend to have better functionality than the 11S type (Barac et al., 2010). Therefore, preparing protein ingredients with a low L/V ratio may be functionally advantageous.

2.3. Protein isolate production methods

Different extraction methods produce protein concentrates or isolates with different final compositions that significantly influence the functional properties of the product (Stone et al., 2015). Various techniques are being tested to obtain the maximum protein yield and avoid negative impacts on protein functionality. Generally, the production of protein-rich fractions (i.e., protein concentrate and isolate) is classified as dry and wet methods.

2.3.1. Dry extraction

Dry fractionation of proteins from starch-rich legume seeds typically involves milling and air classification. The dry processes produce flours, enriched flours (<40~50% protein), and protein concentrates (60-80% protein). The fractionated materials can also be used as a feedstock for wet extraction to increase protein content. Air classification separates dry particles in the flours based on their size, shape, and density within an airstream (Boye et al., 2010a). The process can be repeated several times to improve separation efficiency. In general, dehulling before air classification improves protein purity and decreases the fiber content (Vose et al., 1976). A study done by Coda et al. (2015) showed that the combination of pin mill and air classification of dehulled faba bean seeds produced a light, fine fraction with 51.49% protein and 23.38% starch with high contents of dietary fiber, ash and fat, and a heavy, coarse fraction with 65.82% starch, 16.73% protein and a low level of fat. Meanwhile, according to Vose et al. (1976), the protein content of the fine fraction prepared from faba bean was significantly higher than that reported for cereals and legumes with high lipid content. It was

also in agreement with Tyler et al. (1981) that the method of combining pin millng and air classification was suitable for producing protein and starch-rich fractions from legume seeds that are low in fat. Generally, dry fractionation is advantageous over wet methods because of its lighter impact on the native structure of the proteins and a lower requirement for energy and water. However, since each fraction is contaminated with the other, relatively low protein purity results from dry processing (Boye et al., 2010a; Tyler, 1984).

2.3.2. Wet extraction

Wet extraction of plant proteins involves protein solubilization in suitable solvents followed by centrifugation and precipitation, dependent on the methods employed and the nature of the proteins present in the raw materials. Many factors influence the protein extraction process, such as pH, ionic strength, temperature, and the types of solvent used. Wet extraction of protein is often employed under laboratory conditions to produce protein concentrates (>60% protein) and isolates (>90% protein).

a) Alkaline extraction-isoelectric precipitation

The AE-IP method is based on the effect of pH on protein solubility. At the isoelectric point (pI), where the electrostatic repulsion between proteins is minimal, the proteins show the lowest solubility. Generally, the pI of most plant globulins is around pH 4-5 (Klupšaitė & Juodeikienė, 2015). When dispersed in alkaline solutions (pH 8-11), the proteins regain charges and become more soluble (Klupšaitė & Juodeikienė, 2015). Typically, ground pulse flour is dispersed in water with varying ratios (w:v) from 1:5 to 1:20, and the mixture is adjusted to alkaline pH to allow solubilization (Boye et al., 2010a). After centrifugation, the insoluble portion (e.g., carbohydrates, insoluble fiber, and prolamins) is discarded in the pellet, followed by adjusting the supernatant pH back to or near the pI, where the proteins precipitate out of the solution and get neutralized for further analysis.

Studies have shown that the AE-IP method is the most widely used method to produce legume protein extracts with high protein content (Can Karaca et al., 2011; Han & Hamaker, 2002; Russin et al., 2007; Stone et al., 2015). Chakraborty et al. (1979) prepared protein isolates containing 91.2%, 90.5%, 90.1% and 89.3% protein from Great Northern bean, chickpea, pea, and lentil using AE-IP, respectively. The protein content and yield are easily affected by the extraction conditions, including temperature, time, pH, flour:solvent ratio, nature of the starting material (dehulled/defatted), and equipment parameters (Russin et al., 2007). Faba bean protein

isolates (FPI) extracted by Flink and Christiansen (1973) contained approximately 80-90% protein using a 1:5 flour to solvent ratio, pH 8-10 for solubilization and pH 3.5 for precipitation at 23℃. Using a slightly modified process with the same flour:solvent ratio but different pH and temperature (pH 7-10 and 4-5.3 for solubilization and precipitation, respectively, at 10-20℃), McCurdy and Knipfel (1990) obtained FPI with protein contents of 76.4-94.0%. AE-IP on defatted faba bean flour yielded a 92% protein isolate with a high oil binding capacity when extracted at pH 10.5 and precipitated at pH 4.0 (Vioque et al., 2012). FPI with 76.6% and 84.1% of the protein was reported by Singhal et al. (2016) and Can Karaca et al. (2011), respectively, using the same extraction parameters. Can Karaca et al. (2011) reported that protein isolates prepared using AE-IP had higher overall protein content (85.6%) than that produced by salt extraction (78.4%) for chickpea, lentil, faba bean, soybean, and pea, which agreed with Stone et al. (2015)'s findings on pea protein isolates (PPI). On the other hand, the former study reported that compared to salt extracted isolates, those prepared using AE-IP had higher surface charge, surface hydrophobicity, and solubility, while the latter study reported lower solubility for isolates from AE-IP. The effect on the functional properties was hypothesized as AE-IP produces mostly globulins whereas salt extraction extracts a mixture of globulins and albumins, of which the globulin fraction shows higher surface hydrophobicity (Liu et al., 2008; Papalamprou et al., 2009; 2010).

b) Salt extraction and micellar precipitation

Salt extraction (SE) and micellar precipitation (MP) extract proteins based on their solubility as a function of salt concentration. Protein molecules are structured with hydrophobic cores and hydrophilic surfaces. When the ionic strength within the solution is low (i.e., at low salt concentration), the hydrophilic moieties on the surface interact with the neighboring water molecules and form a layer of hydration around the protein. This phenomenon of "salting-in" improves protein solubility. In MP, the material is dissolved in a dilute salt solution (e.g., 0.1 M NaCl) to solubilize both albumins and globulins, followed by centrifugation to remove the insoluble fraction. A large quantity of cold water is added to the supernatant, substantially lowering the ionic strength and encouraging protein association via hydrophobic interactions. The proteins precipitate out of the solution as micelles to be collected via centrifugation or microfiltration. According to Stone et al. (2015), MP resulted in significantly lower protein yields (30.7-31.1%) for pea than AE-IP (62.6–76.7%), while the protein contents were similar (83.3–86.9% and 81.9–87.8% for MP and AE-IP, respectively). Slightly higher protein contents

for FPI and chickpea protein isolates (CPI) prepared using MP than those by AE-IP were reported by Abdel-Aal et al. (1986). Significantly higher solubility at pH 7.0 for chickpea protein isolate (CPI) prepared by MP (72.5%) than that by AE-IP (60.4%) was observed by Paredes-Lopez et al. (1991), suggesting the proteins were extracted in a more native state by MP (Fuhrmeister & Meuser, 2003).

SE extracts proteins in a similar manner to MP with appropriate choices of salts and concentrations to select for proteins of interest. Typically, the solubilization of the proteins in dilute salt solutions is followed by a clarification step, where the salt is removed by dialysis using a semi-permeable membrane (Can Karaca et al., 2011). The dialysis membrane permits the diffusion of water and salt out of the bag while keeping the larger protein particles inside till reaching a gradient equilibrium (Andrew et al., 2001). The salt extracted FPI had lower protein content (81.98%) than that via AE-IP (84.14%) (Can Karaca et al., 2011), which agreed with Stone et al. (2015) that SE gave the lowest protein content (71.5-79.3%) of PPI but the highest isolate yield (17.4-19.2%) compared to AE-IP and MF. A similar technique used by Bhatty and Christison (1984) on lentil, pea, and faba bean resulted in isolates containing 87%, 91% and 95% protein, respectively, while a low nutritional quality of the isolates was found when fed as the sole protein source in rats' diets. The retarded rat growth may result from the ANF that were extracted along with the proteins, corresponding to the high isolate yield (Murray et al., 1985). A lower degree of denaturation was observed for SE than AE-IP (Paredes-López et al., 1991; Sun & Arntfield, 2010), and SE produced PPI with better functionality, including oil holding capacity, solubility, and foaming capacity (Stone et al., 2015). On the other hand, when a high level of salt is present in the solution, the ions would compete with the proteins for water in the process of "salting-out", disrupting the hydration layer, promoting hydrophobic protein-protein interaction, and decreasing solubility. As a result, the proteins aggregate and precipitate out of the solution. According to Hofmeister (1888), polyvalent salts (e.g., ammonium sulfate) are more effective than univalent salts (e.g., NaCl) in salt precipitation due to the ability to reach high salt concentrations (e.g., 4.1 M) and remain soluble. Followed by a clarification step, the salt can be removed by dialysis, from where the proteins respond to the change in ionic strength and form micelles to be recovered (Boye et al., 2010a).

c) Ultrafiltration

Ultrafiltration (UF) is a pressure-driven membrane filtration process that requires no heat and is often used as an alternative to the isoelectric precipitation (IP) after alkaline

extraction (AE) (Boye et al., 2010a; Klupšaitė & Juodeikienė, 2015). The UF process separates proteins based on the molecular size, ranging from 1,000 to 100,000 kDa, where various molecular weight cut-offs are available to retain proteins of interest (Klupšaitė & Juodeikienė, 2015). Diafiltration (DF) is often combined with UF to improve product recovery and purity, a process in which water is added to dilute the retentate (Singhal et al., 2016). Boye et al. (2010a) found that the pulse (pea, lentil, and chickpea) protein concentrates prepared by UF had a higher overall protein content than those prepared by IP, which agreed with Fuhrmeister and Meuser (2003), whose study showed that protein concentrates of wrinkled pea obtained from UF had higher protein content (70–80%) and lower fat content (2.3%) than those prepared by IP (68% and 3.8%, respectively). Vose (1980) reported the protein content of 94.1% from the UF processed FPI, which was higher than that of PPI (89.5%). It is also reported that UF/DF produces proteins with improved functionality and lower levels of ANF, including protease and amylase inhibitors, lectins, and polyphenols (Fredrikson et al., 2001; Fuhrmeister & Meuser, 2003; Mondor et al., 2009).

2.3.3. Fractionation of 11S and 7S globulins

The enriched protein fraction after wet extraction is often subjected to further purification. To quantify individual proteins (e.g., 11S and 7S globulins from pulses) with high degrees of homogeneity, several methods have been developed, including selective precipitation, chromatography, SDS-PAGE, ultracentrifugation, and UF (García et al., 1997; Leslie et al., 1983; Thanh & Shibasaki, 1976; Wolf et al., 1962; Wu et al., 2000). Depending on the characteristics of the proteins (e.g., size, shape, charge, hydrophobicity, and affinity to certain substances), purity of the samples, extraction methods, and the desired level of purification, appropriate techniques are selected to ensure effective quantification. For example, a common method to fractionate proteins is precipitation by increasing ammonium sulphate concentration, and the proteins are collected in order based on the differential solubility in salt solutions. According to Osborne and Harris (1907), vicilin and legumin fractions of pea were obtained using repeated precipitation at 60% and 80% saturation, respectively. Selective precipitation can also be achieved by controlling the pH in the extracting solvent, where the protein mixtures are precipitated depending on their isoelectric points. To study the structure and function of the individual proteins, it is important to ensure an adequate amount of purified fraction is obtained from the method of choice.

2.4. Functionality of legume proteins

The functionality of proteins is of great importance when it comes to the formulation of food products, as they are closely related to the sensory, physicochemical, and mechanical characteristics of the products, therefore affecting consumer preference and market competitiveness. The nature of the raw material, extraction methods, and processing conditions can have significant impacts on protein functionality.

a) Protein solubility

The protein solubility is an essential parameter in evaluating functionality, as other functional attributes are highly dependent on how well the protein interacts with water. Factors including the hydrophilic/lipophilic balance and the pI of the protein, the pH, ionic strength, and the temperature in the liquid medium determine how soluble the proteins can be. Generally, the higher the surface hydrophobicity of a protein, the greater the protein-protein interaction, the less soluble in the solution (Jiang et al., 2015). As mentioned above, at pI, where the net charge of the molecule is close to zero, the protein exhibits the least electrostatic repulsion towards neighboring protein molecules, therefore minimal solubility. Likewise, when there is high ionic strength in the solution, the salt ions screen the charges around the protein surface, leading to protein aggregation. On the contrary, when the pH is away from the pI, or the ionic strength of the solution is low, the protein molecules become repulsive, thus remaining stable and soluble in the solution. In addition, protein solubility increases with temperature till the denaturation point where the protein unfolds and exposes its hidden hydrophobic regions and aggregates.

Similar patterns for FPI, PPI, and SPI were reported by Fernández-Quintela et al. (1997), with low solubility observed at the pH range 4.0-6.0 around the pI of the proteins. Maximal solubility was obtained at pH 8.0 and 9.0 for SPI and FPI, respectively, while the PPI showed much lower solubility at alkaline pH values. According to Can Karaca et al. (2011), at pH 7.0, CPI, FPI, lentil protein isolate (LPI), and PPI prepared using AE-IP had solubility values of 91.20%, 89.65%, 90.73% and 61.42%, respectively, which were significantly higher than those prepared by SE. The solubility of the pulse proteins was found to be positively and negatively correlated with their surface charge and hydrophobicity, respectively, except for that of CPI. A similar solubility value of FPI prepared using AE-IP of 85%, was found by Johnston et al. (2015), which was lower than that of CPI (94%) and LPI (90%). The solubility of air-classified faba bean protein concentrate (FPC) was reported to range between 82% and 88% by Martinez

et al. (2016), while that of the commercial FPC was 91%, similar to that of the commercial pea protein concentrate (PPC) (92%). An average of 81% for FPI prepared using AE-IP was found by Singhal et al. (2016), significantly higher than that of the commercial PPI (20%) and SPI (31%). The authors concluded that spray drying for the preparation of the commercial samples might result in lower protein solubility than those produced by freeze-drying in laboratory settings.

b) Water and oil holding capacity

Water (WHC) and oil holding capacity (OHC) are important parameters related to the texture, flavor profile, and mouthfeel of food products such as soup, baked goods, ground meals, and processed meat (Shevkani et al., 2015; Sreerama et al. 2012). The ability of the proteins to bind and retain water and oil against gravitational separation within the food matrix prevents the loss of quality during processing and storage (Kiosseoglou & Paraskevopoulou, 2021). WHC and OHC are defined as the amount of water or oil absorbed by the known weight of proteins (g/g), respectively. WHC measures a sum of absorbed water (unfreezable) and retained water (freezable) held by a protein matrix structure, of which the latter one has a more profound contribution (Damodaran, 2008). Retained water is also referred to as physically entrapped water or entrapped bulk phase water, often held between the capillaries of protein aggregates within the matrix (Schnepf, 1992). On the other hand, absorbed water, i.e., vicinal water and multi-layer water, binds to the protein surface via water-ion and water-dipole interactions as well as water-water and water-protein hydrogen bonds (Schnepf, 1992). Water binding can also occur with carbohydrates in the sample (Kinsella & Melachouris, 1976). In short, WHC reflects the ability of a protein matrix to hold water primarily via physical entrapment with relatively minor influences from the hydrophilic/hydrophobic balance, amino acid composition, and charge distribution on the protein surface. Likewise, OHC assesses associations between lipids and hydrophobic, non-polar amino acids on a protein surface and the ability of proteins to physically entrap oil within the matrix. Generally, the OHC for pulse protein isolates is higher than that of the corresponding flours due to the partial denaturation resulting from protein extraction that exposes the hydrophobic regions, favoring oil-binding (Kaur & Singh, 2007; Tiwari & Singh, 2012; Vioque et al., 2012).

The WHC of PPI prepared by AE-IP was significantly higher than that produced by SE, according to Stone et al. (2015), and vice versa for OHC. The WHC was also reported to be higher for AE-IP-produced PPI than that by UF (Boye et al., 2010b). The WHC and OHC for

FPI prepared using AE-IP was 2.6 and 2.3 g/g, respectively, reported by Vioque et al. (2012), while much higher OHC (5.7 and 6.12 g/g) was found by Singhal et al. (2016) and Eckert et al. (2019) using the same protein extraction method. In the latter two studies, the OHC of FPI was substantially higher than that of PPI (1.1 and 3.2 g/g, respectively), which agreed with Fernandez-Quintela et al. (1997) that the FPI had the highest OHC, followed by PPI and SPI, while the WHC was similar for FPI and PPI. However, both WHC and OHC of commercial FPC (0.9 and 1.2 g/g, respectively) measured by Martinez et al. (2016) were similar to those of commercial PPC (0.8 and 1.1 g/g, respectively). It is important to consider different surface properties of pulse cultivars and genotypes, and the methodology differences in measuring WHC and OHC.

c) Foaming

Foams are comprised of a continuous water phase and dispersed air bubbles that would spontaneously separate in the absence of a surfactant (Schwenke et al., 1983). Due to the high solubility of air in water, the primary mechanism of foam destabilization is Ostwald ripening, a process in which the mass transfer occurs from small, dispersed droplets to larger ones (Lifshitz & Slyosov, 1961). Various food applications use proteins to stabilize foams, such as desserts, confectionery, whipped topping, ice cream, and leavened bakery goods (Townsend & Nakai, 1983). The surface-active nature of proteins allows the reduction of surface tension by forming viscoelastic protein films around the air bubbles (Dickinson, 1992). Foaming capacity (FC) is the ability of a protein to generate foams upon homogenization at a given concentration, whereas foaming stability (FS) is the ability of that protein to retain the foam volume as a function of time.

Foam formation with proteins involves several stages. Firstly, the proteins are solubilized within the solution and transported from the bulk phase to the interface primarily via diffusion. Then the proteins penetrate the interface and undergo surface denaturation to re-orient structure and effectively reduce surface tension. A cohesive, viscoelastic film is formed to encapsulate air bubbles and prevent instability. During the initial diffusion stage, protein solubility plays an essential role in determining the rate of diffusion and the availability of protein for film formation (Yang et al., 2009). On the other hand, average hydrophobicity influences the affinity of a protein to the interfacial space and the adsorption process (Barac et al., 2010; Damodaran, 2005). Structural flexibility is also critical in surface denaturation and interaction with other proteins or non-protein molecules upon film formation (Damodaran,

2005; Wilde, 2000). These intrinsic characteristics are related to a protein's suitability as a foaming agent and affect FC. Generally, factors that influence FC do not necessarily apply to FS and should be considered separately. For example, small and flexible proteins such as egg and whey proteins tend to experience easier anchorage at the interfacial layer and unfold faster, whereas larger globulins found in legumes denature at a slower rate (Lam et al., 2017). However, because of their bulky polypeptide chains and intramolecular bonds, globulins could instead form much stronger and more homogenous films with greater surface viscoelasticity and prevent coalescence of air bubbles, even though they could be less surface-active to begin with (Damodaran, 2005; Gharsallaoui et al., 2009; Wilde, 2000).

As a function of pH, both FC and FS improved in the acid and alkaline regions compared to lower values near the pI for pigeon pea protein concentrates, according to Akintayo et al. (1999), resulting from higher solubility of the proteins. In the same study, the increase in ionic strength and protein concentration enhanced FS by reducing the electrostatic repulsion between adsorbed proteins on the interface. Similar results were obtained by Singhal et al. (2016) wherein the FS of FPI prepared using AE-IP was positively correlated with the protein surface charge. The higher surface charge favors the protein-solvent interaction over that of protein-protein, leading to greater structural flexibility of the proteins surrounding the air bubbles. FC and FS of FPI in their study were 162%, higher than that of PPI (150%) and SPI (157%), and 65%, slightly higher than that of PPI (56%) and SPI (58%), respectively. Similar results on FPC were obtained by Martinez et al. (2016), ranging between 122-154% for FC and 71-80% for FS. FS was reported by Fernández-Quintela et al. (1997) to be 77%, 93% and 94% for FPI, SPI, and PPI prepared using AE-IP, respectively, while the ability of the proteins to generate foams was reported as foam expansion (FE) with values of 15%, 20% and 15% for FPI, SPI, and PPI, respectively. On the other hand, superior foaming properties of FPI prepared using UF compared with that of smooth-seeded yellow pea and soybean were reported by Vose (1980). The extraction method, cultivar, and the foaming method employed may account for the differences between the results obtained by the two studies.

d) Emulsifying

Emulsions are colloidal systems created by mechanical agitation of two immiscible liquids such that one is dispersed in a continuous phase of the other as small droplets (McClements, 2005). Typically, food emulsions include oil-in-water (O/W) (e.g., milk, mayonnaise, and coffee creamer) and water-in-oil (O/W) (e.g., butter and margarine)

dispersions. Such dispersion systems are thermodynamically unstable and require the addition of emulsifiers to lower the interfacial tension. One may say that the principles behind emulsions and foams are similar because they both are dispersions of a hydrophobic fluid (i.e., air or oil) within a hydrophilic liquid. However, the two systems differ from one another in many qualities. For example, phase density difference (-10^2 kg/m^3) and droplet diameter (10^{-6} m) are less for emulsions than that of foams (-10^3 kg/m^3 and 10^{-3} m, respectively) (Walstra & Vliet, 2008). Coupled with the insolubility of oil in water (compared to the high solubility, 2.1 vol.%, of air in water), emulsions resist phase separation relatively easier than foams do (Walstra & Vliet, 2008). The instability could also occur during the initial stage of foam formation due to longer characteristic time scales for foams and spontaneous Ostwald ripening. The lack of such potential instability in emulsions makes the investigation of emulsifying properties less complicated than foaming (Walstra & Vliet, 2008). We could also assume that the general differences in volume fraction (∅) and the resulting morphology of emulsion droplets and air bubbles could lead to variations in stabilizing effects. Therefore, as compared with foams, different protein requirements to make and stabilize an emulsion are expected. Proteins that are good foaming agents are not necessarily good at emulsifying, and an emulsifier that is suitable for making small droplets does not always provide long-term stability, or vice versa (Damodaran, 2008).

Various tests have been introduced to investigate emulsifying properties. For a few examples, based on the turbidity of diluted emulsions, the emulsifying activity index (EAI, m^2/g) represents a protein's ability in a dilute solution to form an emulsion with a large quantity of oil and is an estimate of the interfacial area stabilized per unit weight of protein. The emulsifying stability index (ESI, min) measures the ability of the protein to impart strength to the emulsion against changes over a defined time period (Pearce & Kinsella, 1978). The EAI is related to the ability of the protein to adsorb at the interfacial space, while ESI relates to the consistency of the interfacial film over time. A more simplified approach in determining the emulsifying properties of proteins involves emulsion capacity (EC, g/g) and emulsion stability (ES, also called creaming stability, CS), which measures the amount of oil emulsified per g of protein and the ability of the proteins to resist creaming, respectively (Boye et al., 2010a; Singhal et al., 2016).

It has been repeatedly reported that the 7S globulins show better emulsifying characteristics than the corresponding 11S globulins, which has contributed to their higher solubility and surface hydrophobicity (Boye et al., 2010a; Dagorn-Scaviner et al., 1987;

Kimura et al., 2008; Koyoro & Powers, 1987). Although less surface-active, the 11S globulins with highly ordered native structures give strong and cohesive interfacial films and stabilize the emulsions more efficiently (Graham & Phillips, 1976). However, the 7S globulins with higher solubility in the continuous phase can easily migrate onto the interface during emulsification and disturb the molecular rearrangement process of the 11S globulins, affecting their adsorption rate (Can Karaca et al., 2011; Dagorn-Scaviner et al., 1987). Similarly, the albumins also show better emulsifying properties due to fewer structural constraints (Sathe & Salunkhe, 1981).

The pH dependence of emulsifying properties was observed to resemble that of protein solubility, with higher values found in the acid/alkaline regions and lower values located at pH near pI due to the changes of charge repulsion, solubility, and structural flexibility (Shevkani et al., 2015). Such dependency was found to be more evident for EAI than for ESI in the work of Barac et al. (2015a) on PPI as the factors mentioned above largely govern the diffusion and adsorption of protein particles during the initial stages of emulsification, while the latter attribute relies more on the film properties. The emulsifying properties are influenced by extraction methods as well. Stone et al. (2015) found that the EC of PPI prepared by SE was higher than AE-IP, while the ES was high for all isolates. However, Can Karaca et al. (2011) reported that the EC was not different between the methods, while ES was higher for isolates produced by AE-IP than SE. They also showed that the EAI and ESI of legume protein isolates (FPI, PPI, LPI, and CPI) were lower for those extracted by SE than those by AE-IP, indicating that salt extracted proteins were less effective at forming emulsions. It was concluded that proteins extracted by AE-IP had higher surface charge and solubility and could form smaller droplets and stabilize the emulsion longer than those by SE. The ES of FPI was higher than that of PPI and lower than that of CPI and LPI produced using AE-IP, while the values were similar among pulses when prepared using SE (Can Karaca et al., 2011). The ES for air classified FPC prepared by Martinez et al. (2016) was at an average of 83%, ranging from 74% to 90%, while the commercial FPC had an ES of 92%, similar to that of PPC. High stability was also reported for AE-IP-produced FPI (94%) by Singhal et al. (2016). The superior stability of the emulsions formed by both freeze and spray-dried FPI were reported by Cepeda et al. (1998) upon heating at 80℃ for 30 min.

2.5. Antinutritional factors present in legumes

ANF are naturally generated compounds found in plants that reduce the bioavailability

of nutrients in the human body upon consumption (Fekadu Gemede, 2014). On the other hand, these non-nutritive compounds are often viewed as bioactive with health benefits to potentially prevent chronic diseases. However, depending on the plant origin, chemical structure, concentration, and diet pattern, the consumption of such substances could negatively affect the digestion and assimilation of nutrients and induce troublesome or even toxic effects to susceptible individuals (Gulewicz et al., 2014).

Phenolic compounds are water-soluble substances composed of one or more hydroxyl groups bound to an aromatic ring. Due to the ability of their hydroxyl groups to donate hydrogen, phenolic compounds are metal ion chelating antioxidants in the prevention of oxidative stress-associated diseases (e.g., cancer, diabetes, osteoporosis, and inflammatory disorders) (Dai & Mumper, 2010; Pereira et al., 2009). However, due to such chemical structures, phenolic compounds strongly interact with proteins by either binding or precipitation, resulting in reduced protein digestibility (Bai et al., 2018). Pigmented pulse seeds such as faba beans contain high amounts of phenolic compounds in their seed coats, of which the condensed tannins (CT), also called the proanthocyanidins, account for approximately 70-80% of the total amount (Nasar-Abbas et al., 2009; Sharan et al., 2021). Tannins are polymeric flavonoids that form insoluble complexes with both enzymes and non-enzymatic proteins, especially those that are proline-rich, and have also been reported to limit the availability of vitamins A and B_{12} (Gulewicz et al., 2014; Wang et al., 1998). Therefore, quantification of total phenolic compounds (TPC), particularly the CT, is of great importance when assessing the nutritional value of pulses. A significant relationship between the seed tannin content and the seed coat color of faba bean was reported by Oomah et al. (2011) with darker pigmented cultivars containing generally more tannins than the lighter ones.

Phytic acid (PA), or phytate when bound to a mineral, is ubiquitous among plant seeds and grains and is the main storage form of phosphorus in plants (Schlemmer et al., 2009). PA typically accumulates in the protein bodies of endosperm during seed development till maturity and represents 60–90% of total phosphorus in grain cereals and legumes (Lott et al., 2000; Lott & Buttrose, 1978). The high negative charge of phytates impacts the absorption of micronutrients by chelating metal ions, such as iron, zinc, calcium, magnesium, and copper (Fekadu Gemede, 2014). As humans lack phytase, metal cations linked to PA are not available upon digestion, leading to nutritional deficiencies (e.g., anaemia), particularly for those whose diets mainly consist of legumes (Gulewicz et al., 2014; WHO, 2021). PA also binds proteins, limiting the function of digestive enzymes, and further negatively impacts the absorption of

proteins, starch, and lipids (O'Dell & De Boland, 1976). In the literature, raw soybean has always been a rich source of PA, while that of yellow pea is lower (Adamidou et al., 2011; Chitra et al., 1995; Mohamed et al., 1991; Shi et al., 2018; Ravindran et al., 1994; Wang et al., 2008).

Oligosaccharides, for example, the raffinose family oligosaccharides (RFO), including raffinose (trimer), stachyose (tetramer), and verbascose (pentamer), are often investigated as undesirable compounds in grain legumes. The RFO are synthesized via the sequential stacking of α-galactosyl residues to a sucrose molecule (Peterbauer et al., 2002). These low molecular weight, water-soluble sugars are α-(1-6)-galactosides that cannot be hydrolyzed in the monogastric systems of humans (Gulewicz et al., 2014). For this reason, they are included as part of the dietary fiber family, of which the compounds enter the colon area almost intact to be fermented by the gut microflora and exert prebiotic activity (Goyoaga et al., 2011; Mitsuoka, 1996). However, the anaerobic fermentation of the oligosaccharides leads to the production and buildup of gases such as CO_2, H_2, and CH_4 and causes flatulence, especially for people with intestinal problems (Berrios et al., 2010). The main RFO in faba bean has been reported to be stachyose and verbascose, while verbascose was a minor oligosaccharide in peas and was absent in soybeans in the study of Han and Baik (2006).

Vicine and convicine (V-C), a class of species-specific compounds found in the cotyledons of faba beans and other *Vicia* species, are responsible for favism, a type of anemia due to biochemical abnormalities of blood cells, in glucose-6-phosphate dehydrogenase (G6PD) deficient individuals (Salim-Ur-Rehman et al., 2014; Vioque et al., 2012). Upon ingestion, these pyrimidine glycosides are hydrolyzed in the large intestine by microbial β-glucosidase into their aglycones, divicine and isouramil from vicine and convicine, respectively, inducing oxidative damage (McMillan et al., 2001). The typical symptoms of favism are acute (5-24 h after bean consumption) and include headache, dizziness, nausea, vomiting, stomach pains, and fever (Hill, 2003). In severe cases, the symptoms could lead to hemoglobinuria and potential deaths (Hill, 2003). It was estimated that G6PD deficiency impacts 400 million people worldwide, hinting at the importance of developing and commercializing low or zero V-C cultivars of faba beans (Ray et al., 2015).

In addition, other bioactive yet antinutritive compounds found in pulses have been repeatedly reported in the nutritional assessment of the grains. Enzyme inhibitors in pulses typically include protease inhibitors and amylase inhibitors, of which trypsin inhibitors and chymotrypsin inhibitors are the most encountered ones in raw seeds (Fekadu Gemede, 2014).

By mimicking protein substrates, the inhibitors bind to various residues of the proteases (e.g., lysine, serine, and arginine residues of trypsin, and leucine, phenylalanine, histidine, and tyrosine of chymotrypsin) and prevent protein digestion in the gut (Dantzger et al., 2015). Saponins are a class of surface-active compounds that are bitter and can negatively affect the bioavailability of certain minerals and vitamins (Southon et al., 1988). Lectins bind sugars and proteins, causing agglutination of red blood cells *in vitro* and disrupting nutrient absorption through the intestinal wall (Champ, 2002; Savelkoul et al., 1992). Oxalates chelate minerals (e.g., calcium) and may cause the formation of kidney stones with high levels of intake (Ross et al., 1999).

To reduce ANF levels in pulses, various techniques have been applied. Genetic improvements were shown to decrease the content of CT and V-C in faba beans (Crépon et al., 2010). Post-harvest processing in the reduction of ANF has also been addressed. For example, Alonso et al. (2000) reported that dehulling of faba and kidney beans significantly reduced CT and polyphenol levels, while extrusion was the most efficient method to eliminate the activity of trypsin, chymotrypsin and α-amylase inhibitors without altering the protein content. Germination was also shown to lower phytate and PA concentrations in chickpeas (Chitra et al., 1995; Hemalatha et al., 2007). Fermentation of faba beans caused a decrease of V-C contents and significantly reduced trypsin inhibitor activity and CT content (Coda et al., 2015). Other thermal treatments, such as boiling, roasting, microwave, and infrared heating reported by Khattab and Arntfield (2009), had produced significant decreases in CT, PA, trypsin inhibitor activity and oligosaccharides compared to the raw seeds of cowpea, pea, and kidney bean.

2.6. Protein quality

Proteins are essential nutrients for the development and maintenance of the human body. They serve as the building blocks for the body tissues and supply energy by providing essential amino acids (EAA) required for growth. As alternatives to traditional animal proteins, legumes provide sustainability and health benefits. However, most legume proteins are deficient in one or more EAA compared with those from animals. The consumption of adequate amounts of high-quality protein is essential in meeting the metabolic requirement of the human body. Determined by the amino acid (AA) composition and the protein digestibility, the protein quality can be evaluated using various methods.

The protein efficiency ratio (PER) is an *in vivo* protein quality evaluation method

defined as the ratio of weight gain for growing rats to their protein intake from 10% protein diets for 28 days (Alsmeyer et al., 1974). The *in vivo* digestibility was referred to as the "true digestibility" to give higher values than *in vitro* methods (Swaisgood & Catignani, 1991). According to Friedman (1996), many studies use adjusted PER, in which the actual PER is adjusted relatively to casein, a reference protein with PER of 2.5. Typically, low-quality proteins have PER values below 1.5, and those above 2.0 are considered good to high quality (Friedman, 1996). In the study of Vioque et al. (2012), three theoretical PER values for FPI were calculated to be close to 3.0, indicating a high nutritional value of the proteins comparable to that of chickpea (2.8). The adjusted PER is multiplied by the quantity of protein present in a Reasonable Daily Intake (RDI, g/250 mL serving) to calculate the protein rating, an official method of Health Canada established in "Food and Drug Regulations" (Government of Canada, 2018). For protein content claims in Canada, food products with protein ratings ranging from 20.0 to 39.9 are considered as "source of protein", and the ones with protein ratings equal to or greater than 40.0 are claimed to be "excellent source of protein" (Nosworthy et al., 2017b). The protein ratings of cooked Canadian split yellow peas and chickpeas were 20.01 and 30.44, respectively (Nosworthy et al., 2017b). However, the PER technique is not directly related to protein digestibility in human nutrition, as rats have higher requirements for SAA (Marinangeli & House, 2017). Besides being time-consuming and expensive, bioassays also have poor precision and reproducibility, limiting industrial use (Friedman, 1996).

For Nutrition Facts-labeling purposes, the protein digestibility corrected amino acid score (PDCAAS) can be used to estimate the PER for the protein rating calculations whenever the PER is not available (Government of Canada, 2018). The PDCAAS is an internationally standardized method recommended in evaluating global protein quality by the FAO/WHO in 1991 (FAO/WHO, 1991). The PDCAAS incorporates EAA into protein digestibility evaluation and calculates amino acid scores (AAS) by relating the mg limiting amino acid (LAA) per g protein in the sample to the reference pattern set by the FAO based on the nutritional needs of a 2-5-year-old child (Hughes et al., 2011). The true fecal digestibility (TFD%) using a rat bioassay was then multiplied by the calculated AAS to give the final PDCAAS. It is a chemical score with rapid and straightforward measurements and a direct relationship to human protein requirements. However, since there is no LAA in complete proteins such as egg, casein, whey, and soy, these proteins score 1.0 in PDCAAS without considering the differences in EAA between them (Hughes et al., 2011; Schaafsma, 2005). According to Ma et al. (2017), protein digestibility is adversely affected by ANF present in legumes, leading to reduced bioavailability,

which is another factor that the PDCAAS method does not consider. The TFD %, AAS and PDCAAS for cooked Canadian split yellow peas and chickpeas were 87.94% and 85.02%, 0.73 and 0.61 (with tryptophan as the LAA), and 64.3% and 51.9%, respectively (Nosworthy et al., 2017b).

Considering the issues with bioassays, the PDCAAS is often reported with the *in vitro* protein digestibility (IVPD), which involves the enzymatic hydrolysis of the proteins with controlled pH and temperature, mimicking human digestion (Swaisgood & Catignani, 1991). The IVPD, AAS and IV-PDCAAS for raw flours from faba bean and yellow pea were 78.0% and 78.6%, 0.72 and 0.79, and 56.2% and 62.1%, respectively, with tryptophan as the LAA (Setia et al., 2019). Comparable to that in SPI, the lysine content in FPI was high, suggesting good nutritional quality, except for the greater deficiency for SAA and tryptophan (Sosulski & McCurdy, 1987; Vioque et al., 2012). The IVPD of faba bean flour (70.8%) has also been reported to be similar to that of kidney bean (68.1%) (Alonso et al., 2000). An IVPD assay using only trypsin as the digestive enzyme on the protein-rich faba bean flours resulted in an average of 74.2%, slightly lower than the original flour and the starch-rich flour portions (Coda et al., 2015). Low protein digestibility for both spray and freeze-dried FPI was reported in the study of Cepeda et al. (1998) by measuring the quantity of tyrosine release when the protein was hydrolyzed with trypsin.

3. COMPARATIVE EVALUATION OF THE FUNCTIONALITY OF FABA BEAN PROTEIN ISOLATES WITH MAJOR LEGUME PROTEINS IN THE MARKET (STUDY 1)

3.1. Abstract

In this study, the physicochemical and functional properties of protein isolates prepared from three faba bean cultivars (Fabelle, Malik, and Snowbird), a yellow pea cultivar (CDC Amarillo), and a soybean cultivar (AAC 26-15) were evaluated and compared. The alkaline extraction followed by the isoelectric precipitation (AE-IP) method was used to prepare protein isolates from dehulled legume seeds. The physicochemical analyses included proximate composition (for both flours and isolates), legumin:vicilin (L/V) ratio, surface charge, and surface and interfacial tension, and the functionality tests were protein solubility, water (WHC) and oil holding capacity (OHC), foaming capacity (FC) and stability (FS), and emulsifying activity (EAI) and stability index (ESI). Higher than that of pea (23.1%), the faba bean seeds had protein contents of approximately 31.4% and a small amount of fat (~1.2%). The protein yield was >70% on average for the legumes, and the effect of reduced water usage during AE-IP was significant when lowering the amount of water from 1:10 to 1:6 flour:solvent ratios, whereas the 1:8 ratio did not change the protein yield. Except for surface charge that was higher for faba, the legume protein isolates (FPI, PPI, and SPI for faba bean, pea, and soy, respectively) were largely comparable in surface tension, protein solubility, and OHC. Different from SPI, the interfacial tension, WHC, FC, FS, EAI, and ESI were also overall similar between FPI and PPI. Among faba bean cultivars, Snowbird was more abundant in protein with a higher proportion of legumins, better at stabilizing emulsions while having a lower emulsifying activity than the other two cultivars. Protein composition had a limited impact on the functional properties of FPI when assessing using the L/V ratios. In conclusion, the functionalities of FPI broadly resembled those of PPI while being comparable or higher to those of SPI, suggesting the potential in replacing major legume proteins as functional ingredients in product formulations.

3.2. Introduction

Legumes are good protein sources with protein contents generally higher than most cereals (Biswas & Gresshoff, 2014). As legume proteins tend to be limiting in sulfur-containing amino acids (SAA) while having adequate amounts of lysine, legumes are often consumed in combination with cereal grains by people in many parts of the world for their protein needs (Nosworthy et al., 2017b). Presently, the market for plant protein has been growing at an unprecedented rate with a significant increase of plant-based alternatives to traditional animal proteins. A growing number of consumers are opting for non-meat diets due to the health-promoting benefits and sustainability of plant consumption. New products incorporating plant protein are being formulated to embrace these opportunities. Protein functionality is of great importance in product formulation, as they are closely related to the sensory, physicochemical, and mechanical characteristics of the products, therefore affecting consumer preference and market competitiveness. For example, ingredients high in protein and water and oil holding capacity are suitable for developing plant-based meat analogues because of their ability to retain moisture and fat and reduce purge loss (Kyriakopoulou et al., 2021). Protein ingredients with adequate solubility and good emulsifying properties are ideal for formulating plant-based milk alternatives (Vogelsang-O'Dwyer et al., 2021). It has been suggested that the functionality of proteins is affected by their composition, i.e., the abundance and proportion of legumin (11S) and vicilin (7S), two of the major types of legume storage proteins (globulins) (Koyoro & Powers, 1987; Shevkani et al., 2019). The two protein types generally differ in size, structure, and amino acid composition, consequently enabling them to show distinct functional attributes. For example, proteins that are rich in vicilin are generally more soluble than the ones containing more legumin due to the higher polarity of amino acids and lower molecular weight of the former (Barac et al., 2015a; Kimura et al., 2008; Koyoro & Powers, 1987). Poor foaming and emulsifying properties are often linked to a high content of legumin, as this protein type, compared to vicilin, has more structural constraints from its disulfide bonds, restricting interfacial activities (Barac et al., 2010; Koyoro and Powers, 1987).

Faba bean (*Vicia faba* L.), as food, feed, and green manure, is widely grown in many parts of the world (Köpke & Nemecek, 2010). The consumption of this cold season crop as food is quite common in the mid-Eastern region, while in Europe and North America, the production of faba bean aims primarily for the market of animal feed (Bilalis et al., 2003; Wei, 2019). Faba bean has also been increasingly used as a rotational crop to improve soil quality due to its high nitrogen-fixing capacity compared to other pulses (Etemadi et al., 2018). The

seeds of faba bean are high in protein (26.4-37.4%, higher than that of pea, lentil, chickpea, and beans) and fiber (6.4-8.4%) while low in lipids (~1.8%), making them ideal for producing protein-rich ingredients (Bhatty, 1974). Due to the ideal cultivation conditions (e.g., large production capacity and cold weather) in western Canada, the provinces of Alberta, Saskatchewan, and Manitoba are emerging as faba bean producers in recent years (McGill et al., 2016). Currently, the main end-use of Canadian faba bean is for the domestic feed market (Khazaei et al., 2021). A smaller portion is being exported as food while facing challenges from lead producers such as Australia and Europe (McGill et al., 2016). Within Canada, the food use of faba bean is restricted to the market for ethnic foods with the finite potential to grow (McGill et al., 2016). One way to increase Canadian faba bean utilization is to produce commercially valuable fractions such as protein-rich ingredients for their use in food formulations. To date, the value-added application of faba bean ingredient fractions (e.g., protein isolates) is still in its infancy, as the plant was considered a minor crop until quite recently (Khazaei et al., 2021). Therefore, a scientific knowledge gap concerning new faba bean cultivars grown in Canada is present. To fully take advantage of the emerging opportunities, further utilization of Canadian faba beans requires an updated understanding of the quality attributes of recent market classes. In addition, the current legume protein market is dominated by soy, followed by pea and several niche types (Bashi et al., 2020). Accordingly, the functional evaluation of faba bean needs to be coupled with a direct comparison with the major players to obtain a clear picture of how they compete.

In this study, protein isolates prepared from three recent faba bean cultivars (Fabelle, Malik, and Snowbird) were investigated for their physicochemical properties (proximate composition, legumin:vicilin ratio, surface charge, and interfacial tension) and functionality (protein solubility, water and oil holding capacity, foaming capacity and stability, and emulsifying activity and stability index) and compared to yellow pea (CDC Amarillo) and soy (AAC 26-15) for those quality attributes. The analyses also included the effect of reduced water usage during protein extraction on the protein yield and the impact of protein composition (legumin:vicilin ratio) on the protein functionality. The cultivars Fabelle (medium seeded, regular tannin, and low vicine and convicine), Malik (large seeded and regular tannin), and Snowbird (small/medium seeded and low tannin) were specifically selected based on the current market segments (CFIA, 2021; Saskatchewan Pulse Growers, 2021). The study's findings provide insight into the utilization potential of faba bean protein isolates as functional ingredients in food formulations compared to yellow pea and soy.

3.3. Materials and methods

3.3.1. Materials

Seeds of faba bean cultivars Fabelle and Malik were provided by AGT Foods and Ingredients (Saskatoon, SK, Canada), and Snowbird by W.A. Grain & Pulse Solutions (Innisfail, AB, Canada). Certified seeds of yellow pea (CDC Amarillo) and soybean (Cdn #1, Variety AAC 26-25, Non-GMO & IP) were provided by Greenleaf Seeds (Tisdale, SK, Canada) and Huron seeds (Clinton, ON, Canada), respectively. The crop year for all seeds was 2018. Faba bean and yellow pea seeds were dehulled and milled at the Canadian International Grains Institute (CIGI, Winnipeg, MB, Canada). Soybean seeds were coarsely milled, defatted, and further milled. All legume flours passed through 250-micron sieve. Except for sodium dodecyl sulfate, Tris, and glycine, which were of electrophoresis purity, all chemicals used were of reagent grade and purchased from Sigma-Aldrich (Oakville, ON, Canada) or VWR (Mississauga, ON, Canada). Milli-Q^{TM} (Millipore Corporation, Burlington, MA, U.S.A.) water was used for protein extraction and all analyses.

3.3.2. Preparation of protein isolates

Prior to the extraction of protein isolates, all flours were defatted using a modified method of L'hocine et al. (2006). In brief, the flour samples (~600 g) were mixed with hexane (1:3, w:v) at room temperature and stirred for 40 min using a magnetic stir plate at 500 rpm under a fume hood. The mixture was vacuum filtered through Whatman #1 filter paper (Whatman International Ltd., Maidstone, United Kingdom) and solids were recovered. The process was repeated for two additional times. The defatted flour samples were air-dried overnight in the fume hood and stored at room temperature until further use. The protein isolates were prepared by alkaline extraction followed by isoelectric point precipitation (AE-IP) based on the method of Boye et al. (2010a). To summarize, defatted flour samples (~350-400 g on a dry weight basis) were dispersed in water (1:10, w:v) and adjusted to pH 9.5 with 1.0 M NaOH under continuous stirring (500 rpm) at room temperature for 1 h. The mixture was centrifuged at 4500 × *g* (Sorvall RC-6 Plus centrifuge, Thermo Scientific, Asheville, NC, U.S.A.) for 10 min at 4°C and the supernatant was recovered. Using 1.0 M HCl, the supernatant collected was adjusted to the respective isoelectric point (pI) of each legume cultivar (pH 4.45, 4.47, 4.93, 4.75 and 4.68 for Fabelle, Malik, Snowbird, yellow pea, and soy, respectively) previously determined using a Zetasizer Nano-ZS90 analyzer (Malvern Instruments,

Westborough, MA, U.S.A.), stirred for another 30 min at room temperature and centrifuged again under the same conditions (4500 × *g* for 10 min at 4°C). The protein pellets were collected and stored at –30°C until freeze-dried (Labconco FreeZone 6 freeze dryer, Labconco, Kansas City, MO, U.S.A.) and stored at 4°C. The effect of water reduction on protein yield was assessed by dispersing the flour (faba bean cultivar Fabelle only) samples with two additional flour:solvent ratios (w:v; 1:6 and 1:8) followed by the same procedure presented above.

3.3.3. Physicochemical properties

a) Proximate analysis

The crude protein (%N × 6.25), moisture, lipid and ash contents for the flours and isolates were determined according to AOAC Official Methods 920.87, 925.10, 920.85 and 923.03, respectively (AOAC, 2003). Except for soy flour, which was defatted, all other legume flours being measured for their proximate composition were non-defatted.

b) Determination of L/V ratio

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to identify the legumin:vicilin (L/V) ratio in all protein isolates under reducing conditions. SDS-PAGE was performed according to a modified Laemmli method (1970) using a 15% T separating gel at pH 8.8 and a 4% T stacking gel at pH 6.8. In short, a 30 μL aliquot of 1% (w/w) protein sample was added to 30 μL of 2x SDS-PAGE sample buffer (20 mM Tris-HCl buffer at pH 6.8, 10% SDS solution, 2% β-mercaptoethanol, 50% (v/v) glycerol and 0.01% bromophenol blue) and vortexed for 20 s. After heating in an 85°C water bath for 10 min, samples were centrifuged at 12,000 × *g* for 5 min. Samples were run in an MGV-202 Vertical Mini-Gel System (CBS Scientific, San Diego, CA, U.S.A.) for ~1.5 h at 120 V and 40 mPa using a Power Source 300 V Electrophoresis Power Supply (VWR, Mississauga, ON, Canada). Molecular weight markers used were Prestained Protein Ladder (BLUelf, FroggaBio Scientific Solutions, Toronto, Ontario, Canada) ranging from 5-245 kDa. After electrophoresis, the gel was fixed in gel fixing solution (5:1:4 methanol: glacial acetic acid: water, v:v:v) overnight, stained with 0.1% Coomassie Blue for 2 h, and destained for 4 h with destaining solution (3:6:1 methanol: water: glacial acetic acid, v:v:v). The protein bands were imaged by an EPSON Perfection V750 Pro scanner and quantified using implied densitometry using Image J software (National Institutes of Health, Bethesda, MD, U.S.A.). The protein bands were measured via volume, which is determined by the sum of pixel intensity for all pixels in each section. The

L/V ratios were calculated from the sum of the relative percentages of 11S and 7S protein fractions.

c) Surface charge (Zeta potential)

The surface charge of the protein isolates was determined according to the method of Can Karaca et al. (2011). In brief, electrophoretic mobility (U_E) was measured for 0.05% (w/w) protein solutions (~1 mL) at pH 7.0 (stirring overnight) using a Zetasizer Nano-ZS90 analyzer (Malvern Instruments, Westborough, MA, U.S.A.). Zeta potential (ζ) was calculated as a function of U_E based on Henry's equation:

$$U_E = \frac{2\varepsilon \times \xi \times f(\kappa\alpha)}{3\eta} \quad \text{(Eq. 3.1)}$$

where ε is the permittivity (F (Farad)/m), $f(\kappa\alpha)$ is a function related to the ratio of particle radius (α) and the Debye length (κ), and η is the dispersion viscosity (mPa·s). A value of 1.5 for the Smoluchowski approximation $f(\kappa\alpha)$ is assumed for the present study.

d) Surface and interfacial tension

Surface and interfacial tension were measured between 0.25% (w/w) protein solutions (pH 7 and stirring overnight) and the air interface, and between the protein solutions and canola oil, respectively, using a Du Noüy ring and a semi-automatic tensiometer (Lauda TD2, GmbH and Co., Lauda-Königshofen, Germany). The surface and interfacial tension between water (without protein) and the air interface, and between water (without protein) and canola oil were included as controls. Surface and interfacial tension (γ, mN/m) was determined using the equation below:

$$\gamma = \frac{Fmax}{4\pi R\beta} \quad \text{(Eq. 3.2)}$$

where F_{max} is the maximum force measured, R is the radius of the Du Noüy ring (20 mm for this study) and β is the correction factor based on the ring dimensions and the liquid densities.

3.3.4. Functional properties

a) Protein solubility

Protein solubility for all isolate samples was determined using the method of Stone et al. (2015) with slight modifications. In brief, 0.2 g protein (based on weight protein content within the dried powder) was dispersed in 19 mL water and adjusted to pH 7.0 with 0.5 N

NaOH in a 30 mL beaker. The solutions were stirred using a magnetic stir plate at 500 rpm for 1 h at room temperature. Total solution weight was brought to 20.0 g with water and allowed to rest for 10 min to foster precipitation. An aliquot (~10 g) of the solution above the precipitate for each sample was centrifuged at 4,180 × g for 10 min at room temperature using a VWR clinical centrifuge 200 (VWR International, Mississauga, ON, Canada). The protein content of the supernatant (~5 g) was determined using a micro-Kjeldahl digestion and distillation unit (model 6030000, micro-Kjeldahl digestor; and Rapid Distillation Glassware, Labconco, Kansas City, U.S.A.). Percent protein solubility was calculated by dividing the protein content in the supernatant by the protein content in the initial sample (×100%).

b) Foaming capacity and stability

Foaming capacity (FC) and stability (FS) for each isolate were measured according to Liu et al. (2010) with slight modifications. In brief, 1.0% (w/w) protein solutions were prepared and adjusted to the desired pH (3.0, 5.0, 7.0) with 0.1 or 0.5 N HCl or NaOH, followed by stirring for 1 h at 500 rpm and room temperature. Fifteen (15.0) mL (V_{li}) of the protein solution were transferred into a narrow 400 mL glass beaker (inner diameter = 69 mm, height = 127 mm, as measured by a digital caliper) and foamed using an IKA T10 basic ULTRA-TURRAX Homogenizer (IKA Werke GmbH & Co. KG, Staufen im Breisgau, Germany) equipped with an S-10 N-10G probe at speed 5 for 5 min. The fixture blade was placed just below the air-water interface to ensure maximum foam formation. The liquid mixture after foaming was immediately transferred into a 100 mL graduated cylinder (inner diameter = 26 mm; height = 25 cm) with the foam volume recorded at time zero (V_{f0}) and after 30 min (V_{f30}). FC and FS were calculated as below:

$$\%FC = \frac{V_{f0}}{V_{li}} \times 100 \quad \text{(Eq. 3.3)}$$

$$\%FS = \frac{V_{f30}}{V_{f0}} \times 100 \quad \text{(Eq. 3.4)}$$

c) Emulsifying activity and stability index

The emulsifying activity (EAI) and stability index (ESI) of the protein isolates were determined by the method of Pearce and Kinsella (1978). In brief, 0.25% (w/w) protein solutions were prepared and adjusted to the desired pH (3.0, 5.0, 7.0) with 0.1 or 0.5 N HCl or NaOH, followed by stirring for 1 h at 500 rpm and room temperature. Five (5.0) g of protein

solution and 4.0 g of canola oil were measured into a 50-mL centrifuge tube and homogenized as previously described for foaming properties. Immediately after homogenization, a 50 μL aliquot of the emulsion was collected from the bottom of the tube and diluted with 7.5 mL of 0.1% (w/v) SDS. The diluted emulsion sample was then vortexed on high speed for 10 s before being measured for absorbance at 500 nm using a Genesys 10 UV-visible spectrophotometer (Thermo Scientific, Madison, WI, U.S.A.) with plastic cuvettes with 1 cm path length. After 10 min, another 50 μL aliquot of the emulsion was collected from the same tube and diluted with 7.5 mL of 0.1% (w/v) SDS before being measured for absorbance accordingly. EAI and ESI were calculated as below:

$$EAI = \frac{2 \times 2.203 \times A_0 \times N}{c \times \varphi \times 10000} \quad \text{(Eq. 3.5)}$$

$$ESI = \frac{A_0}{\Delta A} \times t \quad \text{(Eq. 3.6)}$$

where EAI (m^2/g) is an estimate of the interfacial area stabilized per unit weight of protein based on the turbidity of the diluted emulsion, ESI (min) is a measure of stability over a defined time period, A_0 is the absorbance of the diluted emulsion immediately after homogenization, N is the dilution factor (×150), c is the weight of protein per volume (g/mL), φ is the oil volume fraction of the emulsion, ΔA is the change in absorbance between 0 and 10 min (A_0–A_{10}) and t is the time interval, 10 min.

d) Water and oil holding capacity

Water (WHC) and oil holding capacity (OHC) were measured using a modified method of Stone et al. (2015) by suspending 0.5 g of protein in 5.0 g of water/oil (canola) in a 50 mL screw-capped centrifuge tube at room temperature without adjusting pH. Samples were vortexed on high speed for 10 s every 5 min for a total of 30 min and centrifuged at 1,000 × *g* for 15 min at room temperature using a VWR clinical centrifuge 200 (VWR International, Mississauga, ON, Canada). The supernatant was decanted, and remaining pellet was weighed for calculations of WHC and OHC according to the equations below:

$$WHC/OHC = \frac{Wet\ Sample\ Weight - Dry\ Sample\ Weight}{Dry\ Sample\ Weight} \quad \text{(Eq. 3.7)}$$

where the *wet sample weight* is the water or oil absorbed sample weight.

3.3.5. Statistical analysis

Protein extraction and isolate preparation were performed in triplicate for each defatted

flour. Except for the proximate analysis for the flour samples, of which the measurement was made in triplicate from each flour, all other measurements were made twice on each triplicate isolate extraction. Data represent the mean ± one standard deviation (n=3). Statistics were done using SPSS software (version 28.0, SPSS, Chicago, IL, U.S.A.). A one-way analysis of variance (ANOVA) along with a Tukey's Post-hoc test was performed to test differences between samples. A two-way ANOVA was conducted to examine the effects of cultivar, pH, and their interactions on the physicochemical and functional properties. A Pearson correlation (*r*) analysis was done to identify significant correlations between the physicochemical and functional properties of legume protein isolates, and between the attributes and L/V ratio of faba bean protein isolates at pH 7.

3.4. Results and discussion

3.4.1. Physicochemical properties

a) Proximate analysis

The proximate composition, on a dry weight basis, of faba bean (FF), yellow pea (PF), and soybean flours (SF) and isolates are given in Table 3.1. With the highest protein content being 34.35% for Snowbird among the faba bean cultivars, the mean protein content of FF (31.44%) was higher than that of PF (23.10%) and lower than that of SF (56.42%). The protein content of SF was overestimated because the flour used for analysis was defatted, and fat usually makes up a significant portion of seed weight (~18-22%) of soybeans (Malaki Nik et al., 2010; Stone et al., 2019). The protein isolate of Snowbird also had the highest protein content of 97.41% among all five legumes, while SPI gave the lowest (87.58%). The mean protein content of FPI (93.81%) was similar to that reported by Singhal et al. (2016) using a similar extraction method (93.9%). High protein content has been reported to be associated with low tannin content for faba beans by Crépon et al. (2010) and Micek et al. (2015), which agreed with present findings on the low tannin cultivar Snowbird. Ranging from 1.11-1.35% for PF and FF, the lipid levels were low, except for SF with a 1.92% lipid content on a moisture-free basis, even after defatting. The lipid content was significantly reduced in the isolates (<0.3%) due to the defatting pre-treatment before the AE-IP extraction process. The ash content for FPI and PPI increased after protein extraction, while the value was lowered for SPI due to the nature of the sample material (defatted flour). According to Sosulski and McCurdy (1987), the use of acid and alkali during the wet extraction process leads to the accumulation of salts, which in turn raises the level of ash in the resulting isolates.

Table 3.1. Proximate composition of legume flours and isolates and protein yields.

		Moisture (%)	Protein (%, d.b.)	Lipids (%, d.b.)	Ash (%, d.b.)	Protein yield (%)
Flour	Fabelle	7.43 ± 0.04^{d}	29.34 ± 0.88^{c}	1.15 ± 0.03^{c}	3.62 ± 0.09^{b}	ND
	Malik	7.81 ± 0.01^{c}	30.64 ± 0.18^{c}	1.35 ± 0.04^{b}	3.21 ± 0.05^{c}	ND
	Snowbird	8.14 ± 0.03^{b}	34.35 ± 0.54^{b}	1.14 ± 0.05^{c}	3.06 ± 0.18^{c}	ND
	CDC Amarillo (pea)	9.84 ± 0.03^{a}	23.10 ± 0.85^{d}	1.11 ± 0.01^{c}	2.49 ± 0.02^{d}	ND
	AAC 26-15 (soy*)	6.52 ± 0.04^{e}	56.42 ± 0.46^{a}	1.92 ± 0.03^{a}	6.53 ± 0.03^{a}	ND
Isolate	Fabelle	6.72 ± 0.36^{a}	92.67 ± 1.81^{b}	0.15 ± 0.01^{b}	4.49 ± 0.22^{a}	74.35 ± 0.70^{a}
	Malik	4.57 ± 0.14^{c}	91.34 ± 0.56^{b}	0.16 ± 0.01^{b}	3.90 ± 0.25^{b}	69.77 ± 0.59^{bc}
	Snowbird	4.23 ± 0.08^{c}	97.41 ± 0.25^{a}	0.13 ± 0.01^{b}	3.07 ± 0.06^{cd}	73.00 ± 0.08^{a}
	CDC Amarillo (pea)	5.22 ± 0.19^{b}	91.79 ± 0.38^{b}	0.15 ± 0.01^{b}	3.50 ± 0.05^{bc}	67.80 ± 0.41^{c}
	AAC 26-15 (soy)	5.39 ± 0.12^{b}	87.58 ± 0.41^{c}	0.26 ± 0.03^{a}	2.88 ± 0.15^{d}	71.85 ± 2.21^{ab}
WRE	1:10 (w:v)	6.80 ± 0.03^{a}	91.90 ± 0.99^{a}	ND	ND	74.96 ± 1.03^{a}
	1:8 (w:v)	6.62 ± 0.02^{b}	92.44 ± 1.00^{a}	ND	ND	73.42 ± 0.89^{a}
	1:6 (w:v)	6.63 ± 0.01^{b}	88.72 ± 0.85^{b}	ND	ND	70.28 ± 0.71^{b}

*Defatted SF; WRE - water reduction extractions from Fabelle; ND - not determined

Data with the same superscript letter are not significantly different ($p>0.05$).

Protein yields by AE-IP (Table 3.1) were different ($p<0.01$) among the legumes, ranging from 67.80-74.35%. One of the many challenges the plant protein industry faces in the further utilization of legumes is the production cost of value-added ingredients, such as protein isolates. Processing technologies such as the typical two-step wet extraction are costly with high water usage, which translates to the high price of the formulated products and a reluctant population of consumers (Green, 2019). To examine the effect of reduced water usage on the protein yield, isolate from the faba cultivar Fabelle was extracted at three different flour:solvent ratios (w:v). The extraction was performed once before the isolation step. The proteins were extracted at similarly high yields with ratios of 1:10 (74.96%) and 1:8 (73.42%), while further lowering the amount of water to 1:6 reduced the extractability and yield of protein (70.28%). Reduced water usage means reduced alkali, and consequently acid, to maintain the desired pH for solubilization and recovery of the proteins, respectively, hence a lighter environmental impact besides reduced cost. The 1:8 ratio would be desirable to keep the cost down while maintaining production quality. However, variations should be expected when scaling up a bench-top practice into industrial operations (Hansen, 2020).

b) Protein composition of protein isolates

SDS-PAGE was performed under reducing conditions to investigate the L/V ratio of protein isolates for all legumes (Figure 3.1, Table 3.2). Relative migration distances of bands to the molecular weight markers were used to estimate the molecular weight of the targeted bands. Band identification was based on published findings of faba bean, pea, and soy proteins in the literature (Barac et al., 2010; Chen et al., 2019; Singhal et al., 2016; Warsame et al., 2020; Yang et al., 2016). Major legumin (L, 11S) and vicilin (V, 7S) bands were identified as follows: α-legumin (39 kDa), β-legumin (21, 19, 17 kDa) and vicilin (52, 47, 33, 29 kDa) for faba bean, and α-legumin (41 kDa), β-legumin (20, 18, 17 kDa) and vicilin (50, 48, 33, 31 kDa) for yellow pea. For soy isolate, acidic (44, 41, 37 kDa) and basic (19 kDa) subunits of glycinin (11S) and β-conglycinin (7S) (77, 69, 51 kDa) were used.

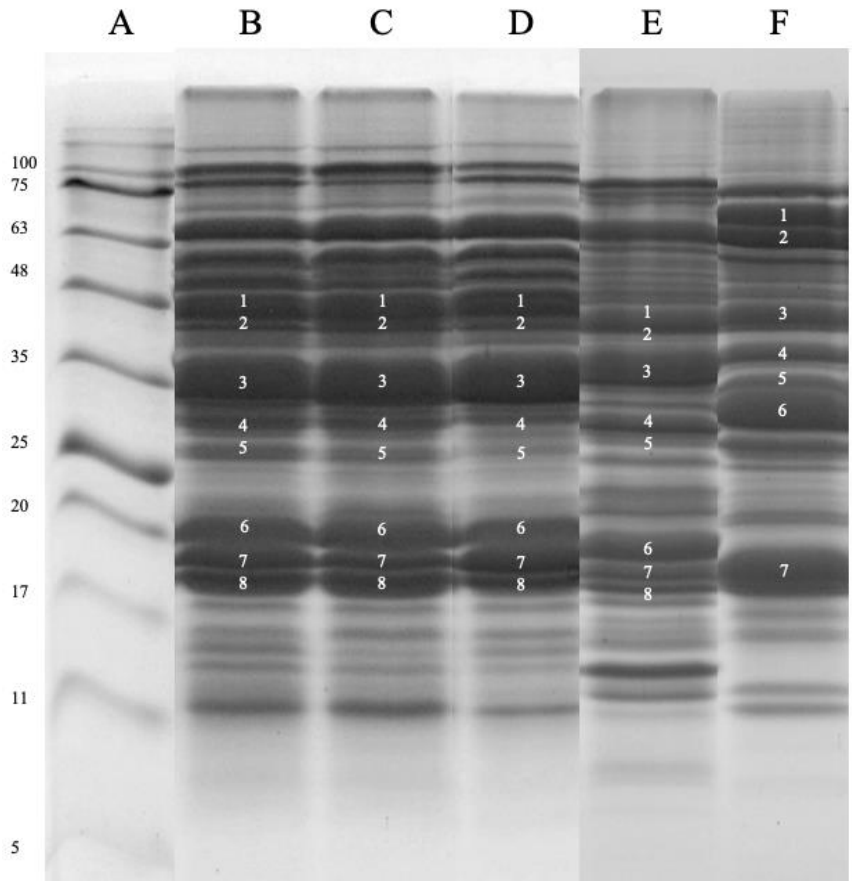

Figure 3.1. Representative samples of SDS-PAGE (reducing conditions) on legume protein isolates. Lanes: (A) molecular weight marker with values in kDa, (B) Fabelle, (C) Malik, (D) Snowbird, (E) CDC Amarillo (pea), and (F) AAC 26-15 (soy). Numbers on lanes of FPI and PPI (B-E): α-legumin (3), β-legumin (6-8), and vicilin (1-2, 4-5). Numbers on the lane of SPI (F): acidic (4-6), basic (7) subunits of glycinin (11S), and β-conglycinin (7S) (1-3).

Table 3.2. L/V ratio and physicochemical characteristics of legume protein isolates.

	L/V ratio	**Zeta potential (mV)**	**Surface tension (mN/m)**	**Interfacial tension (mN/m)**
Fabelle	2.68 ± 0.21^{b}	-41.9 ± 0.6^{c}	54.7 ± 0.4^{a}	13.2 ± 0.1^{bc}
Malik	2.66 ± 0.05^{b}	-42.5 ± 0.7^{c}	54.0 ± 1.0^{a}	12.9 ± 0.1^{bc}
Snowbird	3.22 ± 0.07^{a}	-41.0 ± 0.1^{c}	54.3 ± 1.3^{a}	13.5 ± 0.2^{b}
CDC Amarillo (pea)	2.00 ± 0.03^{c}	-34.4 ± 0.6^{a}	54.4 ± 0.9^{a}	12.6 ± 0.5^{c}
AAC 26-15 (soy)	1.40 ± 0.03^{d}	-38.4 ± 0.6^{b}	54.7 ± 1.2^{a}	14.3 ± 0.3^{a}

Data with the same superscript letter are not significantly different ($p>0.05$).

The L/V ratios were higher for FPI (2.68, 2.66, and 3.22 for Fabelle, Malik, and Snowbird, respectively) than for PPI (2.00) and SPI (1.40) (Table 3.2). The L/V ratios for FPI were within the range of 2.1-5.40 from literature (Gatehouse et al., 1980; Martinez et al., 2016; Singhal et al., 2016). Several factors could influence the L/V ratio of protein isolates, namely the cultivar (i.e., genotype), the environment (i.e., location and year) in which the crop was grown, and the protein extraction process and parameters. For example, Martinez et al. (2016) observed that faba bean cultivars grown at two field plot locations showed significant differences in the L/V ratio of protein concentrates produced by air classification. In addition, reporting an increase in L/V ratio after protein extraction from FF, Singhal (2015) suggested that AE-IP might have selectively precipitated more legumin due to a combined effect of pH, salt type and ionic strength. Hence, it was suggested that by adjusting the processing conditions, wet protein extraction could be optimized to produce protein fractions with altered functionality (Singhal, 2015). A Pearson correlation (*r*) was used to assess the relationship between the L/V ratios and physicochemical and functional attributes for FPI at pH 7. It is obvious on the gels that the yellow pea and soybean protein compositions were quite different from that of faba bean. Therefore, comparing L/V ratios among different crops would be meaningless, and yellow pea and soybean were excluded from the proposed correlations.

c) Surface charge

At pH 7, proteins of faba, pea and soy in the present study were all negatively charged (Table 3.2) as it is above the isoelectric point of most legume proteins (~pH 4.5), where the aspartate and glutamate residues carry a net negative charge on the protein surface (Johnston et al., 2015). No significant difference ($p>0.05$) was observed between faba bean cultivars with a mean zeta potential of -41.8 mV, which was more negative than that of pea (-34.4 mV) and soy (-38.4 mV). The surface charge of FPI and SPI was similar to those reported by Johnston et al. (2015) while being overall higher than those from other studies (Freitas et al., 2017; Martinez et al., 2016; Singhal et al., 2016). Surface charge measurements of proteins are influenced by the surface electrochemical properties and solvent conditions (Wongsagonsup et al., 2005). Therefore, besides extrinsic factors such as protein extraction (e.g., AE-IP vs. air classification) and sample preparation (e.g., in water vs. in buffer) conditions, intrinsic factors associated with cultivar differences, including protein composition, conformation, and amino acid profile, may contribute to the variability of the results. For instance, Can Karaca et al.

(2011) reported that protein isolates produced by AE-IP had a slightly higher surface charge as compared to those by salt extraction ($p<0.01$), while protein source was also a critical factor.

Generally, with a high surface charge (above +30 mV or below -30 mV), protein particles stabilize a solution due to strong electrostatic repulsion, whereas with values between +/- 30 mV, protein-protein aggregation would be favored, resulting in low solubility and a tendency towards precipitation (Guldiken et al., 2021; Kumar & Dixit, 2017; Lam et al., 2017). The overall high surface charge of the investigated legume protein isolates suggested good solubility in solution at pH 7. For FPI, the zeta potential was positively correlated with the L/V ratio ($r=0.847$, $p<0.01$), indicating that with less vicilins present, proteins become less charged, in agreement with Singhal et al. (2016). Vicilin is more abundant in negatively charged amino acids, including glutamic acid and aspartic acid, contributing to a much more hydrophilic profile than the legumin fraction (Koyoro & Powers, 1987; Lam et al., 2017; Rubio et al., 2013).

d) Surface and interfacial tension

Surface and interfacial tension measure the free energy required to increase the interfacial area by a unit amount, and the lower the energy the smaller the droplets and more stable systems can be achieved (Damodaran, 2005; Damodaran, 2008). The surface and interfacial tension between water and air, and water and oil interfaces, respectively, for all legume proteins were investigated (Table 3.2). Legume or cultivar difference was not observed ($p>0.05$) for surface tension (~54.5 mN/m), while SPI showed higher (14.3 mN/m) interfacial tension than PPI and FPI (12.6-13.5 mN/m). For both water and air, and water and oil systems, the addition of legume proteins effectively decreased surface (71.5 mN/m) and interfacial tension (24.3 mN/m) from the control measurements (without protein) by approximately 17.1 mN/m and 11.0 mN/m, respectively. The decrease in tension was similar to that reported for most proteins at both interface types (15 mN/m) (Damodaran, 2008). The magnitude of interfacial tension reduction was the least for SPI, suggesting that, compared to pea and faba, soy protein may not be as flexible to undergo a rapid conformational change at the interface and effectively lower interfacial tension.

No significant correlation ($p>0.05$) was found between the L/V ratio and surface tension for FPI, while interfacial tension measurements were positively correlated with the L/V ratio ($r=0.726$, $p<0.05$). Interfacial tension measurements reflect the surface activity of a protein, which, in general, is linked with the molecular flexibility, mobility and ability of that protein to interact through hydrophobic and electrostatic interactions and hydrogen bonding

(Damodaran, 2005; Kinsella, 1979). In this case, a high legumin content was linked with a small reduction in interfacial tension, suggesting low surface activity for legumin, possibly due to its rigid structure based on disulfide linkages, which hinders conformational change at the interfacial space. The high molecular weight and low charge profile of legumin may also cause low solubility in solution, limiting diffusion. According to the findings of Dagorn-Scaviner et al. (1987) on the adsorption behaviors of purified pea globulins on soybean oil droplets, the initial kinetic step of interfacial tension reduction was more rapid for vicilin than for legumin, which was mainly diffusion dependent.

3.4.2. Functional properties

a) Protein solubility

Protein solubility is the relative percentage of protein in solution to the total amount of protein present in the starting material under a set of given conditions (e.g., different pH and ionic strengths). Protein solubility at pH 7 for legume protein isolates ranged from 71.8-80.9% for FPI and was 65.8% for PPI and 81.6% for SPI (Figure 3.2). No cultivar difference was observed in the solubility of faba bean samples. The isolate of Fabelle was similarly soluble to SPI, while the samples of Malik and Snowbird had comparable solubility to both pea and soy. The mean solubility for FPI (75.7%) was comparable to those obtained with AE-IP and a similar solubility method in the study of Singhal et al. (2016).

Solubility is mediated primarily by the balance of surface hydrophilic and hydrophobic groups that influence the magnitude of protein-solvent interactions (Hall, 1996). The surface charge, which reflects the electrostatic potential of protein particles, is often a strong indicator of how soluble that protein could be. However, no correlation was observed between solubility and zeta potential in the present study, in agreement with Lam et al. (2017). Despite the insignificant correlation in the present study, the PPI was less soluble than SPI and the isolate prepared from Fabelle and had a zeta potential closest to zero (-34.4 mV), suggesting that a lower surface charge may partially contribute to reduced solubility. Solubility was positively correlated ($r=0.521$, $p<0.05$) with interfacial tension, indicating that highly soluble proteins were less capable of reducing interfacial tension at the water/oil interface.

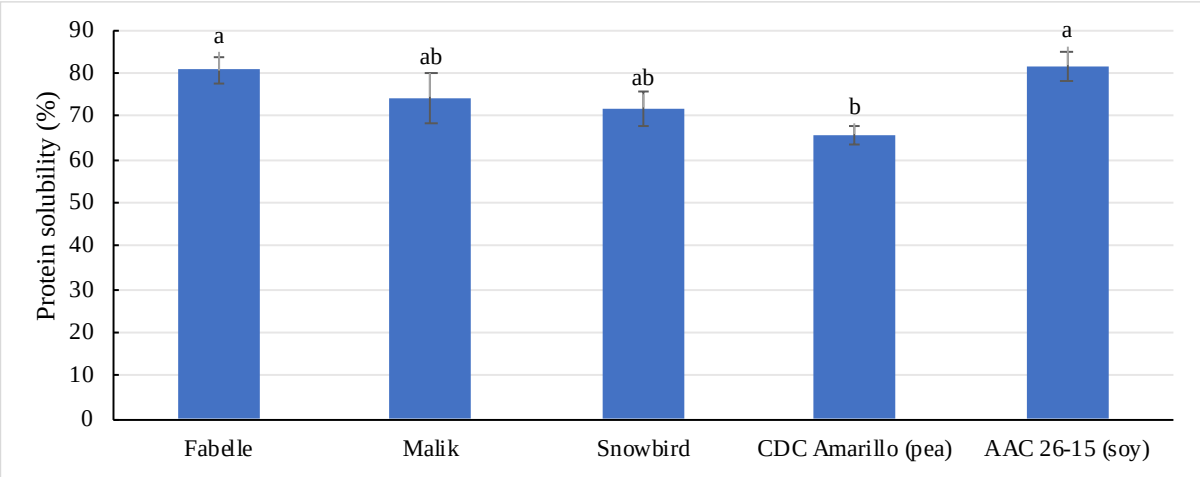

Figure 3.2. Protein solubility of legume protein isolates at pH 7. Data with the same superscript letter are not significantly different ($p>0.05$).

There was no significant relationship ($r=-0.459$ yet $p>0.05$) observed between solubility and L/V ratio for FPI. Our finding agreed with Martinez et al. (2016) and Singhal et al. (2016), while others had reported significant correlations (Barac et al., 2015a; Barac et al., 2015b; Lam et al., 2017). As Singhal (2015) suggested, when evaluating functional properties, other than protein composition reflected by L/V ratio, complications might arise from many aspects, both intrinsically and extrinsically, affecting protein unfolding and ultimately altering the relatability between data sets. Limited sample size based on three faba bean cultivars in the present study could have also reduced the accuracy and significance of the correlations.

b) Water and oil holding capacity

The WHC and OHC for all legume protein isolates are shown in Figure 3.3. WHC was 1.87 g/g on average for FPI, 1.93 g/g for PPI, and 1.62 g/g for SPI. The WHC of SPI was significantly lower than that of PPI and two cultivars of FPI (Malik and Snowbird). No significant difference was observed among legumes for OHC with an average of 1.63 g/g. Our findings on WHC agreed with those by Fernández-Quintela et al. (1997), of which FPI and PPI had similar values, whereas SPI yielded a significantly lower WHC. WHC reflects the ability of a protein matrix to hold water primarily via physical entrapment with relatively minor influences from the hydrophilic/hydrophobic balance, amino acid composition, and charge distribution on the protein surface (Damodaran, 2008; Schnepf, 1992). The lower WHC of SPI indicates that soy protein may not be as flexible as faba or pea protein to form protein aggregates or complexes and physically entrap water molecules. WHC and OHC obtained in

the present study lay in the lower range of those reported in the literature for legume proteins (0.5-5.0 and 1.1-5.7 g/g or equivalent units, respectively) (Boye et al., 2010a; Bühler et al., 2020; Keivaninahr et al., 2021; Martinez et al., 2016; Singhal et al., 2016; Sosulski & McCurdy, 1987; Vioque et al., 2012; Żmudziński et al., 2021). WHC/OHC is often investigated '*as is*', and since the isolates in the present study were produced by AE-IP and freeze-dried without being neutralized, measurements were done at a pH close to the proteins' pI where proteins are the least flexible to form matrices and physically entrap water and oil, hence low WHC and OHC (Ogara et al., 1992).

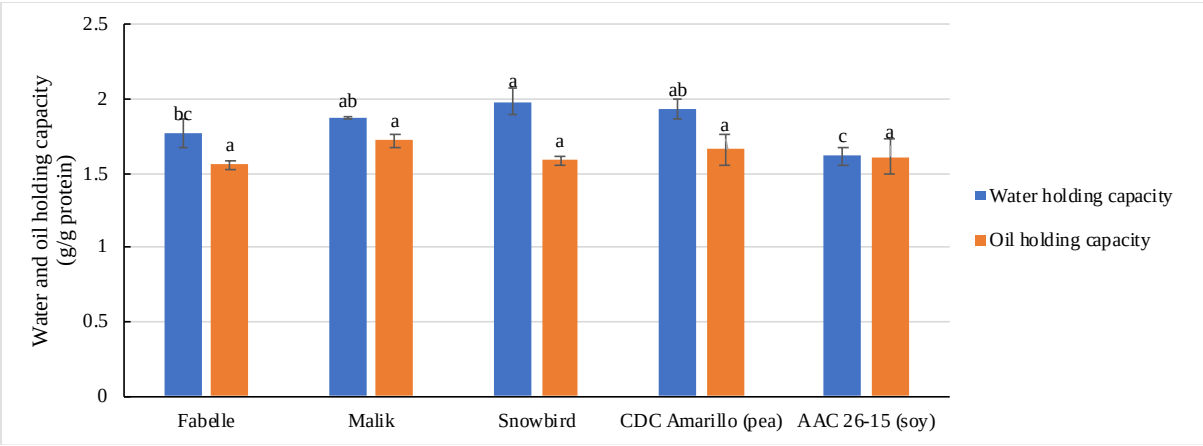

Figure 3.3. Water and oil holding capacity of legume protein isolates. Data with the same superscript letter for each functionality are not significantly different ($p>0.05$).

c) Foaming capacity and stability

The foaming properties of investigated legume protein isolates are given in Figure 3.4. Effects from both cultivar and pH, as well as their interaction (cultivar × pH), were significant ($p<0.01$). Except for Malik with similar FC values at pH 5 and 7, all cultivars showed both their lowest FC and FS at pH 5, exhibiting a U-shape often observed for protein solubility as a function of pH. Our findings on pH agreed with the works of Barac et al. (2010), Schwenke et al. (1983) and Shevkani et al. (2014) on protein isolates sourced from faba bean, pea, and amaranth, respectively. The highest FC of each sample was at pH 3 for isolates of Malik (300%), Snowbird (311%), and soy (249%), whereas similar values were observed for Fabelle (~292%) and yellow pea (~327%) at pH 3 and 7. On the contrary, FS was highest at pH 7 for isolates of Fabelle (78%), yellow pea (71%) and soy (38%), while that of Malik (~68%) and Snowbird (~55%) produced similarly stable foams at pH 3 and 7. Overall, SPI showed relatively poor FC and FS across the pH range, while FPI showed good foaming properties, comparable to PPI,

dependent on pH. For instance, at pH 7, the average FC for FPI was 231%, comparable to that of SPI (230%) and lower than that of PPI (325%), while the mean stability of foams generated from FPI was 68%, similar to that of PPI (71%), higher than that of SPI (38%). At pH 7, the FC of 231% from FPI obtained in the present study was higher than those reported by Martinez et al. (2016) and Singhal et al. (2016) (179% and 162%, respectively), whereas FS was similar (68% vs. 64% and 65%, respectively).

At pH 7, a positive correlation ($r=0.734$, $p<0.01$) was found between FC and zeta potential. Our findings suggested that FC increases with a zeta potential closer to zero, contradicting Shevkani et al. (2015) and Singhal et al. (2016) that a high magnitude of surface charge increases protein solubility, hence enhancing foaming properties. One thing to be noted here is that at pH 7, a moderate surface charge (~22.1 mV) was obtained in Singhal et al. (2016)'s study, while isolates produced in the present study had an average of -39.6 mV (Table 4), which was considered as high. According to Damodaran (2008), the formation of interfacial films could be interfered with by the high charge density of a protein, as strong electrostatic repulsion hinders protein-protein interactions during film-formation. Belitz et al. (2004) mentioned that apart from good solubility and hydrophobicity, an ideal foaming agent should also show a small net charge in terms of the pH of the food. The above statements suggest that a moderate reduction in surface charge could potentially improve foamability for highly charged protein extracts investigated in our study. No other significant correlations were found between the foaming and physicochemical properties, not even for solubility, which agreed with Barac et al. (2010), whereas it had been reported to be positively correlated to FC repeatedly (Lam et al., 2017; Shevkani et al., 2015; Stone et al., 2015). Molecular flexibility and hydrophobicity may have played a more important role here.

A strong negative correlation existed between L/V ratio and FS at pH 7 ($r=-0.814$, $p<0.01$) for FPI, suggesting low foaming properties for the legumin fraction. Naturally, compared with vicilin's intrinsic advantages in terms of size, charge, and structure, legumin shows inevitable limitations in surface activities. Even though large, rigid proteins like legumin could theoretically form thicker and more cohesive films with time, their ability to effectively stabilize foams would face barriers arising from slow conformational change (Dagorn-Scaviner et al., 1987; Damodaran, 2005; Damodaran, 2008). Similarly, Koyoro and Powers (1987) found that foams of green pea legumin showed lower FS, even though the surface hydrophobicity of the fraction was approximately 150% greater than that of vicilin.

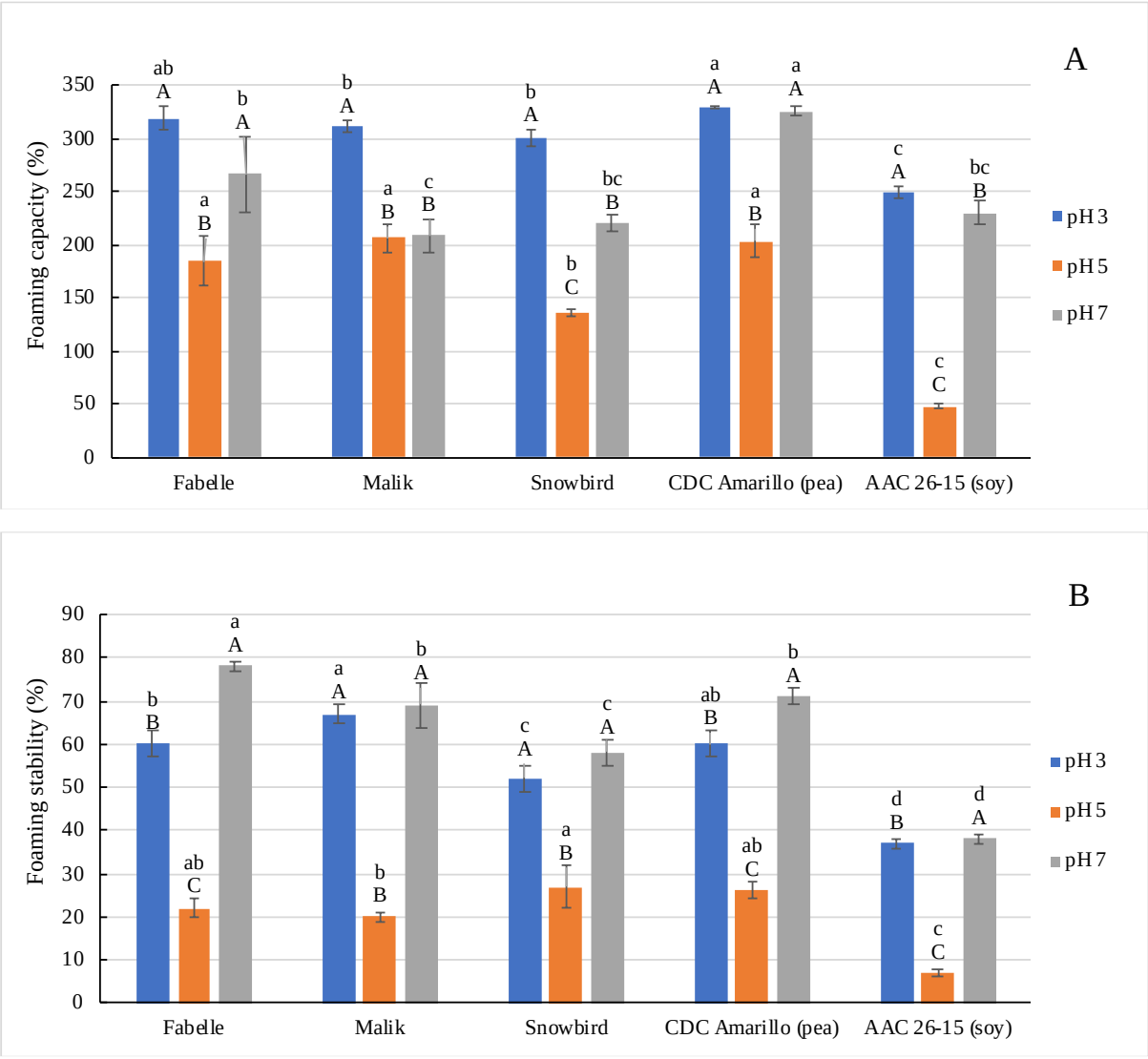

Figure 3.4. Foaming capacity (A) and stability (B) of legume protein isolates. Data with the same superscript letter are not significantly different ($p>0.05$); small letters represent significance between legumes at the given pH whereas capital letters represent significance between pH values within a legume.

d) Emulsifying activity and stability index

The EAI (m^2/g) is an estimation of the interfacial area stabilized per unit weight of protein, while ESI (min) is a measure of the ability of the protein to impart strength to the emulsion against changes over a defined time period (Pearce & Kinsella, 1978). The EAI is related to the ability of the protein to adsorb at the interfacial space, while ESI relates to the consistency of the interfacial film over time. The emulsifying properties of legume protein isolates are shown in Figure 3.5. Significant impacts of pH, cultivar and pH × cultivar were observed for both attributes ($p<0.01$). Like foaming, the lowest EAI was found at pH 5 for all

legumes (9, 9, 7, 6, and 6 m^2/g for Fabelle, Malik, Snowbird, pea, and soy, respectively); However, high ESI was established at this pH (31, 24, 53, and 49 min for Fabelle, Malik, Snowbird, and soy, respectively), except for that of pea (25 min). The emulsifying properties at pH 3 and 7 were more legume source dependent. For example, EAI at pH 3 was higher than at pH 7 for Snowbird (18 and 12 m^2/g for pH 3 and 7, respectively) and soy (29 and 9 m^2/g for pH 3 and 7, respectively), while the opposite was true for others. The pH-dependency of emulsifying properties that resembles protein solubility has been repeatedly reported in the literature (Barac et al., 2011; Chang et al., 2015; Fuhrmeister & Meuser, 2003; Shevkani et al., 2014; Shevkani et al., 2015). The increase in emulsifying properties at a pH below and above the pI is due to the protein's enhanced solubility, charge, and flexibility. Such pH dependency was more evident for EAI than for ESI as the factors mentioned above largely govern the diffusion and adsorption of protein particles during the initial stages of emulsification, while the latter attribute relies more on film properties than on pH (Barac et al., 2015a).

In comparison between legumes, at both pH 5 and 7, SPI had the lowest EAI (5 and 9 m^2/g for pH 5 and 7, respectively) and highest ESI (49 and 45 min for pH 5 and 7, respectively), whereas, at pH 3, the legume produced the least stable emulsions with the largest interfacial area (29 m^2/g and 15 min for EAI and ESI, respectively). The low emulsifying activity for SPI could be related to its high interfacial tension and a lack of conformational flexibility. On the other hand, the relatively compact globular nature of soy protein conveys stability to the emulsions by Pickering stabilization, where the proteins act as solid particles (Tang, 2017). The emulsifying properties for PPI were intermediate at pH 5 and 7 (EAI of 6 and 24 m^2/g, and ESI of 25 and 17 min, for pH 5 and 7, respectively), comparable to those of Fabelle (EAI of 9 and 31 m^2/g, and ESI of 31 and 13 min, for pH 5 and 7, respectively) and Malik (EAI of 9 and 31 m^2/g, and ESI of 24 and 13 min, for pH 5 and 7, respectively), whereas at pH 3, it had the least EAI (14 m^2/g) and highest ESI (33 min), which was the opposite of SPI. Among FPI, Fabelle and Malik had similar emulsifying profiles, while the emulsifying activity and stability were lower and higher, respectively, for Snowbird, especially at pH 5 and 7. The low EAI and high ESI for Snowbird compared with those for other faba cultivars may be due to its high L/V ratio (Table 3), as a high legumin content could hinder emulsification while aiding in the stabilization of oil droplets. Roughly speaking, those that were good at making emulsions by providing high interfacial area (i.e., high EAI) did not stabilize the emulsions for long (i.e., low ESI) at a given pH and vice versa.

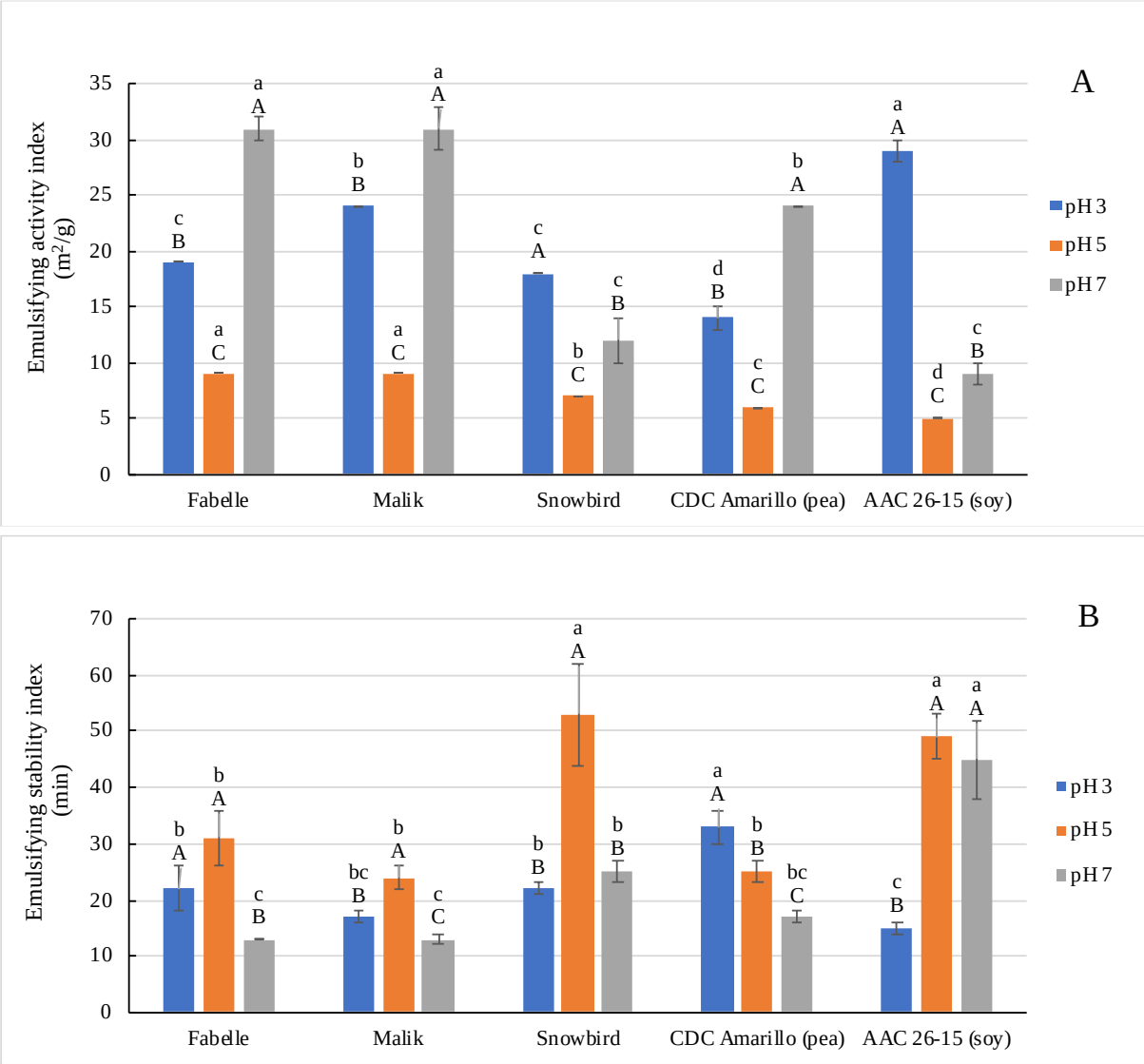

Figure 3.5. Emulsifying activity (A) and stability (B) index of legume protein isolates. Data with the same superscript letter are not significantly different ($p>0.05$); small letters represent significance between legumes at the given pH whereas capital letters represent significance between pH values within a legume.

At pH 7, interfacial tension was negatively correlated with EAI ($r=-0.725$, $p<0.01$) and positively correlated with ESI ($r=0.857$, $p<0.01$), suggesting a linkage between low surface activity and low emulsifying activity as well as high stability. Our finding agreed with Chang et al. (2015) that the higher the interfacial tension value (i.e., lower effectiveness at reducing interfacial tension), the stronger and thicker the viscoelastic films. In their study, the surface hydrophobicity of PPI, SPI, and LPI was significantly reduced with increasing pH (from pH 3), and the proteins at pH 7 were unable to form an interconnected network to stabilize the interfacial space, even though they were good emulsifiers in the early stages of emulsification.

For faba beans, at pH 7, the L/V ratio was negatively and positively correlated with EAI ($r=-0.932$, $p<0.01$) and ESI ($r=0.882$, $p<0.01$), respectively. The relationship between ESI and L/V ratio in the present study agreed with that found by Barac et al. (2011). As discussed previously, legumins are less able to undergo rapid conformational change but are more effective in building strong and cohesive films. On the other hand, in the section on foaming, we discussed why the film-forming ability of the legumin fraction might be limited by slow adsorption. Noting that foams are more prone to instability than emulsions, especially during the initial stage of foam formation, rapid structural change of protein particles may be more critical in their case as compared to emulsions (Walstra & Vliet, 2008).

3.5. Conclusions of Study 1

The primary goal of this study was to provide an updated evaluation of the functionality of protein isolates prepared from recent faba bean cultivars (Fabelle, Malik, and Snowbird) grown in Canada in comparison to those of major legume proteins in the market (yellow pea and soy). The flours prepared from dehulled legume seeds were included in the evaluation of the proximate composition. The crude protein content of faba bean flours (29.34-34.35%) was lower than soy (56.42%) and higher than pea (23.10%), agreeing to literature findings that faba bean is a high protein yielding crop that is suitable for protein ingredient fractionation. Snowbird had the highest protein content among the faba bean cultivars. The protein composition analysis showed that faba proteins (2.66-3.22) are higher in legumin-type proteins than pea (2.00) and soy (1.40), especially for Snowbird with the highest L/V ratio among faba bean cultivars. The zeta potential was significantly higher (more negative) for FPI, while the surface and interfacial tension of FPI were largely similar to those of PPI. In terms of the functional properties, the protein solubility and OHC were generally comparable among legumes, while the WHC, foaming, and emulsifying properties of FPI resembled more of PPI with noticeable differences from SPI. Speaking of cultivar differences, again, Snowbird had higher ESI and lower EAI values (depending on pH), presumably due to its distinct compositional and physicochemical properties as compared to Fabelle and Malik. In short, FPI had overall comparable or higher functionalities than PPI and SPI. The findings suggest that the protein isolates prepared from the faba bean cultivars would be promising candidates to be formulated as functional ingredients in food products.

In addition to the primary goal, the effect of reduced water usage during AE on the protein yield and the impact of protein composition on the functionality of FPI were

investigated on Fabelle. By lowering the amount of water used from 1:10 to 1:8 flour:solvent ratios, the protein yield did not significantly decrease (73.42-74.96%), whereas further reduction to 1:6 lowered the extractability and the yield of protein (70.28%). The finding could be useful to protein ingredient manufacturers regarding cost reduction. In the case of protein composition, it was hypothesized to have a relationship with the physicochemical and functional attributes. However, only few correlations were observed between the L/V ratios and the quality attributes of FPI (+ for zeta potential, interfacial tension, and ESI, and – for FS and EAI). On the one hand, many extrinsic factors would affect functional results, for example, the processing history of samples and variations in experiment setup, which could be significant for foaming and emulsifying. On the other hand, limitations were present when assessing protein composition using the L/V ratio, as the actual abundance of each protein fraction in the samples was not accounted for, and a numerical difference may not reflect a meaningful change in functionality. Future studies should include separate extraction of 7S and 11S proteins and functional evaluation of individual protein fractions to obtain a more direct comparison.

3.6. Linkage to Study 2

As discussed previously, further utilization of Canadian faba bean ingredients requires a comprehensive understanding of recent cultivars' functional and nutritional values. One of the major factors that limit the faba bean applications is the presence of ANF that affects the digestion and assimilation of nutrients or induces troublesome or even toxic effects to susceptible individuals upon consumption. In addition, a similar or better amino acid composition is desirable to compete with major legume proteins in food applications, given that faba bean is inherently high in protein. In the following study, the same faba bean cultivars covering the current market classes selected for Study 1 were evaluated for ANF levels and protein quality to provide insight into their utilization potential from a nutritional perspective.

4. COMPARATIVE EVALUATION OF THE NUTRITIONAL VALUE OF FABA BEAN FLOURS AND PROTEIN ISOLATES WITH MAJOR LEGUMES IN THE MARKET (STUDY 2)

4.1. Abstract

The study was conducted to investigate the utilization potential of Canadian-grown faba bean protein ingredients from a nutritional perspective. The flours and protein isolates of faba bean cultivars Fabelle, Malik, and Snowbird were compared to yellow pea (CDC Amarillo) and soybean (AAC 26-15) for their content of antinutritional factors (ANF) and protein quality. Levels of total phenolic compounds (TPC), condensed tannins (CT), phytic acid (PA), vicine and convicine (V-C), and oligosaccharides (i.e., the raffinose family oligosaccharides, or RFO) were quantified, and protein quality was evaluated based on amino acid (AA) composition, amino acid score (AAS), *in vitro* protein digestibility (IVPD), and *in vitro* protein digestibility corrected amino acid score (IV-PDCAAS). The legume protein isolates were prepared by alkaline extraction followed by isoelectric precipitation (AE-IP) from dehulled seeds, and due to dehulling, all samples were low in TPC and CT. With Fabelle being more concentrated than Malik and Snowbird, the faba bean flours (FF) contained more PA than the pea flour (PF), while the faba bean protein isolates (FPI) generally had more PA than both pea (PPI) and soy protein isolates (SPI). Samples of the low V-C cultivar Fabelle had the lowest V-C content among faba beans, followed by the regular V-C type Malik and Snowbird. The oligosaccharide composition was significantly different among legumes with faba bean having less raffinose and stachyose and more verbascose than pea and soy, while the total RFO amount was generally less. The sulfur-containing amino acids (SAA) were limiting in FF and all protein isolates, while tryptophan was limiting in PF. The IVPD of faba bean samples ranged from 72.8-78.8%, which was intermediate compared to that of pea and soy. However, they scored low in IV-PDCAAS (55.8-66.3% and 44.9-49.3% for flours and isolates, respectively) due to the greater deficiency in methionine and cysteine. The faba bean flours showed higher protein quality values than the isolates (Snowbird < Fabelle < Malik), possibly due to the loss of albumins during AE-IP. Future research should focus on the modification of wet fractionation methods to minimize the loss of sulfur-rich amino acids.

4.2. Introduction

In many parts of the world, legumes are commonly grown crops valued for their nutritional values and health-promoting benefits. Legumes are high in protein (generally higher than that of cereals), dietary fiber, vitamins, minerals, and antioxidants, and their consumption is often linked to the risk reduction of several chronic diseases (Biswas & Gresshoff, 2014; Hu, 2003; Singh, 2017). Relatively abundant in lysine while limiting in sulfur-containing amino acids (SAA), legumes are nutritionally complementary to cereals and often consumed together to meet protein needs by many people around the world. Diets consisting mainly of legumes and cereals are common for individuals with non-meat consumption preferences due to religious and ethical reasons, while the increasing consumer awareness of health and environment is also changing the dietary habits of the general population. According to the National Research Council Canada, approximately >40% of the Canadian population are actively adapting their eating habits towards plant-based alternatives (Government of Canada, 2019). Faba bean (*Vicia faba* L.) is a leguminous crop harvested for its edible seeds. Known as high yielding and abundant in protein, faba bean is one of the most globally important legume crops, especially in the mid-Eastern region (Bilalis et al., 2003; Wei, 2019). While being lower than the top producers (e.g., China, Ethiopia, and the United Kingdom), the production of faba bean in Canada is increasing with most cultivation in the prairie provinces, i.e., Alberta, Saskatchewan, and Manitoba (Friesen, 2021; McGill et al., 2016). The high content of protein (~26.4-37.4%) and fiber (6.4-8.4%) and low levels of lipid (~1.8%) of faba bean make it a promising candidate to produce protein-rich ingredients and embrace the current market opportunities for plant-based protein alternatives (Bhatty, 1974).

Despite being successfully applied in various products, further expanding the utilization of legume proteins faces challenges, one of which is the presence of antinational factors (ANF) and other undesired compounds in legume seeds. The consumption of these non-nutritive compounds can lead to reduced bioavailability of nutrients (e.g., proteins and minerals) and may also cause digestive and metabolic issues in susceptible individuals (Fekadu Gemede, 2014; Gulewicz et al., 2014). For a few examples, phenolic compounds refer to a group of metal ion chelating antioxidants that strongly interact with proteins by either binding or precipitation and reduce protein digestibility (Bai et al., 2018). The seed coat of pigmented legume seeds is a rich source of phenolics, of which approximately 70-80% are condensed tannins (CT) (Nasar-Abbas et al., 2009; Sharan et al., 2021). Like phenolics, phytic acid (PA), the main storage form of phosphorus in plants, chelates metal ions and binds proteins

(Schlemmer et al., 2009). As humans lack phytase, metal cations chelated by PA remain unavailable upon digestion, leading to nutritional deficiencies (e.g., anaemia) (Gulewicz et al., 2014; WHO, 2021). Oligosaccharides, for example, the raffinose family oligosaccharides (RFO), including raffinose (trimer), stachyose (tetramer), and verbascose (pentamer), are included in dietary fiber (Goyoaga et al., 2011; Mitsuoka, 1996). Due to the inability to get hydrolyzed in the human digestive system, these sugars go through anaerobic fermentation leading to flatulence, which is especially problematic for people with gastrointestinal problems (Berrios et al., 2010). Specific to faba bean and other *Vicia* species, vicine and convicine (V-C) are responsible for favism, a type of anemia in glucose-6-phosphate dehydrogenase (G6PD) deficient individuals (Salim-Ur-Rehman et al., 2014; Vioque et al., 2012). The onset of acute favism symptoms could lead to renal infections and possible deaths (Hill, 2003).

Presently, the value-added applications of faba bean ingredients (e.g., protein isolates) are still under development. Besides the concerns of ANF that hinder extensive utilization of legumes in general, the scientific knowledge gap in relation to the compositional and nutritional value of new faba bean cultivars in Canada is also a limiting factor. An updated understanding of the newly developed cultivars is crucial in the advanced utilization of Canadian faba bean. Soy and pea are the two predominant plant proteins as well as legume protein sources in the current market, therefore, a comparative assessment of faba bean with these sources is valuable in narrowing the gap existing in scientific information (Bashi et al., 2020). To compete with these major players as food ingredients, a comparable or better amino acid (AA) composition would be advantageous, given that faba bean is inherently high in protein. Soy protein is considered complete as it contains all the essential AA, while pea and faba bean have been reported to be limiting in either SAA or tryptophan with a lower protein quality than soy (Nosworthy et al., 2018). Therefore, with emphasis on the levels of ANF and other undesirable compounds (total phenolics, CT, PA, V-C, and oligosaccharides) and protein quality (AA composition, AA scores, *in vitro* protein digestibility, and *in vitro* protein digestibility corrected amino acid score), the study aimed to provide an updated nutritional evaluation of flours and protein isolates prepared from three faba bean cultivars (Fabelle, Malik, and Snowbird) currently grown in Canada and to compare the quality attributes with those of yellow pea (CDC Amarillo) and soybean (AAC 26-15). The faba bean cultivars were representative of the current market classification based on seed size and tannin, vicine, and convicine contents. The results of the study provide meaningful insight into the utilization potential of faba bean ingredients in food formulations from a nutritional perspective in comparison to yellow pea and soy.

4.3. Materials and methods

4.3.1. Materials

Flours (FF, PF, and SF for faba bean, pea, and soy, respectively) and isolates (FPI, PPI, and SPI for faba bean, pea, and soy, respectively) produced in Study 1 were used. Isolates prepared with the 1:10 flour:solvent protein extraction ratio were tested. All chemicals used were of reagent grade and purchased from Sigma-Aldrich (Oakville, ON, Canada) or VWR (Mississauga, ON, Canada). Milli-Q^{TM} (Millipore Corporation, MA, U.S.A.) water was used for all analyses.

4.3.2. Antinutritional factors

a) Total phenolic compounds

The level of total phenolic compounds (TPC) was determined according to a method of Singleton and Rossi (1965) using the Folin-Ciocalteu assay. In brief, the extraction was done by mixing 1.0 g of sample with 5 mL of 1% (v/v) HCl in methanol in a 50 mL centrifuge tube with constant stirring on a rotating shaker for a total of 2 h. The mixture was then centrifuged at 1,050 × g for 10 min at room temperature using a Model 5804R centrifuge (Eppendorf, Mississauga, ON, Canada). The supernatant was collected in a different tube, and the pellet was re-extracted twice with 5 mL of the solvent each time. The resulting supernatant from all three extractions was combined. Of the pooled supernatant, each sample was determined in duplicate by taking 1 mL of sample and adding it into a 25 mL volumetric flask containing 9 mL of Milli-Q water and 1 mL of Folin-Ciocalteu reagent and inverted to mix. After 5 min, 10 mL of 7% (w/w) sodium carbonate solution was quickly added into the flask and diluted to volume with Milli-Q water. A standard curve of catechin was made by preparing various concentrations (0.0, 0.1, 0.2, 0.3, 0.4 and 0.5 mg/mL) from a 1 mg/mL catechin stock solution with 1% (v/v) HCl in methanol, followed by the same procedure as for the samples. After the incubation at room temperature for exactly 90 min, the absorbance of the samples was measured at 550 nm using a Genesys 10-S spectrophotometer (Thermo Scientific, Madison, WI, U.S.A.). The content of TPC was calculated as below and expressed as mg catechin equivalent (CE) per 1 gram of sample material on an *as is* basis:

$$TPC\ content = \frac{sample\ Abs - intercept}{slope} \times \frac{15\ mL\ of\ extract}{1\ gram\ of\ sample} \qquad \text{(Eq. 4.1)}$$

b) Condensed tannins

CT were quantified according to the method of Price et al. (1978). In brief, 0.2 g of the sample was extracted in 10 mL absolute methanol with constant stirring for 20 min, followed by centrifugation at 3,000 × *g* for 10 min using a VWR Clinical 200 centrifuge (VWR International, Mississauga, ON, Canada). The supernatant was recovered, and 1 mL each was transferred into 3 tubes. A working reagent was prepared fresh daily by mixing a stock solution of 1% vanillin in methanol with 8% HCl in methanol at 1:1 (v:v). In a 30°C water bath, 5 mL of the working reagent was added into the first 2 tubes containing sample supernatant, while 5 mL of 4% HCl was added into the third one in a 1-min interval. Following incubation for exactly 20 min, the absorbance was measured at 500 nm using a Genesys 10-S spectrophotometer (Thermo Scientific, Madison, WI, U.S.A.). A fresh (prepared daily) catechin solution was used to construct a standard curve. In short, 3 mg of catechin was dissolved in 10 mL methanol, after which 0, 0.1, 0.2, 0.3, 0.4 and 0.5 mL of the solution was transferred into a set of 6 tubes and adjusted to 1 mL with methanol. Another set of tubes was prepared in the same way. In a 30°C water bath, 5 mL of the working reagent was added into one set of tubes, while 5 mL of 4% HCl was added to another in a 1-min interval. The standard solutions were incubated and measured for absorbance using the same procedure described above. The level of CT was calculated as below and expressed as mg CE per 1 gram of sample material on an *as is* basis:

$$CT\ content = \frac{Abs_1 - Abs_0 - intercept}{slope} \times \frac{10\ mL\ of\ extract}{0.2\ g\ of\ sample} \qquad \text{(Eq. 4.2)}$$

where Abs_1 represents the absorbance of samples containing the working reagent and Abs_0 refers to the absorbance of samples containing HCl.

c) Phytic acid

PA was quantified using a Phytic Acid (Phytate)/Total Phosphorus Assay Kit (cat. no. K-PHYT 05/19) (Megazyme, Bray, Co. Wicklow, Ireland). The results were expressed as mg/g sample material on an *as is* basis.

d) Vicine and convicine

The contents of V-C for FF and FPI samples were quantified based on the methods of Quemener et al. (1982) and Marquardt et al. (1983) with modifications described by Wei et al. (2021). Sample extraction was done by intermittent mixing with 0.1 N NaOH (1:30 w:v with

uridine as the internal standard) at 40°C for 90 min. The sample mixture was then centrifuged for 10 minutes at 14,000 × *g* at 22°C, and the supernatant was recovered. To collect the protein depleted supernatant, the supernatant was adjusted to pH 3.5 with 1 N HCl and centrifuged further under the same conditions. The clear supernatant was filtered through a 0.45 μm PVDF syringe filter into HPLC vials. The quantification was done at Agriculture and Agri-Food (AAFC, Saskatoon, SK, Canada). Extract separation was performed using a Waters 2695 Alliance HPLC with an Atlantis T3 column (3 μm, 4.6 × 100 mm column, maintained at 30°C), a Waters 2998 Photodiode Array Detector, a Waters 2424 Evaporative Light Scattering Detector, and a Empower 3 Software (Waters Corporation, MA, U.S.A.) with the sample injection volume of 5 μL, flow rate of 0.8 mL/min and the eluate monitored at 273 nm. Solvent A (0.1% (v/v) formic acid in water) and solvent B (0.1% (v/v) and formic acid in acetonitrile) were used for the separation with a total run time of 35 min, gradient elution followed as 0-8 min 100% solvent A, 8-22 min 30% A and 70% B and 22-35 min 100% A. Vicine eluted at 4.8 min, and convicine at 5.2 min. An external standard curve prepared with vicine (Sigma-Aldrich, 10-350 μg/mL in acetonitrile) was used to quantify both compounds. The results were expressed as mg/g sample material on an *as is* basis.

e) Oligosaccharides

The oligosaccharides including raffinose, stachyose, and verbascose for legume flour and isolate samples were extracted using water (Xu, 2017) and quantified at AAFC (Saskatoon, SK, Canada) according to the procedure of Wei et al. (2021). The separation and detection of the oligosaccharides in the extract was done with an Acquity UPLC BEH Amide column (1.7 μm, 2.1 × 100 mm, maintained at 85°C) and an Evaporative Light Scattering Detector. Sample injection volume was 5 μL. Solvents used in the gradient elution mode were 95% acetonitrile, 5% water with 0.1% triethyl amine by volume (solvent A) and 30 % acetonitrile, 70% water with 0.1% triethyl amine by volume (solvent B). At a maintained flow rate of 0.3 mL/min, the elution started with 90 % A and 10% B to reach 87% A and 13% B at 5 min, then reached 55% A and 45% B at 20 min and completed at 25 min reaching 90% A and 10% B. Mixed standards of D-arabinose, raffinose, stachyose, and verbascose were prepared to obtain external calibration curves for each sugar (0-400 μg/mL). The final results were expressed as mg/g sample material on an *as is* basis.

4.3.3. Protein quality

a) Amino acid composition

The AA composition of all flour and isolate samples was determined at the University of Manitoba (Winnipeg, MB, Canada). All AA, except for methionine, cysteine, and tryptophan, were quantified according to AOAC Official Method 982.30 (2005) with a 24 h hydrolysis using 6 N HCl. Methionine and cysteine were oxidized with performic acid before the acid hydrolysis, according to AOAC Official Method 985.28 (2005). The AA compositions of both sets of hydrolysis were determined using an AccQ-Tag Ultra C18, 1.7 µm column on a Shimadzu UPLC system. The content of tryptophan was measured by alkaline hydrolysis, followed by procedures described in ISO protocol 13904 (International Organization for Standardization, 2016). For quality control purposes, a soy flour standard (NIST 3234) was hydrolyzed alongside all AA samples.

b) Amino acid score

The amino acid score (AAS) refers to the ratio of 1 g of the target protein to the reference protein. The reference AA composition for a 2-5-year-old child in mg/g protein was recommended by FAO/WHO (1991) as: Histidine, 19; Isoleucine, 28; Leucine, 66; Lysine, 58; Methionine + Cysteine, 25; Phenylalanine + Tyrosine, 63; Threonine, 34; Tryptophan, 11; Valine, 35. The AAS represents the lowest score among all the AAs in the samples.

$$AAS = \frac{amino\ acid\ content\ of\ test\ protein}{reference\ AA\ pattern} \quad \text{(Eq. 4.3)}$$

c) In vitro protein digestibility

The *in vitro* protein digestibility (IVPD) was measured using a multi-enzyme pH drop assay based on the method of Tinus et al. (2012). In brief, an enzyme solution was prepared by mixing 31 mg chymotrypsin (bovine pancreas ≥ 40 units/mg protein), 16 mg trypsin (porcine pancreas 13,000–20,000 BAEE units/mg protein) and 13 mg protease (*Streptomyces griseus* ≥ 15 units/mg solid) with 10 mL water. The enzyme solution was kept at 37°C and adjusted to pH 8.0±0.05 with 0.1 M NaOH or HCl. The flour/isolate suspensions were prepared by mixing 62.5±0.5 mg protein with 10 mL water and stirred at 37°C for 1 h before being adjusted to pH 8.0±0.05 with 0.1 M NaOH or HCl. With the addition of 1 mL of the multi-enzyme solution, the pH of the protein solutions was recorded every 30 s for 10 min to calculate the IVPD based on the equation below:

$$IVPD\ (\%) = 65.66 + 18.10 \times \Delta pH_{10\,min} \quad \text{(Eq. 4.4)}$$

where $\Delta pH_{10\,min}$ refers to the change in pH from time zero to the end of the 10-min period.

d) In vitro protein digestibility corrected amino acid score

The *in vitro* protein digestibility corrected amino acid score (IV-PDCAAS) was determined by multiplying the most limiting AAS and IVPD values as follows (Bai et al., 2018):

$$IV-PDCAAS\ (\%) = AAS\ of\ the\ most\ limiting\ AA \times\ IVPD \quad \text{(Eq. 4.5)}$$

4.3.4. Statistical analysis

Similar to Study 1, where the protein extraction was performed in triplicate for each defatted flour, the measurement for each analysis was made in triplicate from each flour, and was made twice on each triplicate isolate extraction. Data represents the mean ± one standard deviation (n=3). Statistics were done using SPSS software. A one-way analysis of variance (ANOVA) was used to test differences between isolates, along with a Tukey's Post-hoc test.

4.4. Results and discussion

4.4.1. Antinutritional factors

a) Total phenolic compounds and condensed tannins

The content of TPC and CT for legume flours and protein isolates are in Table 4.1. Ranging from 1.12 to 2.87 mg CE/g sample material in the flours, the lowest and highest concentration of TPC was found for pea and soy, respectively. Dehulled and defatted flours of faba bean showed intermediate values, with the cultivar Snowbird having the most TPC (2.53 mg CE/g) as compared to Fabelle (2.02 mg CE/g) and Malik (2.10 mg CE/g). For isolates, soy, Fabelle, and Malik had similarly higher values (mean of 3.55 mg CE/g, $p>0.05$) than those of pea and Snowbird (mean of 3.09 mg CE/g, $p>0.05$). The CT content was low (0.02-0.39 mg CE/g) for all flour and isolate samples. The highest concentration in the faba bean samples was found for Fabelle (0.22 and 0.39 mg CE/g for flour and isolate, respectively), followed by the other two cultivars. The faba bean samples contained slightly more CT than pea and soy. Both TPC and CP were found in greater concentrations in the isolates than in the flours, which could be due to the ability of these substances to bind and complex with proteins.

Table 4.1. Concentration of total phenolic compounds, condensed tannins, phytic acid, vicine, and convicine in investigated legume samples.

	Total phenolic compounds (mg CE/g)	Condensed tannins (mg CE/g)	Phytic acid (mg/g)	Vicine (mg/g)	Convicine (mg/g)
Flour					
Fabelle	2.02 ± 0.05^{c}	0.22 ± 0.02^{a}	14.93 ± 0.49^{b}	0.64 ± 0.10^{c}	0.23 ± 0.05^{b}
Malik	2.10 ± 0.02^{c}	0.10 ± 0.02^{c}	10.34 ± 0.09^{c}	4.84 ± 0.67^{b}	3.41 ± 0.03^{a}
Snowbird	2.53 ± 0.09^{b}	0.14 ± 0.02^{b}	10.34 ± 0.06^{c}	8.56 ± 0.04^{a}	3.59 ± 0.61^{a}
CDC Amarillo (pea)	1.12 ± 0.05^{d}	0.02 ± 0.00^{d}	7.29 ± 0.07^{d}	n.d.	n.d.
AAC 26-15 (soy)*	2.87 ± 0.13^{a}	0.11 ± 0.00^{bc}	17.54 ± 0.11^{a}	n.d.	n.d.
Isolate					
Fabelle	3.48 ± 0.09^{a}	0.39 ± 0.06^{a}	35.04 ± 0.60^{a}	0.19 ± 0.01^{c}	0.10 ± 0.00^{c}
Malik	3.62 ± 0.12^{a}	0.27 ± 0.04^{b}	27.72 ± 1.12^{b}	2.40 ± 0.02^{b}	1.45 ± 0.02^{b}
Snowbird	2.98 ± 0.13^{b}	0.21 ± 0.03^{b}	20.80 ± 0.34^{d}	3.16 ± 0.06^{a}	1.57 ± 0.03^{a}
CDC Amarillo (pea)	3.19 ± 0.11^{b}	0.17 ± 0.03^{b}	23.29 ± 0.48^{c}	n.d.	n.d.
AAC 26-15 (soy)	3.55 ±0.06^{a}	0.17 ± 0.04^{b}	19.08 ± 0.77^{d}	n.d.	n.d.

Notes:

Flour or isolate data with the same superscript letter are not significantly different ($p>0.05$).

Values are expressed on an *as is* basis.

Abbreviations: Not determined (n.d.).

*AAC 26-15 (defatted SF)

In comparison to literature values, our results on TPC and CT positioned in the lower range (Amarowicz et al., 2004; Baginsky et al., 2013; Chaieb et al., 2011; Jin et al., 2012; Oomah et al., 2011; Stone et al., 2021). For example, in the study of Oomah et al. (2011), where 13 Canadian faba bean genotypes were investigated for their antioxidant activities, Snowbird had 37.76 mg CE/g of TPC and 4.86 mg/g of total tannins (including both hydrolysable and condensed tannins) in the flour. In other studies, the TPC concentration of mature whole faba beans cultivated in Tunisia was reported with a range of 16.98-67.47 mg gallic acid equivalent (GAE)/g sample material (Chaieb et al., 2011), while 6.54 mg/g of CT was found in 'Fatima' faba bean seeds cultivated in Alberta, Canada (Jin et al., 2012). Direct comparison of literature values is difficult, not only due to variations in the standards (e.g., gallic acid vs. catechin) being used for reporting but also the choices of extraction solvent (e.g., acetone vs. methanol) and assay (e.g., Folin-Ciocalteu vs. Prussian blue). On the other hand, due to the protective effect of tannins in plants, their presence is mainly associated to the seed coat with low or negligible amounts in the cotyledons, where other non-flavonoids such as hydroxycinnamic and hydroxybenzoic acids are primarily located (Shahidi & Ambigaipalan, 2015). Since the raw materials used in our study were obtained from dehulled legume seeds, both TPC and CT levels were expected to be low, especially for the latter component. In the work of Coda et al. (2015), flours prepared from dehulled faba bean seeds had 3.86 mg GAE/g of TPC and 0.27 mg CE/g of CT, and 5.37 mg GAE/g of TPC and 0.35 mg CE/g of CT in the later produced protein-rich fraction by air classification. Using a similar extraction procedure and a quantification assay (i.e., vanillin), dehulling of raw faba beans reduced the level of CT from 1.95 to 0.15 mg CE/g in the study of Alonso et al. (2000). According to the market classification on the investigated cultivars, the faba beans in the present study generally differ in the seed tannin content, with Fabelle and Malik having regular tannin levels and Snowbird being the zero or low tannin type (Saskatchewan Pulse Growers, 2021). However, as the hulls were removed before the flour production, such distinct characteristics were not observed.

b) Phytic acid

The content of PA of legume samples is summarized in Table 4.1. The protein isolates contained more PA (19.08-35.0 mg/g) than the flours (7.29-17.54 mg/g), possibly due to a higher occurrence of PA-protein interactions when there were more proteins. According to Chitra et al. (1995), the PA concentration was positively correlated to the sample protein content when a series of legumes (chickpea, pigeonpea, urd bean, mung bean, and soybean)

were analyzed together. In the flours, the PA levels for faba bean (10.34-14.93 mg/g) were between those for pea and soy, with Malik and Snowbird containing the same amount (10.34 mg/g), which was lower than that of Fabelle (14.93 mg/g). In literature, raw soybean has always been a rich source of PA with a content ranging from 13.10-40.67 mg/g, while that of yellow pea is lower, ranging from 6.40-12.0 mg/g (Adamidou et al., 2011; Chitra et al., 1995; Mohamed et al., 1991; Shi et al., 2018; Ravindran et al., 1994; Wang et al., 2008). On the other hand, for the isolates, the lowest PA concentration was found for the soy (19.08 mg/g) and Snowbird (20.80 mg/g), followed by pea (23.29 mg/g), Malik (27.72 mg/g), and Fabelle (35.04 mg/g). It is possible that due to differences in the composition and surface properties of the proteins and their subsequent binding activities with PA, the leaching of the compound by hydration varied among the samples during isolate production (Beleia et al., 1993; Urbano et al., 2000).

The flours of Fabelle, Malik, and Snowbird showed no significant difference in PA levels in the work of Wei (2019), with an average concentration of 17.5 mg/g, higher than those found in the present study. The faba beans in their study were produced in Saskatchewan, Canada and North Dakota, U.S.A., in three different years. In the work of Oomah et al. (2011), the cultivar Snowbird grown at two different locations in Alberta, Canada had an average PA level of 8.90 mg/g in the flour, lower than the 10.34 mg/g found for our dehulled Snowbird seeds. It was suggested that the effects of environmental factors, including the crop year, climate, location, irrigation, and soil conditions (e.g., availability of phosphorus), are significant in PA content (Urbano et al., 2000). On the other hand, PA is mainly present in the globoids, one of the inclusions of the protein body in the cotyledons of legume seeds (Madsen & Brinch-Pedersen, 2020). With little or no contribution from the seed coat, the proportion of PA in the cotyledons is expected to increase with dehulling. In the work of Luo and Xie (2013) on green and white Chinese faba beans, dehulling significantly increased the level of PA in the flour from 8.57 to 9.62 mg/g and from 8.36 to 9.25 mg/g, respectively.

c) Vicine and convicine

V-C are a class of species-specific compounds found in the cotyledons of faba beans and other *Vicia* species. Hence, the quantification of these compounds was not performed on the samples of yellow pea and soy. The V-C contents of FF and FPI varied among cultivars (Table 4.1). In the flours, Fabelle contained very low levels of V-C at 0.64 and 0.23 mg/g, while Snowbird was the cultivar most concentrated with V-C at 8.56 and 3.59 mg/g, respectively. The

flour of Malik had an intermediate vicine content of 4.84 mg/g and a similar amount of convicine to that of Snowbird (3.59 mg/g). Fabelle is a registered low V-C cultivar in Canada, while Malik and Snowbird are regular or high V-C cultivars. Slightly higher values of V-C for the three cultivars were observed by Wei (2019) following the same trend (with vicine concentrations of 1.02, 7.27, and 10.82 mg/g, and convicine concentrations of 0.40, 3.64, and 3.70 mg/g for Fabelle, Malik, and Snowbird, respectively). The low V-C content of Fabelle offers opportunities for faba beans in the consumer food market as it reduces the risk for favism. Currently, the Canadian cultivation of low V-C faba cultivars is in its infancy and is expected to grow (Friesen 2021; Khazaei et al., 2019). In addition to genotype, influences from the environment and its interaction with genotype on the V-C content have been observed in other studies (Khazaei et al., 2019; Pulkkinen et al., 2015).

In the protein isolates, again, the lowest values were found for the isolate of Fabelle (0.19 and 0.10 mg/g for V-C, respectively), followed by Malik (2.40 and 1.45 mg/g for V-C, respectively) and Snowbird (3.16 and 1.57 mg/g for V-C, respectively). The contents of V-C in the isolates were lower than those in the flours. In the work of Olsen and Andersen (1978), FPI produced from mature, dehulled seeds had total amounts of V-C ranging from 0.7 to 2.0 mg/g. The highest and lowest values were found in the samples of air-classified protein-rich fraction and wet extracted isolate, respectively. Similarly, only residual amounts of V-C in FPI extracted by AE-IP were detected by Vioque et al. (2012), representing less than 1% of the amount in the original flour. It has been suggested that wet methods of protein extraction leach out V-C by hydration and the acidification of the medium (Olsen & Andersen, 1978; Sharan et al., 2021).

d) Oligosaccharides

The fermentable sugar contents varied significantly among legume species (Table 4.2). In the flours, the soy sample contained the highest levels of raffinose (9.30 mg/g) and stachyose (51.29 mg/g), while the concentration of verbascose was the lowest (2.73 mg/g). Following soy, the contents of raffinose (3.11 mg/g) and stachyose (25.57 mg/g) in the yellow pea sample were greater than those of faba bean (1.54 and 11.62 mg/g on average for raffinose and stachyose, respectively), while the verbascose (15.66 mg/g) level was also lower (27.78 mg/g on average for faba bean). In the isolates, both pea and soy again had more stachyose (10.68 mg/g on average) and less verbascose (5.69 mg/g for pea and zero for soy) than faba bean (2.78 and 6.81 mg/g for stachyose and verbascose, respectively). In both flours and isolates, no cultivar difference was observed for faba bean sugar content, except for verbascose in flour

that was lower for Fabelle than the other two cultivars. The protein isolates generally contained less sugars than their corresponding flours, especially for stachyose and verbascose with a >70% reduction, while the decrease in raffinose was smaller (~23%) and more species and cultivar dependent. As reported by Vogelsang-O'Dwyer et al. (2020), acid extraction followed by isoelectric point precipitation of dehulled faba beans eliminated the majority of RFO in the starting materials, leaving behind approximately 0.8 mg/g total RFO in the protein isolates. The reduction of these water-soluble sugars by wet protein extraction suggests the potential of producing high purity protein products with low levels of fermentable sugars.

In both the flours and isolates, the order of abundance of RFO in the faba beans was verbascose > stachyose > raffinose, whereas stachyose was the most concentrated one in the samples of yellow pea and soy. Higher contents of stachyose and verbascose than that of raffinose in faba beans have been reported in the literature (Goyoaga et al., 2011; Landry et al., 2016; Ray et al., 2015; Stone et al., 2021; Wei, 2019). Verbascose was reported to be a minor oligosaccharide in peas and was absent in soybeans in the study of Han and Baik (2006). The RFO are synthesized via the sequential stacking of α-galactosyl residues to a sucrose molecule (Peterbauer et al., 2002). There is likely a block in this pathway for yellow pea and soy, as the samples had less verbascose but an accumulation of stachyose, the previous homologue in RFO synthesis (Frıas et al., 2000). It was also hypothesized by Obendorf (1997) that raffinose is synthesized only in the early stages of seed development, after which the synthesis stops and the compound converts to stachyose and verbascose, hence the low content of raffinose in mature legume seeds.

Almost double the values on the RFO levels for FF, Stone et al. (2021) found high contents of raffinose, stachyose and raffinose at 7.2, 21.8 and 56.1 mg/g, respectively, in samples of a Canadian faba bean (cultivar unknown). With no significant difference among the cultivars, Wei (2019) reported similarly high values for Fabelle, Malik, and Snowbird at mean concentrations of 8.79, 23.93 and 52.7 mg/g for raffinose, stachyose, and verbascose, respectively. As mentioned above, in legumes, RFO accumulate during seed development at the expense of sucrose. Consequently, the growing conditions of the plants that influence the life cycle of seeds, particularly the maturation stage, will bring considerable variations in the sugar profiles. For example, Gorecki et al. (1996) observed that when matured at 13℃, yellow lupin seeds had twice the amount of stachyose and verbascose compared to those matured at 28℃.

Table 4.2. Concentration of oligosaccharides found in investigated legume flours and isolates.

	Raffinose (mg/g)	Stachyose (mg/g)	Verbascose (mg/g)	Total RFO (mg/g)
Flour				
Fabelle	1.37 ± 0.14^{c}	11.45 ± 0.10^{c}	24.97 ± 2.44^{b}	37.80 ± 2.38^{c}
Malik	1.59 ± 0.12^{c}	11.50 ± 0.18^{c}	28.49 ± 1.16^{a}	41.58 ± 1.19^{bc}
Snowbird	1.65 ± 0.15^{c}	11.91 ± 0.26^{c}	29.88 ± 0.83^{a}	43.44 ± 1.05^{b}
CDC Amarillo (pea)	3.11 ± 0.29^{b}	25.57 ± 0.65^{b}	15.66 ± 0.36^{c}	44.34 ± 0.74^{b}
AAC 26-15 (soy)*	9.30 ± 0.60^{a}	51.29 ± 2.11^{a}	2.73 ± 0.05^{d}	63.32 ± 2.68^{a}
Isolate				
Fabelle	1.51 ± 0.08^{b}	2.96 ± 0.11^{b}	6.64 ± 0.30^{a}	11.11 ± 0.35^{cd}
Malik	1.47 ± 0.11^{b}	2.88 ± 0.12^{b}	7.17 ± 0.31^{a}	11.53 ± 0.11^{c}
Snowbird	1.45 ± 0.03^{b}	2.51 ± 0.05^{b}	6.63 ± 0.15^{a}	10.59 ± 0.17^{d}
CDC Amarillo (pea)	2.71 ± 0.18^{a}	10.67 ± 0.15^{a}	5.69 ± 0.08^{b}	19.07 ± 0.29^{a}
AAC 26-15 (soy)	1.74 ± 0.14^{b}	10.68 ± 0.55^{a}	0.00 ± 0.00^{c}	12.42 ± 0.53^{b}

Notes:
Flour or isolate data with the same superscript letter are not significantly different ($p>0.05$).
Values are expressed on an *as is* basis
Total RFO: Raffinose, stachyose, and verbascose.
*AAC 26-15 (defatted SF)

Overall, the SF contained the highest total RFO (63.32 mg/g), followed by PF (44.34 mg/g). The FF had total RFO ranging from 37.80-43.44 mg/g, with Malik and Snowbird having similar concentrations to that of PF, while Fabelle was significantly less concentrated with the sugars. On the contrary, the PPI had the largest amount of total RFO (19.07 mg/g), while the content for SPI was much lower (12.42 mg/g). Ranging from 10.59-11.53 mg/g, the FPI contained less total RFO than PPI and SPI. According to Varney et al. (2017), 0.3 g per serving of food is considered the cutoff value of galacto-oligosaccharides to cause flatulence problems for individuals with Irritable Bowel Syndrome (IBS). If a susceptible person consumes a 200 g serving of faba beans, the content of RFO would be more than enough to result in gastrointestinal discomfort. However, since bean flours are not consumed raw, the content of RFO is expected to decline if cooked. Thermal processing breaks down the oligosaccharides into smaller sugar units by heating and hydration. A 41-43% reduction in the total oligosaccharide concentration of whole and split faba bean seeds by soaking and cooking was reported by Stone et al. (2021).

4.4.2. Protein quality

a) Amino acid composition and amino acid score

The AA composition, essential amino acid (EAA) concentration and corresponding AAS of the legume samples are presented in Tables 4.3 and 4.4. Relating to the reference pattern set by the FAO based on the nutritional needs of a 3-5-year-old child, the limiting amino acid (LAA) were methionine and cysteine for FF, with AAS of 0.88, 0.91, and 0.77 for cultivars Fabelle, Malik, and Snowbird, respectively. Tryptophan was limiting in the PF with an AAS of 0.96 while being also limiting in the flours of Malik (0.92) and Snowbird (0.96) to a lesser extent than methionine and cysteine. The SF was not limiting in any AA, with the lowest score being 1.13 for lysine. Soy proteins are complete because they contain all the EAA required for the normal development and maintenance of the human body. In the isolates, the primary LAA were methionine and cysteine for all legumes, including soy. The FPI were more limiting (0.62, 0.63, and 0.58 for Fabelle, Malik, and Snowbird, respectively) in SAA than those of PPI (0.79) and SPI (0.91). Tryptophan was also limiting in the FPI and PPI with slightly higher AAS (0.87-0.94) than SAA. The findings agreed with those reported in the literature, as SAA and tryptophan are the most limiting AA in pulses (Fernández-Quintela et al., 1997; Mertens et al., 2012; Nosworthy et al., 2017b; Nosworthy et al., 2018; Setia et al., 2019; Vogelsang-O'Dwyer et al., 2020). The differences in the LAA profiles between the flours and isolates are likely due

to the loss of sulfur-rich albumins in the protein extraction process (Liu et al., 2008; Kiosseoglou & Paraskevopoulou, 2021; Vioque et al., 2012; Vogelsang-O'Dwyer et al., 2020). Except for the LAA, all other AA were found in moderate to high amounts (relative to the reference pattern), especially valine, isoleucine, histidine, phenylalanine, and tyrosine in the isolates. The non-limiting presence of lysine allows for well-balanced nutrition when the legumes are consumed together with cereals rich in SAA. Similar among cultivars, the EAA were less abundant in FF than PF and SF, whereas the difference was less noticeable in the isolates, except for the SAA.

Table 4.3. Amino acid composition (g per 100 g of sample, on an *as is* basis) of investigated legume flours (a) and isolates (b).

Amino acid	(a) Flour				
	Fabelle	**Malik**	**Snowbird**	**CDC Amarillo (pea)**	**AAC 26-15 (soy)[2]**
CP (%)[1]	27.2	28.2	31.6	20.8	52.7
Moisture (%)	7.4	7.8	8.1	9.8	6.5
Aspartic Acid	3.00	3.29	3.56	2.51	6.45
Glutamic Acid	4.64	4.77	5.50	3.57	10.28
Serine	1.39	1.43	1.62	1.01	2.88
Glycine	1.15	1.20	1.34	0.93	2.28
Histidine‡	0.78	0.80	0.90	0.61	1.60
Arginine	2.64	2.55	3.03	1.66	3.92
Threonine‡	1.00	1.03	1.14	0.82	2.13
Alanine	1.13	1.18	1.26	0.92	2.27
Proline	1.18	1.21	1.39	0.88	2.81
Tyrosine	0.91	0.92	1.05	0.78	1.95
Valine‡	1.32	1.35	1.51	1.03	2.64
Methionine*‡	0.27	0.26	0.27	0.24	0.80
Cysteine*	0.33	0.38	0.33	0.31	0.71
Isoleucine‡	1.20	1.21	1.36	0.88	2.57
Leucine‡	2.08	2.12	2.42	1.52	4.24
Phenylalanine‡	1.20	1.21	1.34	1.02	2.78
Lysine‡	1.80	1.95	1.98	1.50	3.45
Tryptophan‡	0.30	0.28	0.33	0.22	0.86

Amino acid	(b) Isolate				
	Fabelle	**Malik**	**Snowbird**	**CDC Amarillo (pea)**	**AAC 26-15 (soy)**
CP (%)[1]	83.9	88.9	91.8	86.9	87.0
Moisture (%)	6.72	4.57	4.23	5.22	5.39
Aspartic Acid	11.47	13.35	12.21	11.83	11.83
Glutamic Acid	18.05	20.04	19.72	18.29	19.64
Serine	5.37	5.67	5.61	5.24	5.13
Glycine	4.06	4.27	4.14	4.13	3.83
Histidine‡	2.28	2.30	2.56	2.26	2.34
Arginine	9.62	9.79	10.13	9.46	7.46
Threonine‡	3.54	3.66	3.59	3.59	3.55
Alanine	3.92	4.42	4.18	4.01	3.82
Proline	4.48	4.82	4.61	4.31	4.99
Tyrosine	3.61	3.26	3.64	3.82	3.27
Valine‡	4.93	5.23	5.13	5.00	4.49
Methionine*‡	0.67	0.74	0.75	0.98	1.10
Cysteine*	0.64	0.65	0.57	0.73	0.88
Isoleucine‡	4.53	4.98	4.73	4.52	4.48
Leucine‡	8.33	8.98	8.83	8.39	7.65
Phenylalanine‡	4.82	4.96	4.81	5.47	5.18
Lysine‡	6.63	7.23	6.86	7.33	6.41
Tryptophan‡	0.87	0.88	0.88	0.88	1.18

Notes:

[1] CP (crude protein on a wet weight basis)

[2] AAC 26-15 (defatted SF)

*, sulfur amino acid. ‡, essential amino acids.

Table 4.4. Essential amino acid concentration (mg/g protein) and amino acid scores of investigated legume flours and isolates.

	Amino acids								
	THR	**VAL**	**MET + CYS**	**ILE**	**LEU**	**PHE + TYR**	**HIS**	**LYS**	**TRP**
a) Essential amino acids									
Flour									
Fabelle	37	49	22	44	77	78	29	66	11
Malik	36	48	23	43	75	75	28	69	10
Snowbird	36	48	19	43	77	75	28	63	11
CDC Amarillo (pea)	39	49	26	43	73	87	29	73	11
AAC 26-15 (soy)[1]	40	50	29	49	80	90	30	65	16
Isolate									
Fabelle	42	59	16	54	99	100	27	79	10
Malik	41	59	16	56	101	92	26	81	10
Snowbird	39	56	14	51	96	92	28	75	10
CDC Amarillo (pea)	41	58	20	52	97	107	26	84	10
AAC 26-15 (soy)	41	52	23	51	88	97	27	74	14
FAO reference	34	35	25	28	66	63	19	58	11
b) Amino acid score									
Flour									
Fabelle	1.09	1.39	0.88*	1.57	1.16	1.23	1.50	1.15	1.01
Malik	1.07	1.36	0.91*	1.53	1.14	1.20	1.49	1.19	0.92
Snowbird	1.06	1.37	0.77*	1.54	1.16	1.20	1.50	1.08	0.96
CDC Amarilio (pea)	1.15	1.41	1.06	1.52	1.10	1.37	1.55	1.24	0.96*
AAC 26-15 (soy)[1]	1.19	1.43	1.15	1.74	1.22	1.42	1.59	1.13	1.49
Isolate									
Fabelle	1.24	1.68	0.62*	1.93	1.50	1.59	1.43	1.36	0.94
Malik	1.21	1.68	0.63*	2.00	1.53	1.47	1.36	1.40	0.89
Snowbird	1.15	1.60	0.58*	1.84	1.46	1.46	1.47	1.29	0.87
CDC Amarilio (pea)	1.22	1.64	0.79*	1.86	1.46	1.70	1.37	1.46	0.92
AAC 26-15 (soy)	1.20	1.48	0.91*	1.84	1.33	1.54	1.41	1.27	1.24

Notes:

Abbreviations: THR (threonine); CYS (cysteine); VAL (valine); MET (methionine); ILE (isoleucine); LEU (leucine); TYR (tyrosine); PHE (phenylalanine); HIS (histidine); LYS (lysine); and TRP (tryptophan).

*The first limiting amino acid

[1] AAC 26-15 (defatted SF)

b) In vitro protein digestibility (IVPD)

The IVPD of the legume samples are given in Table 4.5. In the flours, the IVPD of PF (74.9%) was significantly higher than that of SF (71.4%) and FF (72.8-73.0%). No significant difference was observed among faba bean cultivars. The IVPD values were comparable to those obtained for the investigated legume flours using similar multi-enzyme assays and pH drop methods (Alonso et al., 2000; Han et al., 2007; Luo & Xie, 2013; Setia et al., 2019). The IVPD was improved in the protein isolates, ranging from 73.8-82.3%, with SPI and PPI being the lowest and the highest, respectively. The lower IVPD values for the flours than their respective isolates are possibly due to other components such as lipids and fiber that may have interacted with proteins and limited digestion (Acton et al., 1982; Luo & Xie, 2013; Vogelsang-O'Dwyer et al., 2020). In addition, the presence of ANF in legume samples is also one of the most discussed factors contributing to reduced protein digestibility (Alonso et al., 2000; Chitra et al., 1995; Guldiken et al., 2021; Luo & Xie, 2013; Ritter et al., 1987; Vogelsang-O'Dwyer et al., 2020). When expressed in mg/g protein, the investigated legume flours had TPC ranging from 5.44-7.45 mg CE/g protein, CT ranging from 0.10-0.80 mg CE/g protein, and PA ranging from 32.78-54.95 mg/g protein, considerably higher than those found for the isolates (3.19-4.03 mg CE/g protein, 0.19-0.45 mg CE/g protein, and 22.30-40.55 mg/g protein for TPC, CT, and PA, respectively). These antinutritive compounds are known to form complexes with proteins and digestive enzymes via hydrogen bonds, hydrophobic and covalent cross-links, and van der Waals activities, inducing changes in protein conformation, solubility, and digestibility (Guldiken et al., 2021; Gulewicz et al., 2014; Ozdal et al., 2013; O'Dell & De Boland, 1976; Wang et al., 1998). Calculated as mg/g protein, we can conclude that the amount of ANF attached to one gram of protein was more in the flours than in the isolates, which could lead to stronger interactions of the compounds with protein, and lower protein digestibility, even though the abundance of the ANF (expressed as mg/g sample material in previous sections) was showing the opposite trend when the sample material was assessed as a whole. Significant and negative correlations were established between the content of PA and IVPD of several genotypes of select legumes, including soy and chickpea, in the work of Chitra et al. (1995), and between TPC and IVPD of navy bean flours in the study of Guldiken et al. (2021). A noticeable reduction in the levels of TPC, CT, PA, and a series of enzyme inhibitors by soaking, germination, and extrusion was reported by Alonso et al. (2000), which was coupled with a significant improvement in the *in vitro* digestibility of both protein and starch in faba and kidney beans. In the present study, the reduction of ANFs including TPC, CT, and PA by wet

protein extraction suggests a promising method to produce highly digestible protein ingredients.

Table 4.5. Limiting amino acid scores and protein quality data of investigated legume samples.

	Limiting amino acid	Amino acid score[1]	IVPD[2] (%)	IV-PDCAAS[3] (%)
Flour				
Fabelle	MET + CYS	0.88	72.8 ± 1.0^{bc}	64.1 ± 0.9^{d}
Malik	MET + CYS	0.91	73.0 ± 0.6^{b}	66.3 ± 0.5^{c}
Snowbird	MET + CYS	0.77	72.8 ± 0.4^{bc}	55.8 ± 0.3^{e}
CDC Amarillo (pea)	TRP	0.96	74.9 ± 0.5^{a}	71.7 ± 0.5^{b}
AAC 26-15 (soy)*	N/A	1.13	71.4 ± 0.1^{c}	80.7 ± 0.1^{a}
Isolate				
Fabelle	MET + CYS	0.62	75.8 ± 0.5^{c}	47.3 ± 0.3^{d}
Malik	MET + CYS	0.63	78.8 ± 0.8^{b}	49.3 ± 0.5^{c}
Snowbird	MET + CYS	0.58	77.9 ± 0.4^{b}	44.8 ± 0.2^{e}
CDC Amarillo (pea)	MET + CYS	0.79	82.3 ± 0.1^{a}	64.8 ± 0.1^{b}
AAC 26-15 (soy)	MET + CYS	0.91	73.8 ± 0.5^{d}	67.2 ± 0.5^{a}

Notes:
Flour or isolate data with the same superscript letter are not significantly different ($p > 0.05$).
[1]Measurements were preformed once.
[2]Measurements were performed in triplicate. Data represent the mean ± one standard deviation (n = 3).
[3]Data represents the product of the limiting amino acid score and IVPD (measured in triplicate). Data represent the mean ± one standard deviation (n=3).
Abbreviations: IVPD (*In vitro* protein digestibility), IV-PDCAAS (*In vitro* protein digestibility corrected amino acid score), CYS (cysteine), MET (methionine), TRP (tryptophan), and N/A (not applicable).
*AAC 26-15 (defatted SF).

c) In vitro protein digestibility corrected amino acid score (IV-PDCAAS)

The IV-PDCAAS ranged from 55.8% to 80.7% for the flours and 44.8% to 67.2% for the isolates (Table 4.5). Despite being more digestible, the protein quality of the isolate samples was lower than that of the flours. It was previously reported that IV-PDCAAS was negatively correlated to the protein content of navy bean flours (Guldiken et al., 2021). Besides the loss of sulfur-rich albumins during alkaline extraction-isoelectric precipitation (AE-IP), the increased protein content in the isolates may have also led to an increase in the level of non-essential AA, and consequently lowered the AAS for calculating the PDCAAS, an indicator of protein quality (Chung et al., 2008). In other words, the PDCAAS, as a chemical score, is mainly dependent on the EAA composition of the test proteins with lesser influence from

digestibility. It does not fully account for the presence of ANF or their reactions that may potentially reduce nutrient bioavailability (Sarwar, 1997). Therefore, the adverse impact of ANF observed previously for protein digestibility was not found here for overall protein quality. Likewise, the soy proteins scored the highest in IV-PDCAAS in both the flour (80.7%) and isolate (67.2%) because of their superior AA composition and high AAS, even though they were less digestible. The yellow pea samples were the 2nd highest in protein quality with IV-PDCAAS of 71.7% and 64.8% for flour and isolate, respectively, followed by faba bean (55.8-66.3% for flours, and 44.8-49.3% for isolates). The faba bean samples had lower protein quality as they were more limiting in SAA than pea and soy, particularly in the isolates with IV-PDCAAS below 50%. Among cultivars, the order of protein quality from high to low was Malik, Fabelle, and Snowbird, corresponding to their AAS. Stone et al. (2019) investigated a variety of Canadian legume flours and reported high protein quality for SF with a mean IV-PDCAAS of 82.2%, comparable to findings in the current study, whereas their results for PF (52.1%) and FF (55.2%) were lower than present findings. In the work of Setia et al. (2019), raw PF and FF had IV-PDCAAS of 62.1% and 56.2%, respectively, with tryptophan being the LAA. Besides plant differences, variations in PDCAAS may arise from factors like cultivation conditions that likely influence the AA composition of grain crops (Eriksen & Mortensen, 2002; Järvan et al., 2012).

4.5. Conclusions of Study 2

The study aimed to compare the nutritional value of recent faba bean cultivars grown in Canada to that of yellow pea and soy, emphasizing the levels of ANF and protein quality of flours and protein isolates. The contents of TPC and CT were low in all samples due to dehulling. Therefore, no clear distinction was observed among faba bean cultivars based on tannin content, and the adverse impact on the protein digestibility was assumed minimal. The PA concentrations in the flours were intermediate for faba bean while sitting in the higher range in the isolates. The cultivar Fabelle was more concentrated with PA than Malik and Snowbird in both flours and isolates. The amount of TPC, CT, and PA (mg/g sample material) were less in the flours, possibly due to the higher protein content of the isolates and the greater occurrence of binding activities between proteins and these compounds. The V-C contents of the investigated faba bean samples agreed with their market classes, of which Fabelle is the low V-C cultivar while Malik and Snowbird are the regular V-C type. The low V-C cultivars like Fabelle can potentially expand faba bean utilization in the consumer food market as it prevents

the onset of favism in G6PD individuals. Cultivar differences were not observed for oligosaccharides with the faba bean samples generally having less raffinose and stachyose but more verbascose than pea and soy. The total RFO was lower in FF than in SF, while in the isolates, the content was significantly less for FPI than for PPI and SPI. A low content of these fermentable sugars makes the bean consumption less problematic for people with irritable digestive systems, whereas their concentrations in our faba bean samples (flours) were still high enough to induce symptoms. V-C and oligosaccharides were reduced in the isolates due to leaching by hydration (during wet protein extraction).

The sulfur-containing methionine and cysteine were the first LAA in FF while tryptophane was limiting in PF, and no LAA was found in SF. However, the SAA were limiting in all isolate samples, including pea and soy, with faba bean being more deficient in them. Tryptophan was also limiting in the flours of Malik and Snowbird and the isolates of faba and pea with higher AAS than the primary LAA. The IVPD of the flours was the highest for PF, followed by FF and SF, and was improved in the isolates due to less intense interactions between the proteins and ANF or other components. However, due to the loss of sulfur-rich albumins during AE-IP, the AAS was lower for the isolates, resulting in lower protein quality than their respective flours. Likewise, despite being relatively easy to digest, the faba bean samples scored low (Malik > Fabelle > Snowbird) in the IV-PDCAAS with significant influences from the AA composition. In short, the faba bean samples in the present study were overall comparable to pea and soy for levels of ANF in terms of TPC, CT, and PA while having generally less oligosaccharides and containing species-specific compounds V-C. However, the investigated cultivars were more limiting in SAA and had lower protein quality than pea and soy, especially in the isolates. The lower protein quality of faba bean flours (Snowbird < Fabelle < Malik) than pea and soy could be improved by blending with cereals, whereas in the isolates, the loss of sulfur-rich albumins (for all legumes, especially faba) may be a challenge and warrant further investigation to modify wet fractionation processes.

5. GENERAL DISCUSSION

The plant protein market has been growing at an unprecedented rate, especially in North America. Protein isolates prepared from a variety of legumes (e.g., soy, pea, and others) are being incorporated into a wide range of food products to substitute animal ingredients and improve their techno-functional qualities. Faba bean (*Vicia faba* L.) is an emerging crop in the Canadian prairies with great potential to produce functional ingredients and compete in the current plant-based market. The underlying goal of this research was to generate an updated scientific knowledge base in relation to recent faba bean cultivars in Canada and provide useful information on the crop's competitiveness as value-added ingredients in the ever-growing market. Three faba bean cultivars (Fabelle, Malik, and Snowbird) were evaluated for their functionality and nutritional value with direct comparison to those of yellow pea (CDC Amarillo) and soybean (AAC 26-15), the two prominent sources of legume protein in the market. The faba bean cultivars were representative of the current market segments, with Fabelle being regular tannin and low vicine and convicine, Malik being regular tannin, and Snowbird being low tannin.

The first study focused on the functional aspect of the protein isolates of faba bean, yellow pea, and soy. The protein isolates were prepared from dehulled legume seeds by AE-IP. Analyses included the proximate composition, L/V ratio, surface charge, interfacial tension, protein solubility, WHC, OHC, FC, FS, EAI, and ESI. The legume flours were also assessed for their compositional information. In agreement with literature findings, the faba bean seeds were high in protein with protein contents ranging 29.34-34.35%, higher than that of pea (23.10%), and low in lipids (1.14-1.35%) (Table 3.1). Such composition of faba beans makes them suitable to produce ingredient fractions (e.g., protein isolates) to be formulated in products for high-protein and low-fat claims. Among the faba bean cultivars, the highest protein content was found for Snowbird (34.35% and 97.41% for flour and isolate, respectively), which was suggested to be a trait linked to low tannin cultivars. In terms of the protein yield, the effect of reduced water usage during AE on the protein recovery by IP was investigated on Fabelle. Compared to 1:6 (70.28%), the flour:solvent ratio 1:8 yielded a similar

level of protein (73.42%) to that of 1:10 (74.96%), which could be meaningful to protein ingredient manufacturers in reducing the production cost.

The protein composition was hypothesized to have a relationship to the functional properties of legume protein isolates due to the distinct characteristics between legumin and vicilin type proteins. To test the hypothesis, the L/V ratio of the legume protein isolates was determined by SDS-PAGE to correlate with their functionality. Significant differences existed among legumes, with L/V ratios being higher for FPI (2.68, 2.66, and 3.22 for Fabelle, Malik, and Snowbird, respectively) than for PPI (2.00) and SPI (1.40) (Table 3.2). As it would not be meaningful to compare L/V ratios among different legumes, PPI and SPI were excluded from the Pearson correlation (r). Nonetheless, only a few of the functional properties were related to the L/V ratios for FPI, including zeta potential, interfacial tension, and ESI that were positively correlated and FS and EAI that were negatively correlated. The lack of correlations was not unexpected, because 1) functional tests may be more sensitive to extrinsic factors than inherent protein composition, 2) a statistical difference in the ratios could be not significant enough to reflect an actual change in functionality, and 3) the relative estimation of ratios often introduces errors. A larger data set with a greater number of cultivars is likely to improve statistical inferences.

In comparison between the quality attributes of FPI, PPI and SPI, the surface tension, solubility, WHC, and OHC were generally similar (Table 3.2, Figure 3.2, and Figure 3.3). The surface charge of FPI was significantly higher than that of PPI and SPI, and the interfacial tension was largely comparable between FPI and PPI, which was lower than that of SPI (Table 3.2). The FC and FS of FPI were overall similar to that of PPI and higher than that of SPI (Figure 3.4). The emulsifying properties were somewhat similar between PPI and the isolates of Malik and Fabelle, while Snowbird had significantly lower EAI and higher ESI dependent on pH (Figure 3.5). The comparably good foaming and emulsifying properties of FPI suggest the potential use of this ingredient in dairy and meat-based products. The high WHC and OHC comparable to those of PPI and SPI suggest the use of FPI to prevent quality loss during processing and storage and maintain sensory characteristics of products such as soup, baked goods, ground meats, and processed meat. The cultivar Snowbird had a few relatively distinct characteristics compared to Fabelle and Malik, presumably due to its high L/V ratio. For example, legumin has more structural constraints than vicilin, making it less capable of rapid conformational changes at the interface (low EAI), while its bulky polypeptide chains and intramolecular bonds could instead form stronger and more viscoelastic films and convey

stability in emulsions (high ESI). To summarize, the FPI had comparable or higher physicochemical and functional attributes than those of PPI and SPI, making it a promising candidate to be formulated as a functional ingredient in food products.

The second study aimed to provide insight into the utilization potential of faba bean from a nutritional perspective. One of the major factors that limit faba bean utilization is the presence of undesired bioactive compounds, while a comparable or better AA composition would be desirable for faba bean to compete with major legume proteins in food applications. Therefore, in Study 2, both flours and isolates prepared in the first study from Fabelle, Malik, Snowbird, yellow pea (CDC Amarillo), and soy (AAC 26-15) were evaluated for levels of TPC, CT, PA, V-C, and oligosaccharides, and protein quality in terms of AA composition, AAS, IVPD, and IV-PDCAAS. Dehulling of the legume seeds removed the majority of TPC and CT, leaving only residual amounts in the flours and isolates (Table 4.1). For this reason, the low tannin characteristic of Snowbird was not evident when compared to the regular tannin types (Fabelle and Malik). The FF contained more PA than PF but less than SF, while the FPI had generally more PA than both PPI and SPI (Table 4.1). Fabelle was more concentrated with PA than Malik and Snowbird in both the flours and isolates. Typically, the TPC, CT, and PA tend to form complexes with proteins and negatively affect their digestion. Therefore, these compounds accumulated in the isolates. However, when protein content was factored in the calculation of their concentrations (using mg/g protein versus mg/g sample), the legume flours had generally more TPC, CT, and PA than the respective isolates. The numbers suggested that the actual amount of ANF attached to one gram of protein was more for the flours than for the isolates with stronger protein-binding activities and possibly hindered digestion.

In contrast to the above compounds, the V-C and oligosaccharides do not interfere with digestion, and their effect on health varies among individuals. V-C are responsible for inducing favism in G6PD deficient patients. The V-C contents of the faba bean cultivars were representative of their market classes (Fabelle < Malik < Snowbird) (Table 4.1). In the literature, there was no established cut-off values of V-C from faba bean consumption to induce favism in G6PD deficient populations, possibly due to the varying severity of the deficiency that is genetically dependent. The safety of low V-C faba bean (Divine) consumption by G6PD deficient patients was confirmed by a clinical trial with a lack of signs of oxidative red blood cell damage or hemolysis within 8 hours of bean ingestion (Gallo et al., 2018). The low V-C characteristic of Fabelle in the present research offers opportunities for expanding the customer base of faba bean products. The oligosaccharides can be either beneficial (as prebiotics) or

problematic, depending on the gastrointestinal conditions of individuals. The fermentation of these sugars in the human digestive tract produces gas and causes flatulence, particularly for people with IBS. The faba bean cultivars had generally similar sugar profiles and less total RFO than pea and soy (Table 4.2), which is advantageous and yet still not sufficiently low to prevent the bloating problems. The V-C and oligosaccharides were less concentrated in the isolates than in the flours due to leaching by hydration during AE-IP. The combination of cultivar selection (low tannin and V-C), dehulling (to reduce tannins and other phenolics), and wet protein extraction (to reduce V-C and oligosaccharides) could be used to produce FPI with an optimized nutritional profile.

The primary LAA were the sulfur-containing methionine and cysteine for FF, tryptophan for PF, and methionine and cysteine again for all legume protein isolates (Table 4.4). The SF was not limiting in any AA as soy proteins are complete, whereas the SAA was limiting in SPI. Tryptophan was also limiting in the flours of Malik and Snowbird and the isolates of all three faba bean cultivars and pea with slightly higher AAS than their primary LAA. Faba bean was less abundant in EAA than pea and soy with Snowbird being more deficient in SAA than Fabelle and Malik. Snowbird, in the previous section on protein composition, had the highest L/V ratio among faba bean cultivars (Table 3.2). However, the theoretically high content of legumin observed in Study 1was not reflected in the AA composition, hinting at the inherent limitations of using this technique to assess protein composition. All samples had high digestibility (>70% of IVPD) (Table 4.5). The adverse impact of ANF (TPC, CT, and PA) on protein digestion was only evident when evaluating their concentrations in mg/g protein (versus mg/g sample), and as discussed above, the flours had lower IVPD values than the respective isolates due to stronger interactions with the proteins.

Nevertheless, the flours had better protein quality (Table 4.5), as IV-PDCAAS does not fully account for the presence of ANF or their negative influence on protein digestion but rather depends strongly on the EAA composition. For the same reason, since the faba bean samples were more deficient in SAA than pea and soy, their protein quality was lower, especially for the isolates (<50% of IV-PDCAAS compared to 64.8% and 67.2% for PPI and SPI, respectively), even though they were comparably digestible. The faba bean samples' IV-PDCAAS was the lowest for Snowbird, followed by Fabelle and Malik, corresponding to their AAS. For the prevention and treatment of acute malnutrition in areas of need, the World Food Program (WFP) of the United Nations' standard for producing the WFP Specialized Nutritious Foods requires them to have a PDCAAS of no less than 70% (WHP, 2022). Assuming this

standard as a baseline value for assessing protein quality, in the present study, only flours of yellow pea (71.7%) and soy (80.7%) met the requirement. To further standardize the evaluation of protein quality, the IV-PDCAAS values were converted to estimate the PER using the formula of *PDCAAS for food × 2.5 = estimated PER for food*, where 2.5 is the PER of casein, a reference protein (Friedman, 1996; Government of Canada, 2022). The estimated PER of the legume samples are shown in Table 5.1. Typically, low-quality proteins have PER values below 1.5, and those with values above 2.0 are considered good to high quality (Friedman, 1996). Except for Snowbird (1.40), proteins in all flour samples were of moderate to high quality, whereas in the isolates, all three faba bean cultivars had low quality proteins (1.12-1.23).

Table 5.1. Protein quality data with protein efficiency ratios of investigated legume samples.

	Limiting amino acid	**Amino acid score[1]**	**IVPD[2] (%)**	**IV-PDCAAS[3] (%)**	**PER[4]**
Flour					
Fabelle	MET + CYS	0.88	72.8 ± 1.0^{bc}	64.1 ± 0.9^{d}	1.60
Malik	MET + CYS	0.91	73.0 ± 0.6^{b}	66.3 ± 0.5^{c}	1.66
Snowbird	MET + CYS	0.77	72.8 ± 0.4^{bc}	55.8 ± 0.3^{e}	1.40
CDC Amarillo (pea)	TRP	0.96	74.9 ± 0.5^{a}	71.7 ± 0.5^{b}	1.79
AAC 26-15 (soy)*	N/A	1.13	71.4 ± 0.1^{c}	80.7 ± 0.1^{a}	2.02
Isolate					
Fabelle	MET + CYS	0.62	75.8 ± 0.5^{c}	47.3 ± 0.3^{d}	1.18
Malik	MET + CYS	0.63	78.8 ± 0.8^{b}	49.3 ± 0.5^{c}	1.23
Snowbird	MET + CYS	0.58	77.9 ± 0.4^{b}	44.8 ± 0.2^{e}	1.12
CDC Amarillo (pea)	MET + CYS	0.79	82.3 ± 0.1^{a}	64.8 ± 0.1^{b}	1.62
AAC 26-15 (soy)	MET + CYS	0.91	73.8 ± 0.5^{d}	67.2 ± 0.5^{a}	1.68

Notes:
Data in this table were adapted from Table 4.5 with an additional column of PER.
Flour or isolate data with the same superscript letter are not significantly different ($p > 0.05$).
[1]Measurements were preformed once.
[2]Measurements were performed in triplicate. Data represent the mean ± one standard deviation (n = 3).
[3]Data represents the product of the limiting amino acid score and IVPD (measured in triplicate). Data represent the mean ± one standard deviation (n=3).
[4]PER = PDCAAS for food × 2.5, where 2.5 is the PER of casein, a reference protein.
Abbreviations: IVPD (*In vitro* protein digestibility), IV-PDCAAS (*In vitro* protein digestibility corrected amino acid score), PER (protein efficiency ratio), CYS (cysteine), MET (methionine), TRP (tryptophan), and N/A (not applicable).
*AAC 26-15 (defatted SF).

However, the IVPD tends to underestimate the true protein digestibility (TPD) (Swaisgood & Catignani, 1991). In general, *in vitro* methods are useful as alternatives to bioassays as they are straightforward, faster, and cheaper with no ethical concerns. However, the *in vitro* analysis is an isolated process and does not account for the physiological factors that simultaneously contribute to protein digestion (Tavano et al., 2016). For example, non-protease enzymes help break down the food particles and allow for an easier passage of the proteases. Even though studies have shown strong correlations between the *in vitro* and *in vivo* results on protein quality, the IVPD and IV-PDCAAS remain conservative estimates of the true value (Nosworthy et al., 2017a; Nosworthy et al., 2018). For this reason, bioassays are required to establish PER for making official protein claims in Canada. According to Wolzak et al. (1981), the sensitivity of the multienzyme assay varies for different types of proteins. Hence, the faba bean proteins examined in the present study could have higher or comparable quality to that of pea when analyzed *in vivo*.

In addition, it is obvious that the differences in protein quality between the isolates and their respective flours were due to the change in the AA composition (Table 5.1). The lower protein quality for the isolates presumably resulted from the loss of sulfur-rich albumins during protein recovery by the AE-IP process and consequently lower AAS. The diminished isolate protein quality poses a challenge for protein ingredient manufacturers, as AE-IP is the most utilized wet method for protein isolation. To avoid albumin loss, SE could be used to extract a mixture of globulins and albumins in a more native state (Liu et al., 2008). However, SE tends to yield less protein, and the high water usage required throughout the process also needs consideration (Stone et al., 2015). The pressure-driven UF could replace the IP after AE or be coupled with other extraction techniques to recover proteins with reduced ANF levels, while the cost would also be a critical factor (Mondor et al., 2009).

6. OVERALL CONCLUSION AND FUTURE STUDIES

To investigate the utilization potential of Canadian faba bean protein ingredients, the present research evaluated the functional and nutritional values of protein isolates prepared from three faba bean cultivars (Fabelle, Malik, and Snowbird) and compared their quality attributes with those of yellow pea (CDC Amarillo) and soybean (AAC 26-15). The research was divided into two studies. In the first study, the protein isolates were prepared from dehulled legume seeds using AE-IP and compared for their physicochemical and functional properties. The proximate composition of dehulled legume seeds confirmed that faba bean is a high protein (29.34-34.35%, higher than pea) crop that is also low in lipids (1.14-1.35%). The surface tension, protein solubility, and OHC were observed to be largely comparable among legume protein isolates, while FPI was more negatively charged than both PPI and SPI. The FPI also had interfacial tension, WHC, foaming, and emulsifying properties that were generally similar to PPI's, while those of SPI varied. The cultivar difference was relatively evident when comparing Snowbird with Fabelle and Malik, as the former one was richer in protein (flour and isolate) and legumin-type proteins, higher in ESI, and lower in EAI. The primary hypothesis, that the protein isolates prepared from the investigated faba bean cultivars are comparable in physicochemical and functional properties to those from yellow pea and soy, was proven to be true. The minor hypothesis, that the reduced water usage during AE will lower the extractability of protein, was partially true as the protein yields were similar when extracting using 1:10 and 1:8 flour:solvent ratios, whereas reducing water usage to 1:6 lowered the values. Protein composition in terms of the L/V ratio was also hypothesized to be related to the functional properties of FPI, whereas very few and weak correlations were established. The findings in Study 1 suggested that the FPI prepared from the investigated cultivars are promising candidates as functional ingredients in food formulations.

The second study investigated the nutritional value of both the legume flours and isolates prepared in Study 1. Dehulling presumably removed most of the TPC and CT in all legume samples. The PA concentration for faba bean was intermediate in the flours while being overall higher in the isolates than pea and soy. Fabelle contained significantly more PA than

the other two cultivars while having the lowest amount of V-C. Not much cultivar difference was observed for the levels of oligosaccharides, whereas among legumes, faba bean had generally less of the sugars than pea and soy. In the flours, faba bean and pea were limiting in the SAA and tryptophan, respectively, while soy had a complete amino acid composition. However, all legume isolates were limiting in SAA, including soy. The faba bean samples were relatively digestible with >70% IVPD values. Nevertheless, they were more limiting in SAA than pea and soy, resulting in lower protein quality, especially for the isolates with <50% IV-PDCAAS. The lowest protein quality was observed for Snowbird, followed by Fabelle and Malik, corresponding to their AAS. Coming back to the hypothesis for Study 2, it was proposed that the flours and protein isolates prepared from the investigated faba bean cultivars are comparable in nutritional values (level of ANF and protein quality) to those of yellow pea and soy. Our results showed that faba bean was indeed largely comparable to pea and soy for TPC, CT, and PA levels, while being lower in fermentable sugars and contained species-specific compounds V-C. However, due to their less balanced protein profile than that of pea and soy, the protein quality was lower for the faba bean samples, particularly for the isolates due to albumin losses by AE-IP.

In conclusion, the comparable or higher functionalities of FPI compared to PPI and SPI offer an advantage in formulating products that require additional or improved functionality. For example, FF and FPI could blend into food products for high-protein and low-fat claims. FPI could also serve as binders in processed meats for its comparably high protein content, WHC, and OHC. However, our second study from the nutritional perspective indicated that faba bean ingredients had lower protein quality with major influences from the AA composition. The lower content of methionine and cysteine in the faba beans could be compensated by blending with cereals, while the loss of SAA in all legume isolates compared with the flours may challenge their utilization in general. Overall, the present research on the representative cultivars of recent Canadian faba bean classes updated the scientific knowledge base in relation to the differences in functional and nutritional properties among the cultivars and how they compete with major legume proteins in the market. The findings of the studies should provide meaningful insights for both faba bean breeders and food product formulators in terms of favorable traits and processing methods to select for and possible applications of faba bean protein ingredients.

Extensive research is still required to aid in the advanced utilization of Canadian faba beans. Stemming from the present findings, future work should focus on the following aspects:

1) Fractionation of the major storage proteins (i.e., 11S, 7S, and 2S) of faba bean and evaluation of the functionality of each protein fraction for a comprehensive investigation into the impact of protein composition on the quality attributes. As discussed in previous sections, using L/V ratio to investigate the proposed relationship between protein composition and functionality had several limitations. The relative estimation of protein ratio also did not allow the comparison of protein composition among legumes. To estimate the functional potential of faba bean protein at the molecular level and understand how it differs fundamentally from pea and soy, the purification of individual protein fractions is required. On the other hand, a study of genotype × environment × agronomic management practices can be conducted on a wider subset of faba bean cultivars to improve the statistical power of the correlations. The results from the proposed study could provide a better understanding of the structure-function relationship of faba bean proteins.

2) A comparative evaluation of nutritional values between raw and cooked faba bean ingredients using *in vivo* testing. Since bean ingredients are not consumed raw, their nutritional quality is expected to change when cooked. In general, soaking and thermal treatments reduce the content and activity of certain ANF and improve protein digestibility. However, their effect on the AA composition seems to be case-specific. Due to the differences in thermal stability among different storage proteins, outcomes of heating vary among species and genotypes (Marsolais et al., 2010; Nosworthy et al., 2018). Moreover, as IVPD tends to yield conservative results, bioassays could be utilized to investigate the true protein digestibility of faba bean ingredients and compare it with pea and soy. It would also be interesting to correlate the change in protein quality after cooking with protein composition.

3) Product development studies on incorporating faba bean, yellow pea, and soy ingredients (flour and protein isolate) coupled with the evaluation of the techno-functional and sensory properties. Given that FPI possesses the ability to hold water and oil, faba bean ingredients could be investigated for their use as binders, fillers, and texture improvers in various meat analogues (Kyriakopoulou et al., 2021). The use of FPI in the development of high-protein and low-fat non-dairy milk could also be exploited based on its comparably good emulsifying properties relative to those of PPI and SPI (Vogelsang-O'Dwyer et al., 2021). The reformulation of food products or the development of new ones should focus on replacing soy ingredients with faba for non-GMO or low-allergenicity claims.

4) Investigation into alternative or modified protein extraction processes to minimize nutrient loss. As discussed before, to reduce albumin loss, several processes (e.g., SE and UF)

could be considered as alternatives to AE-IP while having their own limitations in terms of yield and cost. Efforts could also be made on the modification of the processing parameters during AE-IP (e.g., pH) to recover desired protein fractions. Ultimately, the approach should focus on maintaining or improving the functional and nutritional values of the fractionated product while keeping the cost down for ingredient manufacturers.

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