Novel mild fractionation of faba bean (*Vicia faba* L.) and physicochemical and functional characterization of protein as a food ingredient

by

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ABSTRACT

Alternative planet-friendly protein sources are increasingly acknowledged as a sustainable solution to supplement the global animal-derived protein demand. Among the pulses, faba bean (*Vicia faba* L.) has such potential. The isolation of faba bean protein can minimise the anti-nutritional, flatulence-causing, and beany flavour compounds. Optimization of faba bean fractionation warrants the understanding of the distribution patterns of macro-components within the bean. Dry fractionation methods are a sustainable alternative to energy-intensive alkali extraction but can lead to relatively moderate protein enrichment. Depending on the extraction method, protein functionality may vary; however, such information is limited for faba bean proteins. Therefore, a systematic investigation was performed to characterise the faba beans, evaluate different fractionation techniques, and assess the physicochemical and functional properties of the protein fractions.

In the **first study**, the abaxial (cotyledon-seed coat) and adaxial (cotyledon-cotyledon) topographies of pulses were confirmed to have varietal differences associated with their micromorphological characteristics and the complex cuticle layer on their adaxial surface, which would impact their ease of milling/pearling. In the **second study**, compositional analyses of sequentially pearled fractions of high-tannin (HT, Athena) and low-tannin (LT, Snowbird) faba bean cultivars demonstrated a decreasing trend of protein, ash and total dietary fibre contents from outer to inner layers, which was offset by starch. The 55-57% single-step pearling flour had a higher protein content (LT: 39.1% and HT: 37.4%) compared to whole beans (LT: 30.5% and HT: 29.6%). Aqueous fractionation of the single-step pearling flour resulted in protein and starch isolates and dietary fibre concentrates. The protein and dietary

fibre contents of LT and HT faba beans were positively correlated with the increasing particle size, which was offset by starch (**study three)**. Subjecting the dehulled bean flours to Air-Currents-Assisted Particle Separation (ACAPS) resulted in both protein- and dietary fibre-rich coarse fractions. Aqueous extraction and micellization of the ACAPS-treated coarse fractions (250-500 μm) resulted in protein isolates (LT: 97.7 % and HT: 94.3%).

The impact of different mild wet fractionation conditions was established in the **fourth study** in terms of yield, purity and recovery of faba bean protein concentrates/isolates (PCs/PIs). Although salt-soluble globulins (74-75%) were the primary proteins in faba beans, PIs resulting from dialysis (D) or micellization (M) following water extraction at 35°C (W_{35}) at a solvent/feed ratio of 2 had higher protein contents (LT: >93% and HT: 96%) than PCs from $NaCl_{(aq)}$ solution (1% (w/v)) extraction at 23ºC (S_{23}) followed by D (LT: 79.1% and HT: 81.6%) or alkali extraction followed by acid precipitation (AA) (LT: 87.1% and HT: 86.3%). High levels of naturally occurring minerals in faba beans contributed to the extractability of high-quality PIs by W_{35}, including albumins. W_{35}-PIs were low in raffinose family oligosaccharides, tannins and trypsin inhibitory activity.

The size distribution of the PI particles obtained by W_{35} and S_{23} was similar in water suspensions (**study five**). The pre-dominantly amorphous nature of PCs/PIs was confirmed by XRD. Based on XRD and FTIR analyses, β-sheet structures were the most abundant secondary structures and W_{35} extraction had minimal impact. W_{35}-D-PIs had superior solubility, colour parameters and foam stability compared to the AA-PCs (**study six**). Emulsion stability and foaming and emulsifying capacities of W_{35}-PIs were comparable to those of AA-PCs. S_{23}-D-PCs had relatively limited functional properties, probably due to the interference of salt deposits. AA-PCs had superior hydration capacities and low surface

hydrophobicity, but starch and dietary fibre could have also contributed. The gelation of faba bean protein was temperature-dependent and influenced by the degree of protein denaturation (**Appendix C**). W_{35}-D-PIs had the highest stress and strain at fracture at their minimum gelling concentration and formed the strongest gels. Faba bean protein with konjac glucomannan and canola oil formed more stable networks comparable to that of whole eggs. Thus, the potential of W_{35}-D-PIs for use in vegan liquid egg analogue formulations was demonstrated.

In summary, the findings established that the inclusion of pearling or ACAPS as upstream processing steps prior to wet fractionation of faba beans uses less water than conventional methods and has the potential to create new markets for the underutilised high tannin faba bean cvs. and generate novel "clean label" food ingredients. Overall, the W_{35}-PIs demonstrated superior physicochemical and functional properties compared to S_{23}-PCs and are chemical-free sustainable alternatives to AA-PCs for food applications.

PREFACE

The research reported in this PhD thesis was led by both Prof. Feral Temelli, the principal investigator and Prof. Thava Vasanthan, the co-investigator of the project. This research was financially supported by the Alberta Pulse Growers Commission and the Natural Sciences and Engineering Research Council of Canada (Vanier Canada Graduate Scholarship). This thesis contains original research work carried out by Brasathe Jeganathan under the supervision of Profs. Feral Temelli and Thava Vasanthan, which was written according to the guidelines for the paper-based thesis format of the Faculty of Graduate Studies and Research of the University of Alberta. The thesis comprises nine chapters, and additional work carried out is listed in Annex C. The contributions made by Brasathe Jeganathan and the co-authors to the completion of this work are described here.

Chapter 1 provides a general introduction to the research project and thesis objectives. A version of **Chapter 2** (literature review) of this thesis will be published as Jeganathan, B., Vasanthan, T., & Temelli, F. "Novel mild technologies for the value-added utilisation of faba bean proteins." Brasathe Jeganathan was responsible for writing the original draft of the manuscript based on a thorough literature review on faba bean (*Vicia faba* L.) origin, biology, composition, protein chemistry, fractionation technologies, physicochemical and functional characteristics, and food applications. Feral Temelli and Thava Vasanthan served as advisors and critically reviewed the manuscript.

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https://doi.org/10.1002/cche.10499. Brasathe Jeganathan was responsible for the investigation, methodology, validation, visualization, formal analysis, and writing the original draft of the manuscript. Thava Vasanthan was responsible for conceptualization, supervision, reviewing and editing the manuscript and fund acquisition. Feral Temelli was responsible for conceptualization, project administration, supervision, reviewing and editing the manuscript and fund acquisition.

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Chapter 9 provides overall conclusions and recommendations for future research.

DEDICATION

This thesis is dedicated to my beloved parents Kandiah Jeganathan and Hewadulige Seelawathie Jeganathan, and my husband Samarasinghage Samantha Lankachandra for their love, support, and guidance throughout my academic journey.

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AA	Alkali extraction and acid precipitation
AAS	Amino acid score
ACAPS	Air-currents-assisted particle separation
ALA	Alanine
ARG	Arginine
ASP	Aspartic acid
C	Coarse
CD	Circular dichroism
CYS	Cysteine
D	Dialysis at 4 °C
D_{23}	Dialysis at 23 °C
db	Dry basis
DSC	Differential scanning calorimetry
EDS	Energy-dispersive X-ray spectroscopy
F	Fine
FCW	Fibrous cell wall material
FTIR	Fourier transformed infrared spectroscopy
GAE	Gallic acid equivalent
GLU	Glutamic acid
GLY	Glycine
g	Gravitational force

H	Heat treatment
HIM	Helium ion microscopy
HIS	Histidine
HPLC	High-performance liquid chromatography
HT	High tannin (Athena)
IDF	Insoluble dietary fibre
ILE	Isoleucine
ISO	International organization for standardization
IVPD	*In-vitro* protein digestibility
IVPDCAAS	*In-vitro* protein digestibility corrected amino acid score
LEU	Leucine
LT	Low tannin (Snowbird)
LYS	Lysine
M	Micellization
MET	Methionine
MNP	Micellar non-precipitated protein
MPI	Micellar protein isolate
MWCO	Molecular weight cut-off
P	Proteinaceous material
PB	Protein body
PC	Protein concentrate
PF	Pearling flour
PHE	Phenylalanine

PI	Protein isolate
PSD	Particle size distribution
RFOs	Raffinose family oligosaccharides
RS	Resistant starch
S_{23}	Salt extraction at 23 °C
SC-CO_2	Supercritical carbon dioxide
SDF	Soluble dietary fibre
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SEM	Scanning electron microscopy
SER	Serine
S/F	Solvent/feed ratio
SS	Solubilised starch
TAD	Tangential abrasive dehuller
TAE	Tannic acid equivalent
TDF	Total dietary fibre
THR	Threonine
TIA	Trypsin inhibitory activity
TPP	Total polyphenol
TS	Total starch
TT	Total tannin
TUI	Trypsin units inhibited
TYR	Tyrosine
U	Ultrafiltered and spray dried

VAL	Valine
VP-FESEM	Variable pressure-field emission scanning electron microscopy
w/w	Weight per weight
w/v	Weight per volume
W_{23}	Water extraction at 23 °C
W_{35}	Water extraction at 35 °C
WS_{23}	Extraction with water and 1% $NaCl_{(aq)}$ solution at 23 °C
XRD	X-ray diffraction
ζ	Zeta potential

CHAPTER 1: General introduction and thesis objectives

1.1. GENERAL INTRODUCTION

The global demand for animal-derived protein is expected to double by 2050, with the increasing world population (Boland et al., 2013; FAO, 2009; Henchion et al., 2017). There is a surging demand around the world for alternative plant-based protein sources (Floros et al., 2010), mainly due to the negative environmental impacts of animal-based protein production (Day, 2013; de Boer & Aiking, 2011); health risks associated with high consumption of red and processed meats (Chan et al., 2011; Multari et al., 2015); poor protein quality of staple foods (Acevedo-Pacheco & Serna-Saldívar, 2016; Boye et al., 2010); and the growing demand for vegetarian diets (FAO, 2009; Gandhi & Zhou, 2014). Aligned with the declaration of 2016 as the 'International Year of Pulses' at the 68th General Assembly of the United Nations (UN, 2013) and the protein highway initiative between the USA and Canada, there is more emphasis in North America and the rest of the world to enhance the value-added utilization of pulse proteins. The UN Food Systems Summit held in 2021 has set the stage for global food systems transformation to achieve the Sustainable Development Goals by 2030, which further emphasized the value-added utilization of the nutritional benefits of pulses as part of sustainable global protein supplementation.

Among the pulses, faba bean (*Vicia faba* L.), also known as fava bean or broad bean, is a versatile crop well-adapted to Canadian weather conditions (Multari et al., 2015; Singh et al., 2013). It is a protein-rich (22.4-37.4%), GMO-free, gluten-free, nitrogen-fixing bioresource, which is also a good source of dietary fibre (6.4-8.4%) and bioactive minor compounds (Baginsky et al., 2013; Mayer Labba et al., 2021; Millar et al., 2019; Multari et al., 2015, 2016;

Shi & Nickerson, 2022). Faba beans are low in lipids (<3%) and limiting in sulphur-containing amino acids but high in lysine, which is complementary to cereal-based diets (Bhatty, 1974; Boye et al., 2010; Güzel & Sayar, 2012; Mattila et al., 2018; Tyler et al., 1981). Notwithstanding the positive impact, faba bean is mainly used in the domestic feedstock market and underutilized for human consumption in North America (Khazaei et al., 2021; Multari et al., 2015). Further, improvement is warranted for its use as a food ingredient due to the presence of various bioactives, some of which also have anti-nutritional effects such as tannins, phytic acid, saponins, lectins and trypsin inhibitors (Baginsky et al., 2013; Güzel & Sayar, 2012; Mattila et al., 2018; Multari et al., 2015), which can impede protein digestibility and mineral bioavailability, as well as pyrimidine glycosides, vicine and convicine, which can cause favism upon hydrolysis to their corresponding aglycones, divicine and isouramil (Khazaei et al., 2019; Multari et al., 2016). Faba beans can be broadly classified into low-tannin and high-tannin varieties and the isolation of faba bean protein fractions minimal in the above-mentioned negative factors from both the cultivars, in particular, from the latter is important (Multari et al., 2015) for the value-added utilisation of high-tannin cultivars as techno-functional food ingredients. In addition, the flatulence causing raffinose-family oligosaccharides verbascose, stachyose and raffinose (Güzel & Sayar, 2012; Landry et al., 2016) and off-flavour compounds (Oomah et al., 2013) can limit the use of faba beans as a food ingredient. Although the domestic soaking and cooking methods can significantly reduce and often eliminate the problematic antinutrients (Luo et al., 2009; Multari et al., 2015), it is noteworthy to explore the impact of faba bean protein fractionation methods on the levels of these compounds.

The conventional dry fractionation approaches such as milling and sieving or air classification based on physical properties (i.e., particle size, shape and density) have been

known to result in moderate enrichment in protein content while enhancing the bioactive compounds as well as the anti-nutrients in the resulting protein concentrates (PCs) with more than ~70% protein purity (Saldanha do Carmo et al., 2020; Schutyser et al., 2015). It is important to explore other economically viable dry fractionation techniques as upstream processing steps to separate and concentrate the major faba bean components for successive protein isolation. The optimization of protein fractionation warrants the understanding of the distribution patterns of the macro-components such as starch, protein and dietary fibre within the bean. Micromorphological and elemental information on pulse cotyledon topographies is an essential aspect in identifying grain physiological and structural interactions at cotyledon-seed coat (abaxial) and cotyledon-cotyledon (adaxial) interfaces, which can influence grain quality and processing behaviour in food industry applications; however, such information is lacking in the literature. In addition, the relationship between particle size and inherent physicochemical properties and the resulting techno-functional characteristics of grain flour remains unclear, although, the particle size of flour is a decisive parameter in terms of final product quality. Even in air classification, the cut-off point plays a crucial role, referring to the particle size at which a particle has a 50% chance to move either to the coarse or the fine fraction (Cloutt et al., 1987; Pelgrom et al., 2013).

Alternatively, wet fractionation approaches can be used to obtain protein isolates (PIs) with more than 90% protein content. The pH-shift method, involving alkali extraction followed by acid precipitation, is the most widely used wet fractionation method to isolate legume proteins (Karaca et al., 2011; Langton et al., 2020) based on the impact of pH on protein solubility. The major drawbacks of the conventional wet fractionation methods are the large consumption of water (~87 kg of H_20/kg PI) and energy (~63 MJ/kg protein) as compared to

hybrid fractionation (~35 kg of H_20/kg PI and ~11 MJ/kg protein, respectively), the partial loss of the native techno-functionality of the proteins (Assatory et al., 2019; Berghout et al., 2015; Sun & Arntfield, 2010), and the formation of the undesirable amino acid derivative lysinoalanine (Gould & MacGregor, 1977). Lysinoalanine formed in alkali- and heat-treated soy PI was shown to induce nephrocytomegaly in the pars recta of the proximal tubule in male rats (Karayiannis et al., 1980). Hybrid processing employing a combination of dry and aqueous fractionation has gained attention to enhance protein purity with the expense of less water and energy as compared to wet fractionation alone (Schutyser et al., 2015).

Compared to the conventional pH-shift method, mild fractionation approaches to extract legume proteins are proven to be more resource efficient by using less water and energy and no chemicals (Geerts et al., 2018). Proteins obtained in this manner retain their native properties and exhibit better solubility (Pelgrom et al., 2015). Aqueous salt ($salt_{(aq))}$ extraction is a relatively common method employed to isolate pulse proteins based on the solubility of the major storage proteins (Wang et al., 2010). Nearly 69% to 78% of the total storage proteins of faba beans are salt-soluble globulins, found mainly in the membrane-bound protein bodies (Multari et al., 2015). Consumer demand for clean-label proteins that are low or free of salts necessitates the use of an additional step such as micellar precipitation (Murray et al., 1979) or membrane filtration techniques such as dialysis, diafiltration or ultrafiltration following $salt_{(aq)}$ extraction to remove salts while isolating the proteins (Koros et al., 1996).

The nature of the raw material or the type of the storage proteins present, extraction conditions such as pH, time, temperature, solvent properties and solvent-to-feed (S/F) ratio can influence the physicochemical characteristics (surface hydrophobicity, zeta potential, protein secondary structure conformation changes, thermal properties) of the proteins and the

magnitude to which these characteristics can affect the techno-functional attributes (solubility, hydration, foaming, emulsification and gelation) of the proteins (Jarpa-Parra et al., 2014; Jarpa-Parra, 2018; Karaca et al., 2011; Langton et al., 2020; Ruiz-Ruiz et al., 2012). Thus, following the separation of high-value PIs with the aid of technological advances, it is crucial to fully exploit the physicochemical and techno-functional properties of these PCs/PIs to understand their potential in the development of value-added food products. However, such information is limited for faba bean proteins and, therefore, this PhD thesis research was undertaken with a systematic approach to provide in-depth characterisation starting with the beans to the final proteins as techno-functional ingredients obtained using mild fractionation techniques.

1.2. THESIS OBJECTIVES

The overall objective was to optimize green fractionation approaches to obtain high-value PCs/PIs from faba beans and characterise the impact of these approaches on the compositional, molecular, structural and techno-functional properties of the PCs/PIs to assess their potential for value-added food product applications. To achieve the abovementioned overall objective, it was hypothesized that:

[H1-1] Micromorphological characteristics and the elemental composition of pulse cotyledon abaxial and adaxial surfaces can vary with species and cultivars.

[H1-2] Pearling and air-currents-assisted particle separation (ACAPS) can be used as upstream processing steps in faba bean protein isolation based on the macronutrient (dietary fibre, protein and starch) distribution patterns within faba beans and based on the particle size.

[H1-3] The presence of minerals and changes in solvent/feed ratio, extraction temperature and level of $salt_{(aq)}$ concentration can influence the solubility of proteins in water and thus water alone can be used for faba bean protein extraction.

[H1-4] Water and salt extracted faba bean PCs/PIs by different mild fractionation methods are comparable in their physicochemical and techno-functional properties to those extracted by the conventional pH-shift method.

In order to investigate the above hypotheses, the following specific objectives were addressed in this research. The **specific objectives** were to:

1) Characterise the topographies of the abaxial and adaxial surfaces of faba bean cotyledon in comparison to chickpea, field pea, and lentil cultivars *via* micromorphological and elemental analyses (Chapter 3),
2) Determine the distribution of the macronutrients and ash in faba bean kernels to produce protein-enriched fractions to develop a hybrid fractionation approach to isolate clean-label proteins (Chapter 4),
3) Explore the particle size-based separation of dietary fibre, starch and protein for the hybrid fractionation of clean-label faba bean proteins (Chapter 5),
4) Compare different mild wet fractionation conditions against the pH-shift method to enhance the yield, purity, recovery and quality of the faba bean PCs/PIs (Chapter 6),
5) Characterise the physicochemical attributes of faba bean PCs/PIs obtained using different wet fractionation methods (Chapter 7), and
6) Characterise the functional (techno-functional) properties of faba bean PCs/PIs obtained from different wet fractionation methods (Chapter 8) to assess their potential for different food applications.

CHAPTER 2: Literature review[1]

[1]A version of this chapter will be published: Jeganathan, B., Temelli, F., & Vasanthan, T. Novel mild technologies for the value-added utilisation of faba bean proteins.

2.1. FABA BEAN OVERVIEW

The global demand for animal-derived protein is expected to double (Boland et al., 2013; Henchion et al., 2017) with the increasing world population projected to reach 9.7 billion by 2050 (United Nations, 2019). Although animal husbandry plays a key role to keep up with this demand (Floros et al., 2010), intensified livestock farming will pose an environmental strain in the long run (Boland et al., 2013; Day, 2013; Henchion et al., 2017). Addressing this crisis will necessitate a shift toward planet-friendly high-quality protein sources, which can supply a substantial portion of the global demand (Boland, 2013; Day et al., 2013; Multari et al., 2015).

Broad bean (*Vicia faba* L.), also most commonly known as faba bean, fava bean, horse bean, Windsor bean or tick bean is one of the oldest annual herbaceous legume crops in the world (Bond et al., 1985; Hawtin & Hebblethwaite,1983; Singh et al., 2013). Faba bean is gluten-free and non-GMO, and belongs to the Leguminosae family, which refers to any plant from the Fabaceae family (Allen, 2013). It can be classified as faba major (large broad beans, 1000-2550 g/1000 seeds), faba minor (small tick beans, 150-750 g/1000 seeds) and faba equine (medium horse beans, 450-750 g/1000 seeds) based on seed size (Landry et al., 2016; Pasqualone et al., 2020). While large-seeded beans are generally produced in the Mediterranean countries and are typically used in the form of fresh green vegetable or dry seed; small-seeded beans are often favoured in the United States and Europe and are used as animal feed; and medium-seeded beans are utilised for both human and animal nutrition (Crépon et al., 2010; Duc et al., 2010). However, faba beans are mostly used for animal nutrition as compared to human consumption (Pasqualone et al., 2020).

Faba beans can be classified into two major cultivars, winter-seeded and spring-seeded cultivars (Knott et al., 1990) or into low/zero-tannin and high-tannin cultivars based on their tannin content (CFIA, 2022). Although less is known about its origin, the oldest faba bean seeds were discovered in northwest Syria during prehistoric times, 10,000 B.C. (Multari et al., 2015; Tanno & Wilcox, 2006). In terms of global production, faba beans are the third most valuable feed grain legume, next to soybean and pea (Singh et al., 2013) and the fourth most widely grown cold season pulse crop next to pea, chickpea and lentil (FAO, 2022). Nearly 4.9 million tons of faba beans have been globally produced in 2020 (Sharan et al., 2021). Between 1999 and 2018 faba beans have been cultivated in ~2.5 million ha of land (Merga et al., 2019). Almost 60% of the world's production comes from China followed by other major producers in Northern Europe, Ethiopia, the UK, Australia and France (Semba et al., 2021; Sharan et al., 2021). Faba beans are a relatively new crop in Canada and the first breeding program in Western Canada was initiated in the 1980s at the Crop Development Centre, University of Saskatchewan. Faba bean agronomic activities are fast growing in Alberta in the recent past aligned with the declaration of the International Year of Pulses in 2016 by the United Nations (UN, 2013). The low-tannin cv. Snowbird accounts for almost 89% of the cultivated land in Alberta in 2021, while other high-tannin cultivars (Mallik, Fabelle, Taboar, and CDC SSNS-a) contribute to less than 8% of the cultivated land (Friesen, 2021). Faba bean cultivation in Western Canada is successful due to the plant's adaptation to both wet and cold climate settings (Heneiquez et al., 2017).

Faba beans can withstand harsh winter temperatures through their physiological mechanisms such as the accumulation of free proline and desaturation of membrane-bound fatty acids (Link et al., 2010) as well as decreasing oleic acid while increasing linolenic acid

in leaves and stems (Arbaoui & Link 2008). The most prevalent disease in faba beans is the faba bean rut caused by a fungus that invades the leaf and develops a fusiform vesicle (Emeran et al., 2005). In general, infection with faba bean rust occurs by the second half of the growing season, when pods are mature. If rust epidemics begin early in the season, crop production can be severely impacted (Lapwood et al., 1984). In addition, "chocolate spot disease" can be caused by the development of dark brown blotches by the spores of the common necrotrophic fungus (Villegas-Fernandez et al., 2012). Agricultural management practices such as crop rotation, fertilization, weed management and development of resistant faba bean varieties are essential to mitigate/control pests and disease (Stoddard et al., 2010).

Faba beans are well-adapted to the climate change scenario and a wide array of soil types and pH levels due to their versatile adaptability to thrive under extreme climatic conditions through thick and long tap root systems with profusely branched numerous lateral roots (Multari et al., 2015; Singh et al., 2013). They can solubilize insoluble phosphorus and boost microbial activity in the soil, thus improving the soil's microbiome (Sharan et al., 2021). The cultivation of faba beans has the environmental benefit of a moderate amount of water demand and symbiotic fixation of more atmospheric nitrogen (up to 100 to 200 kg N/ha) than any other legume species under similar soil conditions (Singh et al., 2013). This allows them to be used as cover crops in intensive cereal-dominated crop rotation (Kopke & Nemecek, 2010). Environmental factors play a crucial role in the growth and development of faba beans, and any changes in these factors can have a significant impact on the crop's productivity and yield (Khazaei et al., 2021).

2.2. FABA BEAN COMPOSITION

2.2.1. Nutritional compounds

Faba bean seeds are an excellent source of protein (20-43%, N×6.25) created from a broad range of essential amino acids, including lysine (19.8-74.8 mg/g protein), which is complementary (Ayala-Rodriguez et al., 2022; Mayer-Labba et al., 2021; Millar et al., 2019; Multari et al., 2016) in enhancing the poor protein quality of the cereal-based staple foods (Mattila et al., 2018). They are carbohydrate-rich pulses (51–68%), containing starch as the major carbohydrate (22–45%) (Hoover & Sosulski, 1991). They are also a good source of insoluble fibre (9-25% w/w) (Multari et al., 2016) with only trace levels of soluble fibre (0.6-1.1%) (Mayer-Labba et al., 2002). Faba beans are low in fat (<3%) but rich in minerals and vitamins (Multari et al., 2015 & 2016), in addition to the beneficial polyphenolic antioxidants (Barakat et al., 2017; Turco et al., 2016). The presence of L-3,4-dihydroxyphenylalanine (L-DOPA) has been reported in faba beans, which is used as a drug to cure Parkinson's disease (Multari et al., 2015; Singh et al., 2013).

2.2.2. Bioactive and anti-nutritional compounds

Faba beans are produced on a large scale in many parts of the world; however, from a dietary standpoint, they are underutilized as a food ingredient due to the presence of several anti-nutritional, off-flavour and flatulence-causing compounds (Millar et al., 2019; Multari et al., 2015; Oomah et al., 2013). While the term "anti-nutrient" is commonly used to describe compounds found in plant foods that may reduce the bioavailability or absorption of certain nutrients, it is important to note that some of these compounds can also have positive effects on health. As a result, these compounds can also be referred to as "bioactive compounds".

While the term "anti-nutrient" may be technically accurate in some cases, it is important to recognize that these compounds can have both negative and positive effects on health and that alternative terms, such as "bioactive compounds," may be more appropriate in certain contexts (Popova & Mihaylova, 2019).

Raw faba beans contain enzyme inhibitors (trypsin inhibitors and chymotrypsin inhibitors) and bioactive compounds, some of which may have anti-nutritional effects, such as tannins, phytic acid, lectins and saponins that can impede protein digestibility and mineral bioavailability (Mayer Labba et al., 2021; Millar et al., 2019; Multari et al., 2015; Oomah et al., 2010; Wang et al., 1998); beany flavour compounds that can produce off-flavours (Oomah et al., 2013); raffinose family oligosaccharides (RFOs) such as raffinose, stachyose and verbascose that can cause flatulence (Güzel & Sayar, 2012; Landry et al., 2016); and pyrimidine glycosides, vicine and convicine, which can cause favism in susceptible individuals when hydrolysed to their corresponding aglycones, divicine and isouramil, (Khazaei et al., 2019). However, numerous processing techniques are effective in reducing or eliminating these undesirable components, which can significantly improve the food value of faba beans (Luo et al., 2009; Multari et al., 2015). Only a few *in-vivo* studies have been done to evaluate the impact of anti-nutrients on protein digestibility.

While anti-nutrients found in faba beans have traditionally been viewed as negative, recent research has suggested that they may also have potential health benefits. Tannins, lectins, phytic acid, saponins, vicine, convicine, and other anti-nutrients may have antioxidant, anti-inflammatory, and anti-cancer properties, and may reduce the risk of chronic diseases such as cancer and cardiovascular disease (Multari et al., 2015). Further research is needed to fully understand the health benefits of these compounds and their mechanisms of action.

2.2.2.1. Phytic acid

Phytic acid, also known as phytate when bound to a mineral represents 60–90% of the phosphorus content reckoned to be stored in the protein bodies (Multari et al., 2015; Schlemmer et al., 2009; Zhawar et al., 2011;). Production of phytates takes place during the seed development of grains and it serves as a source of energy and antioxidant for seed germination (Petroski & Minich, 2020). Phytates possess a strong chelating potential and can form complexes with cations such as calcium, potassium, magnesium, zinc, and iron, thereby reducing the bioavailability of these minerals (Boncompagni et al., 2018). This could be beneficial due to the antioxidant potential resulting from the chelating effect of iron and copper as well as the reduced bioavailability of toxic heavy metals (Campos-Vega et al., 2010; Multari et al., 2015).

Phytic acid is often considered an anti-nutrient because of its strong affinity to minerals, protein, and starch, which can negatively impact their digestibility. The phytic acid concentration of faba bean seeds varies with the cultivar but accounts for only ~1% of the seed's dry weight (Multari et al., 2015). Low doses of gamma radiation can considerably reduce phytic acid levels, whereas domestic treatments have more unpredictable effects (Osman et al., 2014). Soaking and dehulling can lower the phytate content; however, heating the soaked seeds can significantly eliminate phytates (Sharma and Sehgal, 1992). Recently, efficient genetic modification approaches have been developed to lower the phytic acid levels of certain crops (Zhawar et al., 2011).

2.2.2.2. Saponins

Saponins are composed of steroidal or triterpene aglycones that are ester and ether-linked mono- or oligosaccharide moieties (Rochfort & Panozzo, 2007). Similar to other legumes, saponins in faba beans feature a triterpene nucleus (Cheeke, 1971). Saponin content varies according to plant age, plant part, cultivar, irrigation conditions, climate, and soil type (Shi et al., 2004). In the past, the potential positive effects of saponins (anti-cancer, anti-inflammatory, and antioxidant properties) were mainly disregarded because of their harmful effects on animal performance. Although they have not been utilized for food purposes due to their harmful effects, there is evidence that they may also have positive benefits on human health (Makkar et al., 1999; Multari et al., 2015). In some types of faba beans, cooking treatments do not eliminate saponins, but they can reduce their concentration. Soaking reduces the level of saponins by 24-81%, germination of the seeds for 24 h reduces the concentration by approximately 30%, and longer periods can result in greater saponin reduction (Patterson et al., 2017; Rochfort & Panozzo 2007; Sharma & Sehgal 1992).

2.2.2.3. Lectins

Lectins are widespread throughout the plant kingdom and can be found in faba beans (Liener, 1974). As they agglutinate red blood cells, they are also known as hemagglutinins or phytoagglutinins (Liener, 1962). Lectins are proteins and glycoproteins, containing between 4% and 10% carbohydrates and makeup between 2% and 10% of the total protein fraction in the majority of legume seeds (Gupta, 1987). They reversibly attach to particular sugars and glycoproteins on the surface of gut wall cells, thus interfering with nutritional digestion and absorption (De Meja & Prisecaru, 2005). However, some studies have suggested that lectins

may have anti-cancer and anti-inflammatory properties. In particular, lectins in chickpeas have been shown to inhibit the growth of cancer cells and reduce inflammation in animal studies (Gupta et al., 2018). Germination is an effective way to drastically reduce lectin levels, but lectins were present even after 48 h of germination (Sharma & Sehgal, 1992). Lectins are thermosensitive; therefore, autoclaving dehulled seeds for 25 min can eradicate them (Sharma & Sehgal, 1992). As a result, lectins have no practical effect on the nutritional value of the food products in which they are present, as they rarely survive food processing.

2.2.2.4. Tannins

Tannins are one of the most prevalent anti-nutritional components of faba beans, which are coming under the category of polyphenols with molecular weight higher than 500 Da (Samtiya et al., 2020). They are chemically assorted into two major categories; hydrolyzable tannins such as ellagitannins, gallotannins and condensed tannins like epicatechin, catechin, epicatechin-3-gallate, epigallocatechin and epigallocatechin-3-gallate (Filho et al., 2017). Moreover, condensed tannins are the most abundantly found plant-based polyphenols in faba beans (Multari et al., 2015). Tannins can form insoluble complexes by binding with proteins and starch-like macromolecules, thereby causing an adverse effect on biological activities (Filho et al., 2017). Furthermore, condensed tannins are known to exert undesirable impacts on the function of digestive enzymes, the bioavailability of nutrients, mineral absorption and protein digestion (Mohapatra et al., 2019). However, tannins may also have potential health benefits, including antioxidant and anti-inflammatory properties and some studies have shown that tannins can reduce the risk of chronic diseases such as cardiovascular disease and cancer (Multari et al., 2015; Wang et al., 1998).

Tannin composition differs significantly amongst standard genotypes of faba beans. The average condensed tannin concentration of conventionally produced faba beans in Europe is between 5 and 10 g/kg dry matter (Vilarino et al., 2009), which are the most abundant phenolic components in faba beans. As they are primarily found in the seed hulls (Helsper et al., 1993), dehulling results in their significant reduction. Tannin concentration is significantly decreased by gamma irradiation and/or heat treatment (Osman et al., 2014).

Tannin molecules operate as chelating agents and form stable complexes with several metal ions, resulting in a decrease in their gastrointestinal absorption and, consequently, nutritional deficits (McDonald et al., 1996; Mila et al., 1996). Tannin-free faba bean species are accessible and simple to obtain through selective breeding. Tannin deficiency is a heritable characteristic that can be selected since it correlates with the white hue of the flower. As tannins inhibit the growth of infectious agents the nutritional value of these cultivars is improved, although they are more susceptible to biotic stress (Cabrera & Martin 1989).

2.3. FABA BEAN PROTEIN QUALITY

Faba beans can produce high levels of protein since they are involved in symbiotic nitrogen fixation (Allen, 2013). The protein content of legume seeds is influenced by both genotype and environment and can vary between variants of a particular species and even between seeds on a single plant (Multari et al., 2015). Similar to other Leguminosae family plants, faba beans accumulate large quantities of proteins during seed development (Duranti, 2006). The primary storage proteins in faba bean seeds are globulins, which are located in the membrane-bound organelles known as protein bodies. These proteins, which survive desiccation during maturation and hydrolysis during germination, deliver ammonia and carbon

skeletons to the developing seedlings (Duranti & Gius, 1997). Enzymatic, regulatory, and structural proteins are present for normal cellular processes, including the synthesis of storage proteins (Millar et al., 2019; Multari et al., 2015).

Globulins are soluble in dilute salt solutions but insoluble in water (Multari et al., 2015; Osborne, 1924). Globulins account for around 70% to 79% of the total seed proteins and are often rich in aspartic and glutamic acids, leucine, and arginine (El Fiel et al., 2002). Globulin levels are followed by albumins (2-20%), glutelins (1-11.5%) and prolamins (2.7-3.5%) (El Fiel et al., 2002). The proportion of the distinct protein fractions in faba beans has been extensively investigated in the past and may vary based on the cultivar, fertilization, growth season, investigation method, and other growth condition-related variables (Multari et al., 2015).

Faba bean globulins consist of two high-molecular-weight proteins, legumin and vicilin, identified by the analytical centrifugation as 11S (300-400 kDa) legumin and 7S (158-180 kDa) vicilin (Wright & Boulter, 1973), respectively. Vicilin is created in the developing seed before legumin (Graham & Gunning, 1970), but legumin (40-45% of the protein) is synthesized at a quicker rate than vicilin (20-25% of the protein) and hence becomes dominant in the mature faba bean seeds (Gueguen & Cerletti, 1994). 11S globulins are hexameric proteins with an approximate molecular weight of 340 kDa made up of six monomers (60 kDa each) connected by the disulphide linkages of cysteine and methionine; whereas, 7S vicilin is a trimeric protein with an approximate molecular weight of 158 kDa made up of three monomers (50-60 kDa each) bound together by hydrophobic interactions (Graham & Gunning, 1970; Multari et al., 2015; Wright & Boulter, 1973). Although convicilin (220-290 kDa) is also

present in faba bean proteins to a minimal extent, whether it is a vicilin subunit or else a separate class of globulin remains a question (Warsame et al., 2020).

In terms of protein quality, as defined by the appropriate proportion of amino acids required by humans, legume proteins lack sulfur-containing amino acids and cysteine and methionine as well as tryptophan but are high in lysine. Cereals, on the other hand, are comparatively deficient in lysine; hence, the addition of legumes to cereals can frequently increase the overall protein quality of mixed diets (Acevedo-Pacheco & Serna-Saldívar, 2016). On the other hand, the presence of enzyme inhibitors can limit the digestibility of proteins by inhibiting the proteolytic enzymes trypsin and chymotrypsin (Gueguen, 1983). The inhibition of these enzymes reduces protein digestibility and, consequently, the ability of the gut to absorb amino acids. Many of these inhibitors are proteinaceous, so cooking, heating, fermentation, and, in some circumstances, germination can deactivate and considerably reduce their inhibitory activity (Multari et al., 2015).

2.4. FABA BEAN FRACTIONATION

The demand for plant-based products is steadily growing with the increasing global population (Boland et al., 2013; De Boer & Aiking, 2011; Multari et al., 2015). This demand has created an imperative shift to plant-based alternative products, especially in the Western world, inflating the popularity of non-GMO pulse grains as a sustainable source of protein as well as dietary fibre (Boland et al., 2013; Henchion et al., 2017; Mayer Labba et al., 2021). Faba bean production is projected to increase over the next decade in Western Canada, with a considerable amount used for functional food ingredient processing as compared to the traditional food and feed markets (Khazaei et al., 2021). However, the presence of flatulence-

causing oligosaccharides and anti-nutritional factors can diminish their food value (Landry et al., 2016; Multari et al., 2015). Therefore, the isolation of faba bean protein fractions free of these negative factors is warranted for their value-added utilisation as functional food ingredients. In addition, the substantially high carbohydrate content can strongly impact the nutritional, functional and sensory attributes of the food products formulated with the incorporation of faba beans (Schutyser et al., 2015). These reasons necessitate making the most of this opportunity, by selling the world more than just raw faba beans (Multari et al., 2015, 2016). The fractionation of faba beans is an essential step needed before their utilisation in value-added food applications (Schutyser et al., 2015).

Conventional pulse protein separation methods can be broadly classified into dry and wet fractionation methods (Assatory et al., 2019; Pelgrom et al., 2013; Schutyser & van der Goot, 2011). Dry fractionation depends on the size, shape and density of the milled particles while the extractability of proteins as well as the changes in their physicochemical and functional characteristics in wet fractionation depend on the nature of the raw material or the type of the storage proteins present and the extraction conditions such as pH, time, temperature, solvent properties and solvent/feed (S/F) ratio (Jarpa-Parra et al., 2014; Jarpa-Parra, 2018; Karaca et al., 2011; Langton et al., 2020; Ruiz-Ruiz et al., 2012). A meta-analysis of two main dry fractionation methods (air classification and electrostatic separation), as well as alkaline extraction based on 41 research studies, showed that the processing conditions affect the purity and recovery of proteins (Allotey et al., 2022). Dry fractionation techniques can yield protein fractions with 60-90% purity, while wet fractionation can result in a protein content above 90% (Boye et al., 2010), depending on the raw material. By definition, protein concentrates (PCs) contain more than ~70% protein (Saldanha do Carmo et al., 2020; Schutyser et al., 2015), while

protein isolates (PIs) should have more than 90% protein purity. However, in the food industry, a protein fraction with a 50-70% protein content can be termed as a PC and a protein fraction with a protein purity above 80% can be referred to as a protein isolate.

2.4.1. Dry fractionation

2.4.1.1. Milling

The milling process of pulses can involve two stages: decortication followed by the splitting of beans to produce split seeds (Wood et al., 2017) and/or the particle size reduction of beans by grinding (Scanlon et al., 2018). Unlike the albuminous cereals, faba beans are non-endospermic (Fig. 2.1) dicotyledons (Anderson, 1949). Structural modification of the endosperm cells with the development of the embryo is associated with the formation of an electron-dense substance closely resembling phenolics. Its association with the polysaccharide-rich wall material leads to the formation of a cementing layer between the phenolic-rich inner surface of the seed coat and the endosperm wall (Yeung, 1990), which can influence the milling behaviour of the beans, depending on whether they are easy- or difficult-to-mill beans (Wood et al., 2011, 2014a).

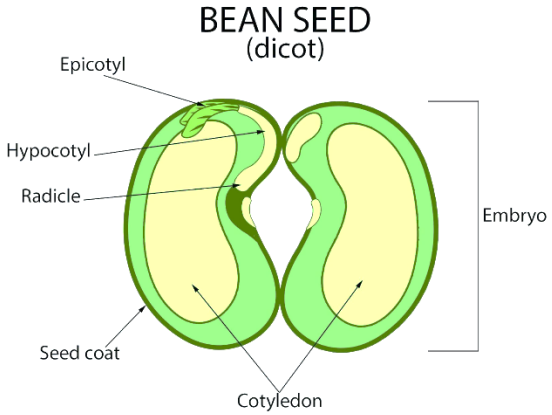

Fig. 2.1. Cross-sectional morphological characteristics of faba bean cotyledon structure (Adapted from Kadam et al., 1989 and redrawn).

Decortication separates the seed coat from cotyledons, which can improve the economic value, sensory quality, cooking time and digestibility of the seeds (Kumar et al., 2022; Saldanha do Carmo et al., 2020; Singh et al., 2013). Dehulling can be achieved by either abrasive dehullers (Wood et al., 2014a, 2014b) for hulls that are tightly attached or attribution-type dehullers for lightly attached hulls (Fernando, 2021). Dehulling facilitates the reduction of anti-nutritional factors abundantly present in the seed coat (Saldanha do Carmo et al., 2020). Soaking and conditioning with water or oil, enzyme or chemical or hydrothermal treatments, micronization and partial germination have been reported as pre-treatments to facilitate decortication (Scanlon et al., 2018) by loosening the adhesion between the seed coat and cotyledon to lessen the breakage of the seed coat and to increase flour quality (Fernando, 2021; Wood et al., 2011). Soaking involves exposing the seed to excess water while conditioning/tempering is the addition of the prescribed amount of water needed to increase moisture content to the desired level for the easy removal of the seed coat during dehulling. Edible oils can also be added, which can penetrate through the pitted seed coat and loosen it from the cotyledon (Opoku et al., 2003) or else dissolve the gums or help weaken the bonds between the seed coat and cotyledon (Tiwari et al., 2007). Dehulling did not significantly improve the techno-functional properties of fine and coarse fractions of faba bean flours according to Saldanha do Carmo et al. (2020), while it did improve according to Fernando (2021).

Dehulling efficiency, split yield, and percentage loss have been used to assess the effectiveness of pulse milling. Dry fractionation mainly depends on the ability of the milling/grinding step to mechanically disengage protein bodies and other cellular compounds such as starch and dietary fibre components. In starch-rich legumes, the cotyledon cells consist

of starch granules (~20 μm) embedded in a matrix of protein bodies (1-3 μm) that are surrounded by a fibre-rich cell wall (Schutyser et al., 2015). The milling treatment needs to be carefully chosen to break up the cotyledon into a suitable particle size without severely damaging starch granules. While pin milling or fine grinding can disengage the starch granules from the protein matrix and disintegrate the constituents into fine particles, extreme fine milling can lead to starch damage and fragments of starch granules similar in size to that of protein bodies, making their separation from protein more challenging (Schutyser et al., 2015). On the other hand, coarse milling can lead to large aggregates of protein bodies, starch granules and cell wall components that can make fractionation more demanding (Pelgrom et al., 2013; Saldanha do Carmo et al., 2020).

2.4.2.2. Pearling

Dehulling and pearling processes can be achieved by abrading the external layers of the grains with general-purpose tangential abrasive dehullers (Yeung & Vasanthan, 2001). While dehulling is associated with the removal of the inedible layers, pearling is associated with the gradual removal of the kernel tissues from the outer to inner layers (Blandino et al., 2015; Liu et al., 2009). Staple grains including cereals, oilseeds, and pulses are good sources of health-beneficial bioactive compounds mainly β-glucan, bioactive peptides, polyphenols, vitamins, minerals, phytosterols and phytoestrogens (Chaturvedi et al., 2011), which are playing a key role in the nutraceutical/pharmaceutical/functional food markets (Augustin & Sanguansri, 2015). Pearling has been widely accepted, especially for cereals (Oomah et al., 1981; Sahay, 1990) to obtain functional food ingredients based on the distribution of nutrients, mainly in barley (Blandino et al., 2015; Liu et al., 2009) and wheat (Hemery et al., 2007; Sovrani et al.,

2012) kernels. Pearling has also been used to remove the saponin-rich outer layers of quinoa (Mattila et al., 2018). However, pearling has been less employed for legumes (Oomah et al., 2010; Opoku et al., 2003; Reichert et al., 1984; Sahay, 1990), except for the exploration of the component distribution of dehulled whole and split chickpeas (Wood et al., 2014b) and the protein and lipid profiles of whole peas (Kosson et al., 1994). Unlike cereals, pulses can split during the dehulling process, which leads to the pearling of both whole and split seeds unless separated (Wood et al., 2014b). However, the morphological characteristics of the cotyledon-cotyledon interface of the bean need to be known for a better understanding of the pearling process.

2.4.2.3. Air classification

Smaller protein-rich fragments from the larger starch granules and fibre-rich fragments are typically separated by milling and air classification of starch-rich legume seeds, which can only lead to relatively modest enrichment in protein content (Schutyser et al., 2015; Schutyser & van der Goot, 2011). Rotor classifiers are mostly utilized for the air classification of finely milled flours. The flour is dispersed and taken into the air classifier chamber by a large airflow into a conical vessel at the top with a rotating classifier wheel containing blades (Schutyser et al., 2015). These blades form a partition zone in which the small and large particles are separated. The drag forces, created by the airflow, and centrifugal forces created by the classifier wheel, together dictates the size of the particles that end up in the fine fraction. Particles on which the drag force exceeds the centrifugal force may pass through the openings in the wheel, and enter the fine fraction (Pelgrom et al., 2013; Schutyser et al., 2015).

Although the relationship between particle size and inherent physicochemical properties, and the resulting functional characteristics of grain flour remains unclear, the particle size of flour is a decisive parameter in terms of the final product quality in air classification (Ahmed et al., 2016). The cut-off point referring to the particle size at which a particle has a 50% chance to move either to the coarse or the fine fraction plays a crucial role in the level of separation that can be achieved with air classification (Cloutt et al., 1987; Pelgrom et al., 2013).

Air classification depends on the differences in the flour particles' size and density to produce a coarse fraction, rich in starch and a fine fraction, rich in protein (Assatory et al., 2019; Scanlon et al., 2018). The particulate and discrete nature of the protein matrix and the large and fairly uniform starch granules (~25-40 mm) in pulse flour as compared to the continuous matrix in cereals containing mixed small- and medium-sized starch (A-, B- and C-type) granules (Vose, 1980) makes the separation of pulse protein (49-70 g protein/100 g dry matter) more efficient by air classification (Cloutt et al., 1987; Pelgrom et al., 2011; Tyler, 1981). Starch granule size mainly determines the potential for dry separation and optimal separation is achievable when the particle size distribution curve of flour and starch granules overlap maximally, with the protein bodies being smaller particles. Faba beans contain large starch granules and can therefore be well separated through air classification (Schutyser et al., 2015). The presence of oil can impair powder dispersibility, thus oilseeds are less suitable for dry fractionation (Sosulski & Youngs, 1979).

Even though air classification can separate the macro-components, the presence of impurities may interfere with the product applications of the fractions enriched in starch and protein (Vasanthan & Bhatty, 1995). However, the protein fractions contain bioactive

compounds at a higher level as compared to the protein fractions obtained *via* wet separation techniques (Saldanha do Carmo et al., 2020).

2.4.2.4. Sieving and air-currents-assisted particle separation

Sieving is the most commonly used and economical dry processing method alternative to air classification. A stack of sieves with decreasing mesh sizes to the top or single sieving steps with increasing mesh sizes can be used. Sieving technology typically employs sieves with openings as small as 100 μm to separate milled particulates based on their particle size. When fine sieves with openings of 100 μm or less are used, clogging tends to occur and this requires slowing down the feeding rate, leading to less throughput. This causes losses in separation efficiency, and with some plant materials, it becomes impossible to continue the sieving operation (Pelgrom et al., 2013; Saldanha do Carmo et al., 2020; Schutyser et al., 2015).

Air-currents-assisted particle separation (ACAPS) is a low-cost patented technology that involves the application of vacuum and high-pressure air pulsing to generate air currents in a high-throughput sieving apparatus (Vasanthan, 2017). These dynamic air currents have enhanced the concentration of barley β-glucan in the coarse fraction retained on a sieve for various food applications, while starch gets concentrated in the fine fraction, with the potential to be transformed into bioethanol (Lu et al., 2020). The applicability of ACAPS technology in pulse fractionation is still not fully explored.

2.4.2.4. Triboelectric separation

Triboelectric separation is an evolving technology mainly used in the mining industry to separate silica from coal (Dwari et al., 2015), as well as in the plastic and pharmaceutical

industries (Landauer & Foerst, 2018; Tabtabaei et al., 2017). It has also been used in the food industry to remove tea stems from leaves (Higashiyama & Asano, 1998) and to separate aleurone and pericarp fractions of wheat bran (Chen et al., 2014). Triboelectric separation can be used to fractionate ground cereals (Hemery et al., 2007; Landauer & Forest, 2018), enrich functional ingredients in rice (Wang et al., 2016), and separate legume nutrients from navy beans (Tabtabaei et al., 2016a, 2016b, 2017) and lupin (Wang et al., 2016).

The triboelectric separation process involves subsequent triboelectrification either by electron or ion transfer followed by the ability of the particles to charge under electric forces (Tabtabaei et al., 2017). Electrification of the particles can be achieved by passing the particles through pneumatic beds where two particles are brought into contact to impart a surface charge to the particles through the transfer of electrons from the surface of one particle to another (Schutyser & van der Goot, 2011; Tabtabaei et al., 2017). The rubbing action of two materials even during impact milling can lead to the exchange of charge as a result of contact or frictional charging (Yang et al., 2022). Although the mechanism behind charge transfer is still under speculation (Yang et al., 2022), many factors including physical, chemical, electrical and environmental characteristics of the material could impact contact charging (Liu et al., 2018). Landauer and Foerst (2018) found that increasing the field strength to 66 kV/m was essential to achieve a maximum of 80% protein purity on the cathode and 90% starch purity on the anode. Although the differences in the particle size facilitate the separation of the protein (~5 μm) from starch (10-40 μm) during air classification (Pelgrom et al., 2015), according to Landauer and Forest (2018), it was possible to separate protein and starch from a very fine particle mixture within the same order of magnitude in size (10 μm) and density using

triboelectric separation. There is potential to make use of the triboelectric separation technique to concentrate faba bean protein, but further research is needed.

2.4.2. Wet fractionation

The conventional wet fractionation method targeting protein isolation includes conventional alkaline extraction (pH 9-10) followed by acid precipitation (AA), which also consumes large amounts of water and energy and often leads to denaturation of the protein (Ruiz-Ruiz et al., 2012; Ruiz et al., 2016). A milder extraction method would involve the use of dilute $salt_{(aq)}$ solution, targeting the solubilization of globulin proteins (Wang et al., 2010).

Following extraction, the isolation of proteins from the extract can be achieved by isoelectric precipitation, heat treatment, micellization, ultracentrifugation, dialysis or diafiltration/ultrafiltration (Boye et al., 2010; Langton et al., 2020; Singhal et al., 2016). Faba bean PIs are conventionally obtained by wet fractionation techniques for incorporation into food product formulations to improve their sensory and nutritional quality (Sharan et al., 2021). Based on Osborne's protein solubility-based classification (Osborne, 1924), more than 70% of faba bean proteins are globulins, which are soluble in dilute salt solutions and are also soluble in dilute alkaline solutions (Multari et al., 2015).

2.4.2.1. Alkaline extraction followed by isoelectric precipitation

The pH-shift method of alkali extraction followed by acid precipitation (AA) is the most widely used wet fractionation method to isolate (~90% purity) legume proteins (Langton et al., 2020; McCurdy & Knipfel, 1990) based on the impact of pH on the solubility of proteins. The proteins can gain negative charges in alkaline solutions and become more soluble due to

electrostatic repulsive forces, which enhances the protein dispersion stability. However, at their isoelectric point, which is between pH 4 and 5.5 for most of the pulse proteins, the net surface charge becomes zero, leading to their precipitation (Boye et al., 2010). Following the dispersion of plant proteins at a solvent/feed (S/F) ratio of 5 or 10 and at the desired alkaline pH, a centrifugation step is essential to remove the insoluble fraction mainly consisting of carbohydrates (Boye et al., 2010; Schutyser et al., 2015; Shevkani et al., 2019). Following the removal of the insoluble fraction, the pH is adjusted to the isoelectric point, during which the proteins unfold, aggregate and precipitate due to the reduction of their surface charge.

Although defatting can significantly improve the separation efficiency of soy proteins, defatting is not a common practice in the isolation of faba bean proteins as their lipid levels are less than 5% (Multari et al., 2015). However, when defatted faba bean flour was extracted at pH 10.5 and precipitated at their isoelectric point 4.0, a 92% pure protein isolate with a high oil-binding capacity was produced (Vioque et al., 2012). Flink and Christiansen (1973) isolated faba bean proteins using an S/F ratio of 5 for alkaline extraction at pH 8-10 followed by isoelectric precipitation at 3.5 at room temperature. However, McCurdy and Knipfel (1990) generated faba bean protein fractions of varying protein purity (76.4-94.0%) following the same principle. Although the S/F ratio was the same, the extraction pH (7-10), temperature and isoelectric point (4-5.3) were different. Depending on the extraction method, the purity, yield as well as physicochemical and functional properties of the proteins changed significantly. Both Singhal et al. (2016) and Karaca et al. (2011) followed the same extraction steps but ended up with 76.6% and 84.1% protein purities, respectively.

The major drawbacks of the conventional wet fractionation methods are the large consumption of water (~87 kg of H_20/kg protein isolate) and energy (~63 MJ/kg protein) as

compared to hybrid fractionation (~35 kg of H_20/kg protein isolate and ~11 MJ/kg protein, respectively), the partial loss of the native functionality of the proteins (Assatory et al., 2019; Berghout et al., 2015; Sun & Arntfield, 2010), and the formation of the undesirable amino acid derivative lysinoalanine (Gould & MacGregor, 1977). Lysinoalanine formed in alkali- and heat-treated soy protein isolates were shown to induce nephrocytomegaly in the pars recta of the proximal tubule in male rats (Karayiannis et al., 1980).

Efficient mild wet processing methods free of alkali and thermal treatments should be explored to cater to the consumer demand for clean-label food ingredients. Alongside purity, the focus should also be placed on the processing conditions, which can influence the physicochemical properties that ultimately dictate the functionality of the proteins.

2.4.2.2. Salt extraction

Compared to the conventional pH-shift method, gentle fractionation approaches to extract legume proteins are proven to be more resource efficient by using less water and energy and no chemicals (Geerts et al., 2018). Proteins obtained in this manner retain their native properties and exhibit better solubility (Pelgrom et al., 2015). Aqueous salt ($salt_{(aq)}$) extraction is a relatively common wet fractionation method employed to isolate pulse proteins based on the solubility of the major storage proteins (Wang et al., 2010). Nearly 69-78% of the total storage proteins of faba beans are salt-soluble globulins, found mainly in the membrane-bound protein bodies (Multari et al., 2015).

The majority of the hydrophobic moieties are buried inside the quaternary and tertiary structures and most of the hydrophilic groups remain on the surface, which are involved in protein-water interactions (Jarpa-Parra et al., 2014; Singhal et al., 2016). When the ionic

strength of a solution is low at low salt concentration, the hydrophilic groups on the surface interact with the surrounding water molecules to produce a hydration layer around the protein. This "salting-in" effect happens at low salt concentrations (e.g., 0.1 M NaCl) and increases the protein-water interactions, resulting in increased protein solubility. This method can be used to solubilize both albumins and globulins and then the insoluble fraction is separated by centrifugation (Ahmed et al., 2012). Karaca et al. (2011) reported that the salt-extracted faba bean PIs had a lower protein content (81.98%) than the AA-PIs (84.14%). A comparable approach employed by Bhatty and Christison (1984) on lentil, pea, and faba beans resulted in isolates containing 87%, 91%, and 95% protein, respectively. Salt-extracted proteins underwent less denaturation compared to AA proteins resulting in PIs with improved functional properties (Paredes-López et al., 1991; Sun & Arntfield, 2010).

In contrast, when a high concentration of salt is present in the extraction solution, the ions compete with the proteins for water, and a process known as "salting-out" occurs, which disrupts the hydration layer, promotes hydrophobic protein-protein interactions and reduces solubility (Singh et al., 2013). In consequence, the proteins clump and precipitate from the solution. According to Hofmeister (1888), polyvalent salts (e.g., ammonium sulfate) are more successful in “salting-out” than univalent salts (e.g., NaCl) because they may attain higher salt concentrations (e.g., 4.1 M) while remaining soluble. Afterwards, the salt can be removed by dialysis, where the proteins respond to the change in ionic strength by forming micelles, which are then retrieved (Boye et al., 2010a).

2.4.2.3. Dialysis and micellar precipitation

Dialysis is clarifying step in which the salts can be removed *via* a semipermeable membrane (Karaca et al., 2011). The dialysis membrane allows water and salt to diffuse out of the bag while retaining bigger protein particles inside until gradient equilibrium is reached (Andrew et al., 2001). During micellization, a large amount of cold water is added to the supernatant, which significantly reduces the ionic strength and promotes protein attachment through hydrophobic contacts. The proteins go through a sequence of dissociation steps to adapt to the new environment (Murray et al., 1979). These steps would then lead to the formation of protein micelles resulting from the accumulation of amphiphilic proteins, which grow in size and number until precipitation occurs (Murray et al., 1979; Shi et al., 2021) and are recovered by centrifugation or microfiltration (Sun & Arntfield, 2010). Paredes-López et al. (1991) showed significantly better solubility at pH 7.0 for chickpea PIs generated by micellization (72.5%) than that by the pH-shift method (60.4%). It is noteworthy that globulins are predominantly captured in the pH-shift method, whereas during salt extraction both globulins and albumins are extracted (Langton et al., 2020; Shi et al., 2021).

2.4.2.4. Ultrafiltration/diafiltration

Ultrafiltration (UF) is a membrane filtration method used with pressure as an alternative to the most commonly used isoelectric precipitation (Boye et al., 2010; Singhal et al., 2016). This method separates proteins according to their molecular weight, which can range from 1,000 to 100,000 kDa, with many molecular weight cut-off membranes available to retain proteins of interest. Diafiltration (DF) is frequently used with UF to enhance product recovery and purity, where water is introduced to dilute the retentate (Singhal et al., 2016). Vose (1980)

observed that UF-processed faba bean PI had 94.1% protein, which was higher than that of pea PI (89.0%). UF/DF yields proteins with increased functionality and reduced levels of anti-nutritional factors, such as protease and amylase inhibitors, lectins, and polyphenols (Fredrikson et al., 2001; Shi et al., 2021).

2.4.3. Hybrid fractionation

For some applications where protein purity is deemed pivotal, hybrid processes involving successive dry and wet separation methods have proven to be more economically viable as compared to wet fractionation alone (Schutyser et al., 2015). Dry fractionation is a more sustainable alternative to energy-intensive wet fractionation. However, for functional food applications demanding high-purity protein ingredients, the development of hybrid fractionation approaches can be sustainable in terms of yielding a high-purity protein fraction with less water and energy usage compared to wet fractionation alone (Schutyser et al., 2015; Schutyser & van der Goot, 2011). The hybrid fractionation approach put forward by Schutyser et al. (2015) involved the fine fraction obtained from air classification suspended in water at a low S/F ratio without the use of any chemicals (alkaline or acid) and no additional dilution took place before spray drying (Pelgrom et al., 2015).

Wet fractionation of the coarse starch-rich but protein-depleted fraction from air classification allowed the recovery of 87% of the total seed proteins (92% of dehulled seeds proteins), which was achieved through the production of two protein concentrates (54 and 61% protein content). However, a major reduction of the anti-nutritional factors was not achieved, rather, the values were equivalent to the amounts present in dry-only or wet-only fractions. This method also consumed 5.5 times less water per kg of extracted proteins, compared to the

traditional one-step wet extraction (Dumoulin et al., 2021). Some anti-nutritional factor contents were also evaluated. Equivalent levels of phytic acid (about 11 mg/g), trypsin inhibitor activity (about 13 trypsin inhibition unit/g) and polyphenols (about 6 mg gallic acid equivalent/g) were observed in the two protein-rich fractions. These levels are mainly equivalent to those found after the usual one-step dry and wet separations.

Having used 98% less water compared to the conventional alkali extraction, the hybrid dry and aqueous fractionation are promising methods for the industry to create value from faba beans more economically and sustainably while minimizing the impact on faba bean's native protein functionality (Dumoulin et al., 2021; Ruiz et al., 2016). However, there is a need to develop simpler and more sustainable dry and wet separation techniques that can be merged to develop hybrid processes to obtain high-value protein isolates.

2.5. FUNCTIONALITY OF FABA BEAN PROTEINS

The naturally occurring bioactive substances enriched or fortified in conventional food products can provide physiological benefits in addition to basic nutrition, and therefore they are referred to as functional foods, focusing on their physiological functionality (Ahmad et al., 2012). On the other hand, the physical functionality of an ingredient relies on how they function in a food product and will be referred to as "functionality" hereinafter. The functionality of pulse proteins is contingent upon the protein composition, which varies depending on the species, cultivar, fractionation method and the ratio of 11S:7S (Shi & Nickerson, 2022). Due to its polar amino acid profile and low molecular weight, a high vicilin concentration is frequently associated with good protein solubility (Barac et al., 2010; Koyoro & Powers, 1987). Vicilin contains more negatively charged amino acids, notably glutamic acid and aspartic acid,

than the legumin fraction, contributing to a considerably more hydrophilic profile (Jarpa-Parra et al., 2014; Koyoro & Powers, 1987; Shi 2021). According to Barac et al. (2010), pure protein fractions of 7S proteins are more functional in food systems, compared to 11S proteins. Consequently, producing protein ingredients with a low legumin/vicilin (L/V) ratio could be beneficial in developing food ingredients with promising techno-functional properties (Shi, 2021; Shi & Nickerson, 2022).

With the globulin fraction exhibiting increased surface hydrophobicity, it was expected that this would have a positive effect on the functional properties (Papalamprou et al., 2009; 2010). Langton et al. (2020) found that the alkali-extracted faba bean protein isolates had a higher surface charge, surface hydrophobicity, and solubility than salt-extracted isolates. Functional properties are a result of the physicochemical phenomena occurring during ingredient or food product storage, processing, and consumption (Jarpa-Parra et al., 2014). They are driven mainly by protein hydration capacity, surface activity (hydrophobicity and charge distribution), and primary, secondary, tertiary and quaternary structures, which together contribute to solubility, wettability, aggregation, interfacial adsorption in colloids (foams and emulsions), and rheological characteristics (viscosity, elasticity, adhesiveness, and gelation) (Jarpa-Parra, 2015; Langton et al., 2020; Liu et al., 2009; Peterson et al., 2002; Singhal et al., 2016). Because food products are multicomponent systems, their functionality should be considered as a result of the interaction of proteins with all the other constituents, including macro- (lipids and carbohydrates), and micro-components (bioactive compounds). These properties can be modified during ingredient production by altering the extraction conditions (Jarpa-Parra et al., 2014). Native pulse proteins resulting from dry, hybrid or mild wet fractionation can exhibit excellent foaming capabilities comparable to those of egg white

because of their albumin-rich composition and good solubility (Day, 2013; Pelgrom et al., 2014; Pelgrom et al., 2015b; Pelgrom et al., 2013). When investigating the foaming characteristics of faba protein concentrates produced by dry fractionation, it was reported that defatting the concentrate significantly increased its foaming characteristics (Boye et al., 2010; Pelgrom et al.,2014). Others also found that faba bean proteins had good foaming properties, however, those proteins were produced using a milder wet isolation method (Day, 2013; Schutyser et al., 2015).

Faba beans contain considerable levels of carbohydrates (40–44%) in addition to protein (Crépon et al., 2010). Protein and starch can be combined to produce mixed gels, which have previously been investigated for various protein and carbohydrate sources (Aguilera et al., 2009). Although the gelation of plant proteins is one of the most important functional properties for various plant-based food applications, limited studies have focused on the gelation properties of faba bean proteins. Functional food ingredients from faba bean proteins have the potential as foaming, emulsifying, and gelling agents that can be used for producing dairy and meat alternatives (Boye et al., 2010; Multari et al., 2015; Singhal et al., 2016). Faba bean proteins have superior functional properties when compared to animal proteins, and even in comparison to other pulse sources (Sosulski & McCurdy, 1987). These functional properties of faba bean ingredients depend on the bean variety, protein secondary structural interactions with other macromolecules and conformation changes (Assatory et al., 2019; Lam & Nickerson, 2013; Liu et al., 2017; Sharan et al., 2021).

2.6. FABA BEAN PROTEIN IN FOOD APPLICATIONS

Pulse PCs/PIs are being used in many food products, including baby foods, bakery products, bean curds, breakfast cereals, food bars, meat analogues, nutrition supplements, non-dairy beverages, and snacks (Boye et al., 2010; Multari et al., 2015; Shevkani et al., 2019; Singhal et al., 2016a). Similar to other pulses, faba beans can serve as a complementary vehicle to improve the nutritional profiles of cereal-based diets and baby foods due to their lysine- and arginine-rich amino acid profile, respectively (Mayer Labba et al., 2021; Millar et al., 2019). However, food products formulated with faba bean protein are still scarce in the food industry due to their limiting functional properties as food ingredients (Boukid & Castellari, 2022; Multari et al., 2015) as affected by the conventional extraction methods. Depending on the targeted final food product, a combination of fractionation approaches can be used to meet the desired properties (Pelgrom et al., 2014, 2015a, 2015b), as the physicochemical properties of the protein fractions can be altered based on the fractionation method that ultimately dictates the functional attributes (Jarpa-Parra, 2018; Jarpa-Parra et al., 2014). Besides the final functional qualities of the proteins, sustainable and scalable approaches should be considered during the selection of suitable processing technologies (Boukid & Castellari, 2022).

Several researchers have studied the fortification of bread with pulse ingredients using whole pulse flours while a few have evaluated the suitability of high protein pulse fractions for these applications (Boye et al., 2010). Abdel-Aal et al. (1987) substituted 15% and 20% of wheat flour with chickpea and faba bean flours, concentrates, and micellar protein mass (57.5-83.8% purity) to understand the suitability of different pulse ingredients in bread formulations. These ingredients were also used as sausage meat extenders at a substitution level of 20-40%.

The faba bean micellar protein mass was favoured over the rest in these product formulations. The bread containing the micellar proteins had a less beany taste and overall acceptable results, emphasizing the suitability of mild fractionation methods in isolating functional protein ingredients suitable for food applications.

Lorenz et al. (1979) were able to produce acceptable bread and cookies by substituting 10% of the wheat flour with air-classified faba bean protein concentrate (68.2% protein). Replacing only 15% of the wheat flour with faba bean PC with a protein content of 61% (Hoehnel et al., 2020) resulted in a medium gluten network, which was stronger than that of lupine and pea protein-based bread. The firmness of the faba bean PC-enriched bread was comparable to the control. Sozer et al. (2019) reported that lactic acid fermentation of faba bean flour did not have a significant impact on the sensory attributes such as crumbliness and springiness of the gluten-free bread, which were comparable to the soft and porous structure of soy flour bread (Sozer et al., 2019). These findings suggest that faba bean PC could be a potential candidate for legume-incorporated bread development.

Substituting 30% of wheat flour with legume flour resulted in a sticky dough, which was hard to extrude into pasta (Multari et al., 2015; Wood, 2009). Durum wheat enriched with 25% faba bean PC/PI resulted in firm pasta; however, the overall taste and flavour were not different from the control (Chan et al., 2019). Substituting semolina with faba bean flour resulted in spaghetti (Gimenez et al., 2012) with a 2.25% protein enrichment for every 10% faba bean flour substitution. Colour alterations of fortified pasta (lower brightness and increased redness) were observed due to the higher mineral content of faba bean flour and the development of Maillard reaction products during the high-temperature drying process. However, substituting 8% of wheat flour resulted in pale bread crust colour probably due to

the low availability of reducing sugars vital for the Maillard reaction (Hsu et al., 1977). In a study by Gimenez et al. (2013), the replacement of corn flour with 30% of faba bean flour enriched the protein content by ~15%. In the same study, the extrusion cooking of pasta dough with 28% moisture content at 100 °C was appropriate to obtain adequate textural characteristics of the final product (Gimenez et al., 2013).

Furthermore, legume proteins have been introduced as binders and extenders to decrease the fat content and enhance the nutritional value and the sensory properties of meat-based food products (Multari et al., 2015; Serdaroğlu et al., 2005). The potential of developing a meat analogue with good sensory attributes using faba bean PCs by extrusion at 130-140 °C was shown by Saldanha do Carmo et al. (2021). However, higher than 5% substitution with lupin flours led to detrimental effects in emulsified meat products, which were influenced by the presence of fibre and oligosaccharides. Therefore, the type of pulse ingredient, whether flour, concentrate or isolate matters in meat product applications. Cai et al. (2001) reported that the bean curds prepared from chickpea and faba bean protein by thermal denaturation and coagulation with calcium and magnesium sulphates resulted in textural properties comparable to that of soy proteins. It was further confirmed that a protein concentration of 2.3–3% using 1.5% calcium sulphate as a coagulant produced the best curd.

Gluten-free pasta made from faba bean had high protein, resistant starch and dietary fibre contents but fewer anti-nutritional factors (Rosa-Sibakov et al., 2016). However, these pasta had less springiness and more cooking loss, but a low glycemic index as compared to commercial gluten-free pasta. Semolina pasta prepared with 10%, 30% and 50% faba bean flour (Tazrart et al., 2016) resulted in lower cooking time but higher dry matter loss. A significant increase in protein, resistant starch, mineral (iron) and *in-vitro* protein digestibility

but a reduction in the glycemic index were observed in pasta formulated with faba bean flour (Chan et al., 2019). The starch present in faba bean four could have led to the formation of protein–starch network, reducing the accessibility of amylase in comparison to the control. Therefore, high protein gluten-free faba bean flour with more fibre, resistant starch and a considerable amount of minerals can be considered a great substitute for the production of gluten-free pasta (Tazrart et al., 2016).

Faba bean protein hydrolysates were efficient as an ingredient to partially substitute egg yolk powder in low-fat mayonnaise formulations (Ouraji et al., 2020). Incorporating hydrolysed faba bean proteins in apple juice formulation altered the sourness, bitterness, saltiness and turbidity (Samaei et al., 2020). However, these alterations did not have an overall significant impact on the sensory perception of these products when compared to the control (Samaei et al., 2020). Infant formulas reformulated by Le Roux et al. (2020) with a partial substitution (50%) of dairy proteins with faba bean proteins resulted in a higher hydrolysis degree (73%) than those containing only whey proteins (50%). Interestingly, the protein digestibility corrected amino acid scores of faba bean formulas (76%) were statistically similar to those of whey protein-only formulas (75%) and higher than pea protein formulas (67%). Therefore, Le Roux et al. (2020) concluded that faba bean proteins could be a good candidate for partial substitution of whey proteins in infant formulas, but robust *in-vivo* studies are required to confirm such conclusions.

Based on the lab-scale experimental findings summarized above, faba bean protein could be considered a sustainable functional food ingredient with the potential to supplement the nutritional requirements of mankind. They have the potential to be utilised in gluten-free foods considering their versatility in being processed as pasta, bread and snacks with promising

organoleptic characteristics similar to traditional wheat-based products (Belghith-Fendri et al., 2016; Multari et al., 2015; Shevkani et al., 2019). However, there is limited research work done on the functional food applications of faba bean proteins. Further work is warranted to narrow down the extraction methods that will best support the functional properties of faba bean proteins necessary for various food product applications.

CHAPTER 3: Micromorphological and elemental characteristics of chickpea, faba bean, field pea and lentil cotyledon topographies[2]

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3.1. INTRODUCTION

There is growing interest in the value-added utilization of pulse protein to partially substitute the increasing animal-based protein demand associated with the increasing world population (Day, 2013; de Boer & Aiking, 2011; Parodi et al., 2018). Although used interchangeably, “legumes” refer to any plant from the Fabaceae (Leguminosae) family, whereas “pulses” signify beans, lentils, peas and other edible dry leguminous seeds from the pods (Allen, 2013; Day, 2013). Legumes can produce high levels of protein since they are involved in symbiotic nitrogen fixation (Allen, 2013). Pulses are not only rich in protein, but those proteins are complementary to cereal proteins (Acevedo et al., 2016; Mattila et al., 2018).

The milling process of pulses can involve two stages concerning value-added pulse ingredient production. It can be the decortication followed by the splitting of beans to produce split seeds (Wood et al., 2017) and/or the particle-size reduction of beans by grinding (Scanlon et al., 2018). Specific seed tissue structure and composition, and interactions at the seed coat-cotyledon interface (abaxial) and cotyledon-cotyledon interface (adaxial) can influence varietal differences observed in the milling process (Scanlon et al., 2018; Schutyser & van der Goot, 2011). On the other hand, dry processing involving sequential pearling has gained attention for the efficient concentration of cereal grain components for subsequent processing based on the distribution of components within the grain, for example, in barley (Blandino et al., 2015; Liu et al., 2009; Vasanthan & Temelli, 2008) and wheat kernels (Hemery et al., 2007). Unlike cereal grains, pulse (legume grain) seeds are comprised of two cotyledons, generally non-endospermic (Anderson, 1949), which have a natural tendency to split while pearling. Therefore, during pearling of pulses, some portion of the pulse grains will be pearled as whole

beans while the rest will be pearled as split beans. More information is warranted on the surface morphology for a better understanding of its impact on pearling.

Structural modification of the endosperm cells concerning their adhesion to the inner surface of the cotyledon seed coat involves the formation of an electron-dense substance closely resembling phenolics. Its association with the polysaccharide-rich wall material leads to the formation of a cementing layer between the phenolics-rich inner surface of the seed coat and the endosperm wall (Yeung, 1990). One of the most adaptive traits during terrestrialization is the formation of a translucent hydrophobic cuticle layer (Buda et al., 2009; Edwards, 1993; Nawrath, 2006) of various polymeric lipid and soluble wax profiles (Zhao et al., 2019) on the aerial epidermis of all land plant species and plant organs (Edwards, 1993).

The cuticle is a highly recalcitrant material capable of protecting against transpirational desiccation and environmental stresses (Yeats & Rose, 2013). The cuticle membrane is mainly composed of cutin and cuticular waxes (Zhao et al., 2019). Cutin is a highly esterified fatty polymer, mainly stemming from a mixture of aliphatic monomers of C_{16} and C_{18} ω-hydroxylated fatty acids, carrying additional hydroxy- and epoxy-groups at mid-chain positions, glycerol, and small amounts of phenolic compounds (Jeffree, 2006; Nawrath, 2006). To date, extensive research has been carried out on the morphological characteristics of the outermost extracellular matrix of the epidermis of terrestrial plants (Bailes & Glover, 2018; Barthlott et al., 1998), chemical constituents of the cuticle and their biosynthesis and critical functional properties (Jeffree, 2006; Nawrath, 2006; Yeats & Rose, 2013; Zhao et al., 2019).

Limited studies have also discovered epicuticular coverings within the legume cotyledon topographies of cowpea (Sefa-Dedeh & Stanley, 1979a), lentils (Hughes et al., 1986), and 18 species belonging to the Fabaceae family (Gandhi et al., 2011; Günes & Ali,

2011) mainly on the cotyledon abaxial surface alongside the characteristics of either the cross-sectional or seed coat morphology. Morphological studies have confirmed the presence of cuticular membrane on the abaxial and adaxial cotyledon surfaces of chickpeas using light microscopy (Wood et al., 2017) and scanning electron microscopy (Wood et al., 2011). However, there is limited evidence to certify that other protein-rich pulses such as faba beans, field peas and lentils, have great potential for value-added pulse protein production (Alcorta et al., 2021; Allen, 2013; Millar et al., 2019; Multari et al., 2015; Multari et al., 2016) also have an adaxial surface that was blanketed by an epidermis consisting of an epicuticular film. To the best of the authors' knowledge, there is no published information on whether this cementing material has a surface elemental composition similar to that of the abaxial surface. Understanding the morphology and surface elemental composition of the abaxial and adaxial surfaces is of utmost importance to interpret the differences observed in pearled bean fractions.

Therefore, the objectives of this study were: a) to characterise the topography of abaxial (cotyledon-seedcoat interface after dehulling), adaxial (cotyledon-cotyledon interface after splitting) and cross-sectional (transverse cut surface of the split cotyledon) surfaces of faba bean (*Vicia faba*, L.) *via* micromorphological and elemental analyses, and b) to apply a similar approach to other protein-rich pulses, including chickpea (*Cicer arietinum*, L.), field pea (*Pisum sativum*, L.), and lentil (*Lens culinaris*, L.) cultivars for comparison purposes. Such characterisation of pulse grains is valuable for process development, targeting pearling, and subsequent protein enrichment.

3.2. MATERIALS AND METHODS

3.2.1. Materials

The pulse grain test samples comprised of cultivars of chickpea (*Cicer arietinum* L.): Desi type, CDC Consul and Kabuli type, CDC Orion; faba bean (*Vicia faba* L.): high-tannin, Athena and low-tannin, Snowbird; field pea (*Pisum sativum* L.): green type, Alaska and yellow type, Carneval; and lentil (*Lens culinaris* L.): green type, CDC Greenstar and red type, CDC Maxim. All these cultivars are registered for production in Alberta under the Agricultural Financial Services Corporation except for the field pea cultivar Alaska, which is an heirloom. Selection criteria included high yield and disease resistance.

Faba bean test samples were recommended and provided by W.A. Grain and Pulse Solutions, Innisfail, AB, Canada. For chickpeas, CDC Orion is the most widely grown high-yielding Kabuli cultivar in Alberta and Saskatchewan, whereas CDC Consul is a high-yielding Desi cultivar. Both have improved Ascochyta blight resistance, and the latter is more suitable for whole, dehulled/split seed markets and milling. CDC Greenstar and CDC Maxim are high-yielding lentil cultivars with resistance to Ascochyta. Both chickpea and lentil test samples were recommended and provided by Dr. Bunyamin Taran, Crop Development Centre, University of Saskatchewan, Saskatoon, SK, Canada. CDC Greenstar is a high-yielding yellow field pea cultivar, whereas Alaska is an early pea variety, obtained from Prof. Jocelyn Ozga's Lab, University of Alberta, Edmonton, AB, Canada.

3.2.2. Preparation of seed sections

The beans were conditioned (1:3, w:w) with Milli-Q water at room temperature (23 ± 1 ˚C) for 2 h, drained, and dried with a paper towel. The seed coats were cut open with a scalpel along the abaxial surface on the dorsal side of the seed, decorticated and split manually (Wood et al., 2017). Faba beans were also fractured into two equal sections by a transverse cut at the midpoint of the longitudinal axis, perpendicular to the cotyledon-cotyledon interface using a jigsaw to examine the internal structure of the cells present. The bottom part was trimmed off with the jigsaw to create a flat surface for mounting purposes.

The samples for scanning electron microscopy (SEM) were dried for 3 h at 30 °C in a forced-air oven (Fisher Scientific, Isotemp 750G Oven, Missouri City, TX, USA). The beans for helium ion microscopy (HIM) were freeze-dried (Labconco 401904, Kansas City, MO, USA) for 48 h. Both samples prepared for SEM and HIM were vacuum-sealed into triple-laminated aluminium packages and stored at room temperature until use.

3.2.3. Micromorphological characterisation

For SEM, carbon-based double-sided adhesive tape was used to firmly fix the cotyledons on aluminium stubs to avoid any movement or vibration. One cotyledon was secured to examine the abaxial surface, whereas the other was mounted to observe the adaxial surface from each test sample. The faba bean transverse cuts were also mounted to comparatively study the abaxial, adaxial and transverse cut surfaces. The specimens were then flushed with compressed air to remove any debris introduced during sectioning or handling. They were gold coated in a SEMPREP 2 sputter coater (Nanotech, London, England, UK) at 1.5 kV for 2 min to prevent charge build-up on the specimen surface. The topography of the

abaxial and adaxial surfaces was examined under a variable pressure-field emission SEM (VP-FESEM, Zeiss Sigma 300, Oberkochen, BW, Germany), located at the Department of Earth and Atmospheric Sciences, University of Alberta. Observations were made at an acceleration voltage of 10.0 kV and viewed and photographed at magnifications of ×250, ×500, and ×2,000. The images were analysed via ImageJ v.1.51a software.

Topographies of faba bean abaxial, adaxial, and transversal cut surfaces were also analysed by HIM (Zeiss Orion Nanofab, Ostalbkreis, BW, Germany), located at NanoFab, University of Alberta. Direct imaging was achieved by using an electron flood gun to neutralise the positive charges accumulated on the sample surfaces. Secondary electron images collected at 10 kV accelerating voltage were analysed *via* Image J v.151j8 software.

3.2.4. Elemental analysis

The elemental analysis on the abaxial and adaxial surfaces was carried out by a Bruker energy-dispersive X-ray spectroscopy (EDS) system with a Bruker dual silicon drift detector, which was installed on the SEM described above. The window area was 60 mm^2 and the resolution was 123 eV. EDS scans were done on three independent spots of a specified region of the gold coated surface following SEM imaging. EDS analysis was performed by "automatic peak identification," and was manually inspected to determine the validity.

3.2.5. Statistical analysis

EDS data are shown as mean ± SD (n=3). Significant differences ($P < 0.05$) between the abaxial and adaxial elemental analyses results were tested using the two-sample t-test. All

these analyses were carried out with the statistical analysis software package (SAS Studio University Version for Windows, SAS Institute Inc., USA).

3.3. RESULTS AND DISCUSSION

3.3.1. Micromorphological characteristics of pulse cotyledon topographies

SEM (Figs. 3.1, 3.2 and 3.4) and HIM (Fig. 3.3) microscopic results confirmed the presence of a hydrophobic thin cuticle material at both cotyledon abaxial and adaxial surfaces of wet pre-conditioned chickpea, faba bean, field pea, and lentil cultivars. Although considered independent from the epidermis (Barthlott et al., 1998; Yeats & Rose, 2013), the cuticle membrane is physically connected and carries an imprint of the underlying epidermal cell pattern, its cell wall junction and middle lamella location. Intra-cuticular waxes are impregnated within the cuticle membrane, whereas epicuticular crystals or films are deposited on the cuticle surface (Yeats & Rose, 2013). The epicuticular micromorphological characteristics observed on the cotyledon abaxial and adaxial surfaces in this study confirmed the presence of an epicuticular film on the cuticle surface.

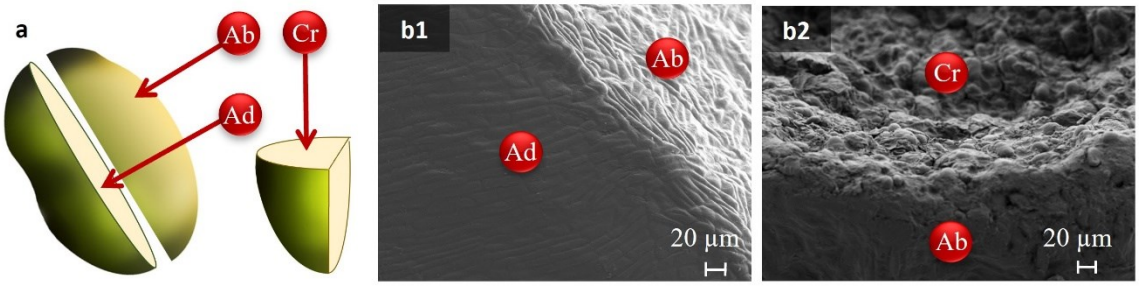

Fig. 3.1. Faba bean cotyledon abaxial, adaxial, and cross-sectional surfaces.

(a) Schematic drawing of the faba bean cotyledon surfaces investigated (abaxial: cotyledon-seed coat and adaxial: cotyledon-cotyledon interfaces, and cross-section: transverse-cut surface). SEM micrographs of (b1) Athena cotyledon abaxial surface adjoining the adaxial surface and (b2) lateral view of Snowbird cross-section. (b1 and b2 magnification: ×250).

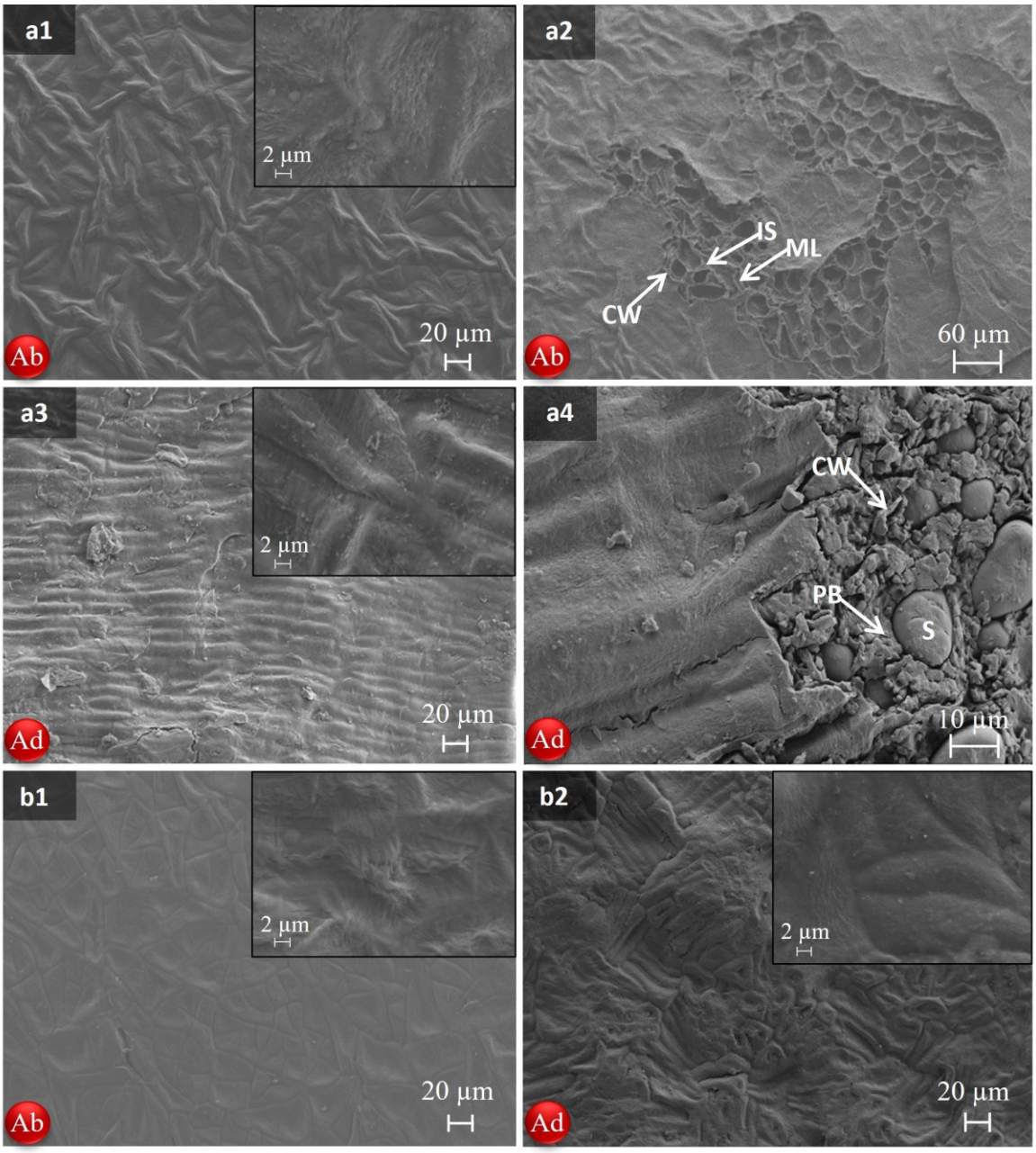

Fig. 3.2. SEM micrographs of faba bean cotyledon abaxial and adaxial surfaces.

(a1) Athena abaxial surface, (a2) Athena abaxial epidermal cell wall (CW), middle lamella (ML) and intercellular space (IS), (a3), Athena adaxial surface, (a4) Athena adaxial surface cementing the endosperm starch granules (S) embedded in a protein matrix (P) with protein bodies (PB) surrounded by a fibrous cell wall (CW), (b1) Snowbird abaxial surface, and (b2) Snowbird adaxial surface. (a1 to b2 magnification: ×250; inserts: ×2000).

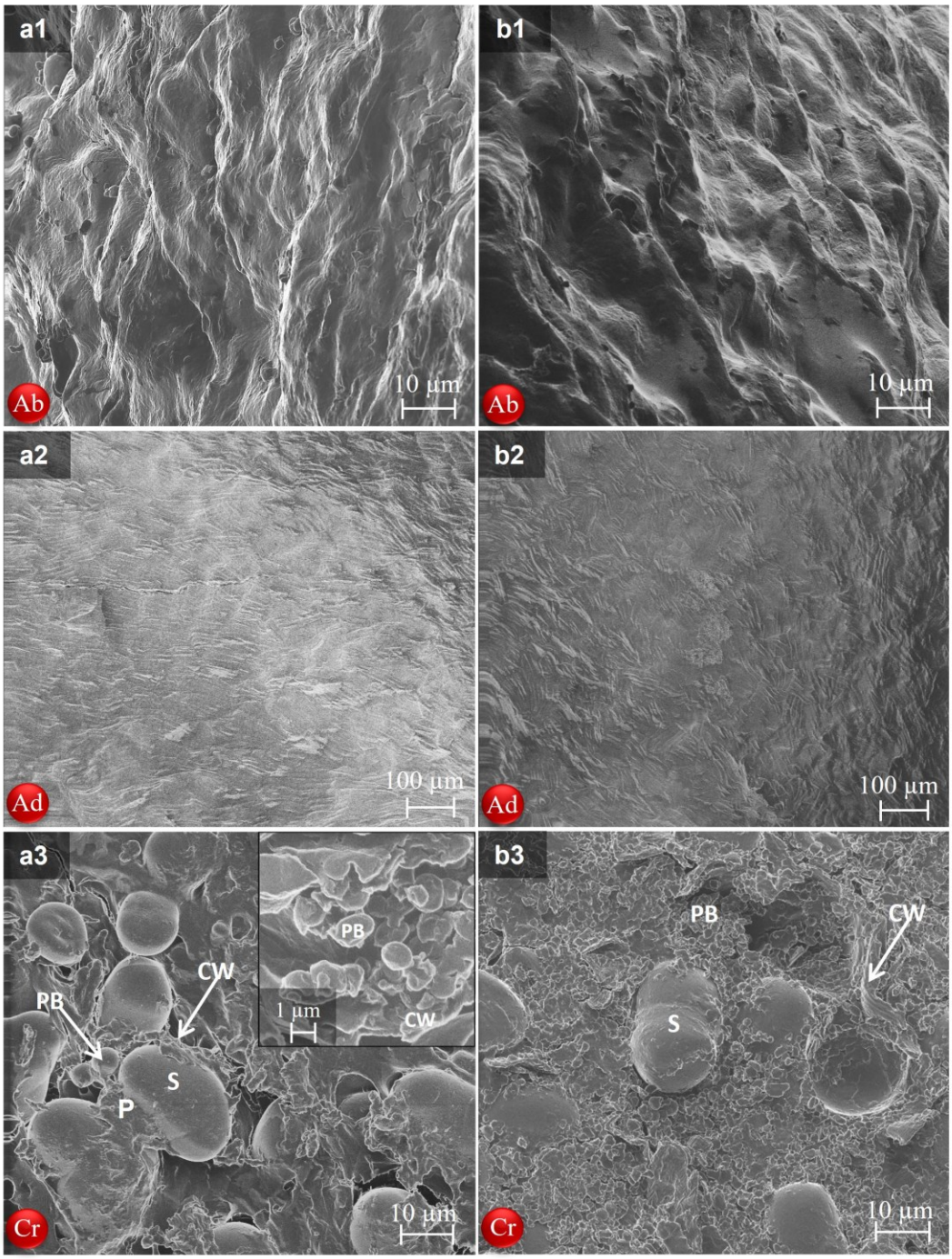

Fig. 3.3. HIM micrographs of faba bean cotyledon abaxial, adaxial and cross-sectional surfaces.

Abaxial (1), adaxial (2) and cross-sectional (3) cotyledon topography of (a) faba bean Athena and (b) faba bean Snowbird. Starch granules (S) embedded in a protein matrix (P) with protein bodies (PB) surrounded by a fibrous cell wall (CW).

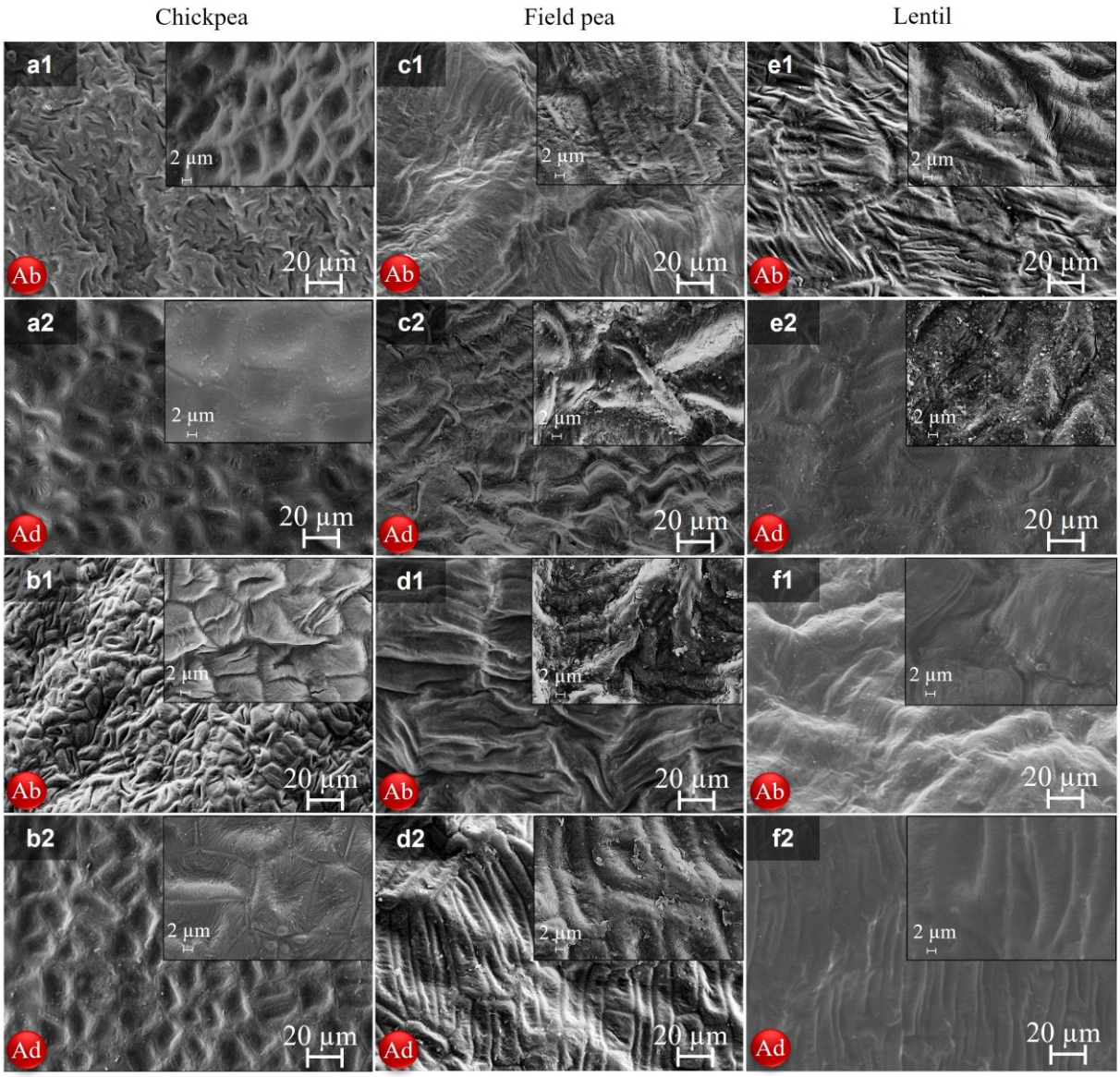

Fig. 3.4. SEM micrographs of chickpea, field pea and lentil cotyledon abaxial and adaxial surfaces.

Abaxial (1) and Adaxial (2) cotyledon topography of (a) Chickpea CDC Consul Desi, (b) Chickpea CDC Orion Kabuli, (c) Field pea Alaska, (d) Field pea Carneval, (e) Lentil CDC Greenstar and (f) Lentil CDC Maxim. (a1 to f2 magnification: ×500; Inserts: ×2000).

The micromorphological characteristics of these cuticular coverings differed depending on the location, whether it is abaxial or adaxial surface, the species and the cultivars. The abaxial and adaxial cotyledon surfaces (Figs. 3.1-3.4) of chickpea, faba bean, field pea, and lentil cultivars selected in this study exhibited gentle undulation of epicuticular wax films covering the underlying epidermal cells, which appeared to be either tabular or polygonal. The 'tabular' cells were approximately rectangular-shaped in their boundary. Polygonal-shaped epidermal cells were mainly three to six-sided shapes. Both tabular and polygonal undulations were projected as either 'flat' or 'rugose'. 'Rugose' cells were either wrinkled, raised or ridged, unlike 'flat' cells. The micromorphology of the epicuticular wax films (Figs. 3.2-3.4) was either 'smooth' with fine sculpturing and no bulges on the surface, 'striate' with a ridged surface, or 'granular' with non-linear bulges.

The schematic sketching (Fig. 3.1a) showcases faba bean cotyledon abaxial, adaxial and cross-sectional surfaces, which were investigated by SEM and HIM. SEM micrographs of the faba bean cv. Athena (Fig. 3.1b1) confirmed the continuation of the epidermal cells from the faba bean abaxial to the adaxial surface. Faba bean-Athena abaxial surface examined through SEM (Fig. 3.2a1) and HIM (Fig. 3.3a) displayed polygonal-shaped epidermal cells. SEM images further confirmed the presence of an epicuticular wax film, which was projected as rugose with generally Y and T-shaped curving edges (Fig. 3.2a1). At times, these curving edges formed small hills. The epicuticular wax sculpturing was striated alongside the narrow valley floor (Fig. 3.2a1 insert) demarcating the underlying epidermal cell wall junctions and middle lamella, which are unveiled in Fig. 3.2a2 due to the gentle peeling away of the thin cuticle film through friction during decortication. The adaxial surface (Figs. 3.2a3, 3.2a4 and Fig. 3a2) epidermis exhibited mostly parallelly aligned, tabular-shaped rugose pleats of different lengths with a

smooth epicuticular wax film. Fig. 3.2a4 confirms the presence of an epicuticular wax film in the cotyledon adaxial surface cementing the cellular components unveiled by either cracking or tearing during the splitting process.

Faba bean cv. Snowbird abaxial surface (Figs. 3.1b2, 3.2b1 and 3.3b1) epicuticular wax film micromorphology was striated. The underlying cell wall and middle lamella were often apparent forming three-to-five-sided polygonal fine grooves. The epicuticular surface appeared rugose due to the sunken centre of many epidermal cells as well as raised small peaks formed by the wrinkling of the cuticle (Fig. 3.2b1 insert). Its adaxial surface (Fig. 3.2b2 and Fig. 3.3b2) consisted of mainly tabular-shaped rugose epidermal cells with thin indented lines revealing the underlying middle lamella and the cell wall. Unlike Athena's adaxial surface, these cells were not parallelly aligned and the epicuticular wax cell micromorphology was also striated and not smooth. Similar tabular-shaped epidermal cells, flat or rugose projections and smooth, striated or granular epicuticular wax micromorphological characteristics were also common in the seedcoat (Zhao et al., 2019) and floral petal abaxial and adaxial surfaces of faba bean (Bailes & Glover, 2018; Zhao et al., 2019) and 175 different species of the family Leguminosae (Cronk, 2009; Ojeda et al., 2019). However, none of the cotyledon topographies showcased the common papillose-shaped conical, knobby, or lobular projections of the legume flower petals, which played a significant role in pollination (Bailes & Glover, 2018; Ojeda et al., 2009).

Cotyledon abaxial and adaxial surfaces have different functions and are physically associated with different structures (Yeats & Rose, 2013). Cuticle elaborations on the abaxial cotyledon surface may increase adherence to the seed coat. Based on the HIM images, it can be confirmed that a highly corrugated abaxial surface with more surface area for adhesion was evident in faba bean cvs. Athena (Fig. 3.3a1) and Snowbird (Fig. 3.3b1), which was comparable

to the findings of Moser (1998). Athena was difficult to split, which may be correlated to the adaxial surface being composed of parallelly aligned tabular-shaped pleats in comparison to Snowbird adaxial surface with randomly aligned tabular-shaped pleats.

The HIM cross-sectional (Fig. 3.3a3) images of Athena inner cotyledon parenchyma cells, revealed the presence of round, elliptical, oval, and bean-shaped starch granules (~15-25 μm) embedded in a proteinaceous matrix containing protein bodies (~1-2 μm) surrounded by a fibrous cell wall. These findings were in agreement with those for the Snowbird inner cotyledon parenchymatous cell and reserve material architecture (Fig. 3b3), inner cotyledon architecture beneath the adaxial surface (Fig. 3.2a4) and the lateral view of the cross-sectional architecture of its inner cotyledon cells (Fig. 3.1b2). Although HIM images were of higher resolution without the need for gold coating and were complementary to the SEM micrographs, HIM ion beam can cause damage (surface sputtering, charge transfer, implantation, and lattice damage) on the sample surface and beneath the surface (Burch et al., 2018), making the technique more challenging. HIM imaging was not carried out for the other samples in this study due to the degassing challenges of the system despite freeze-drying the samples.

The Desi-type chickpea cv. CDC Consul abaxial surface (Fig. 3.4a1) showed protruding four-to-five-sided lines forming polygonal shapes, demarcating the underlying cell wall and middle lamella of the epidermis. The centre of the epidermal cells appeared sunken with wrinkling of the cuticle. In comparison, the adaxial surface (Fig. 3.4a2) also exhibited very similar micro-reliefs but were only slightly sunken in the middle of the cell in comparison to the abaxial surface and exhibited four-to-six-sided polygonal grooves, indicating the underlying epidermal cell walls and middle lamella. The epicuticular wax film of both the abaxial and adaxial surfaces was rugose with striations.

The Kabuli-type chickpea cv. CDC Orion abaxial surface (Fig. 3.4b1) showcased four-to-five-sided irregular polygonal-shaped protruding wide hills. The epicuticular wax film was rugose and wrinkling of the entire cuticle was evident with striations similar to cowpea cv. Adua Ayera (Sefa-Dedeh & Stanley, 1979a). Prominent four-to-five-sided polygonal lines could be observed in Fig. 3.4b1 insert, suggesting the location of the underlying cell wall junctions and middle lamella. The abaxial surface of both chickpea cvs. CDC Consul and Orion showed several ring-shaped deposits arranged in groups. CDC Orion adaxial surface (Fig. 3.4b2) also exhibited micro-reliefs on par with the CDC Consul adaxial surface. However, unlike the Desi type cultivar, the Kabuli type cultivar exhibited wrinkling of the entire cuticle; the ridges showcasing the cell wall were more prominent and the middle of the cells was more shrunken. It is also noteworthy that the Canadian Kabuli-type cv. CDC Orion adaxial surface showcased a very characteristic four-to-six-sided polygonal-shaped epidermis with rugose projections and striated epicuticular wax film comparable to the Australian Kabuli type cultivar (Wood et al., 2017).

Field pea cv. Alaska's abaxial surface (Fig. 3.4c1) showed tabular-shaped rugose epidermal projections. The surface had both narrow ridges and continuous wide hills that were moderately grooved. The epicuticular wax microstructure had striations, which were formed by tiny (<1 μm) grainy structures (Fig. 3.4c1 insert). Thin fine lines suggested the location of the underlying cell wall junctions and middle lamella. The adaxial surface (Fig. 3.4c2) showed tabular-shaped rugose epidermal projections with looped thread-like structures alongside narrow and wide ridges and irregular hills that were moderately grooved (sometimes contained large dents). The overall epicuticular wax microstructure was mainly striated alongside granular projections mostly covering the meandering valleys of varying width (Fig. 3.4c2 insert).

These granular crystal observations had similarities to the Cowpea variety Kaase Market abaxial surface morphologies (Sefa-Dedeh & Stanley, 1979b) and were in agreement with the dispersed prismatic crystals found on the abaxial surface of Australian Desi isoline chickpea cv. Angular (Wood et al., 2017). However, both Desi and Kabuli type Canadian chickpea cultivars (Fig. 3.4a1-b2) did not showcase any of these prismatic/granular crystals, similar to the micromorphological characteristics of Australian chickpea Desi genotypes Amethyst and 9105-33 N (Wood et al., 2017).

Field pea cv. Carneval abaxial surface (Fig. 3.4d1) exhibited tabular-shaped rugose epidermal projections. The epicuticular wax microstructure was grainy with root-like ridges, which were grooved. Some of the ridges were much wider with more fine grooves. The valley floor was narrow with evident tiny lines suggesting the location of the underlying cell wall junctions and middle lamella. The adaxial surface (Fig. 3.4d2) epidermis showed tabular-shaped rugose pleats similar to those of the faba bean-Athena adaxial surface. These were composed of granular epicuticular wax prognostications (Fig. 3.4d2 insert). Thin indented lines revealed the underlying middle lamella and the raised projections suggested the location of underlying cell walls.

Lentil cv. CDC Greenstar abaxial surface (Fig. 3.4e1) displayed tabular-shaped rugose epidermal surface features with straight and some Y- or T-shaped ridges. Epicuticular wax elaborations were mostly striated. Thin lines seemingly identified the location of the underlying cell wall junctions and middle lamella (Fig. 3.4e1 insert), comparable to that of faba bean cv. Athena abaxial (Fig. 3.2a1) and adaxial (Fig. 3.2a3) surfaces as well as Snowbird adaxial (Fig. 3.2b2) surfaces. The valley floor had fine grooves. Its adaxial surface (Fig. 3.4e2) cell walls

formed four-to-six-sided raised polygonal shapes and the underlying middle lamella appeared grooved, covered by a rugose epicuticular wax film.

Although protruding epicuticular wax crystalloids commonly observed in major seed plant cuticle layers (Barthlott et al., 1998) were not detected on any of the abaxial nor adaxial cotyledon surfaces, granular crystal-like structures were observed on the adaxial surface of lentil cv. CDC Greenstar (Fig. 3.4e2) equivalent to the abaxial surface of field pea cv. Carneval (Fig. 3.4d1) and the adaxial surface of field pea cv. Alaska (Fig. 3.4c2). These characteristic grainy structures cannot be considered artefacts of the endosperm nor wax crystalloids as they do not have a distinct orientation towards the surface. Wax crystals are known to stem directly above the anti-clinical walls of the stomatal guard cells (Barthlott et al., 1998) and none of the micro-structural observations on different cultivars closely resembled stomata.

Lentil cv. CDC Maxim abaxial surface (Fig. 3.4f1) displayed tabular-shaped rugose epidermal surface features with some raised lines as well as bumps and grooves forming hills. The observations were very similar to those of the field pea Alaska abaxial surface (Fig. 3.4c1). The epicuticular wax projections were with striations (Fig. 3.4f1 insert). The adaxial surface (Fig. 3.4f2) exhibited a tabular-shaped epidermis that appeared to be rugose similar to the faba bean Athena's adaxial surface (Fig. 3.2a3). However, the epicuticular wax microstructure (Fig. 3.4f2 insert) appeared to be raised with striations and with fine grooves comparable to the faba bean Snowbird adaxial surface (Fig. 3.2b2). Thin lines should be indicating the underlying cell walls and middle lamella.

Field pea cvs. Alaska and Carneval and lentil cvs. CDC Greenstar and CDC Maxim abaxial surface morphological characteristics closely resembled the more random "wide hills" and "narrow valleys" observed on cowpea cotyledon abaxial surfaces, which were not

significantly different from the anatomical characteristics of their internal seed coat surfaces (Sefa-Dedeh & Stanley, 1979b). These complementary patterns at the abaxial interface support the physical interlocking mechanism contributing to the adhesion of the seed coat, which will influence the milling/pearling behaviour during processing.

Gum-like deposits were observed on the adaxial surface of chickpea cvs. CDC Consul and CDC Orion followed by the rest of the cotyledon surfaces to a lesser extent. These adhesive deposits could be present as part of the physical interlocking system between cotyledon-seedcoat and cotyledon-cotyledon interfaces. Mucilages have been confirmed to be present in the endosperm of legumes (Anderson, 1949), whereas both gums and mucilage were reported to be involved in the adhesion at the abaxial and adaxial cotyledon surfaces of chickpea (Wood et al., 2014c). The presence of two relatively flat cotyledon surfaces at the adaxial junction and a more corrugated abaxial surface can be correlated to their functional properties associated with the interlocking mechanism at the cotyledon-seed coat and cotyledon-cotyledon interfaces influencing their milling behaviour (Moser, 1998; Wood et al., 2017). Differences in milling behaviour are correlated to different flour characteristics (Scanlon et al., 2018).

3.3.2. Elemental characteristics of pulse cotyledon topographies

The SEM-EDS analyses enabled the comparative study of the complete array of key elements present on the abaxial and adaxial surfaces of chickpea, faba bean, field pea, and lentil cultivars investigated (Table 3.1). These surface elemental results confirm the presence of C (52.3 ± 1.0 to 63.5 ± 0.5%) followed by O (28.8 ± 1.4 to 39.9 ± 0.6%), K (0.3 ± 0.2 to 3.5 ± 0.9%), Ca (0.5 ± 0.4 to 3.8 ± 0.3%), and Mg (0.5 ± 0.1 to 2.2 ± 0.1%) as the dominating elements on both abaxial and adaxial surfaces of these cultivars. There were no significant

differences ($P > 0.05$) in the mass concentrations (%) of the elemental profiles between the abaxial and adaxial surfaces, except for Ca in chickpea-CDC cv. Orion; C in field pea cv. Alaska; O, K, and Mg in faba bean cv. Athena, Ca and Mg in faba bean cv. Snowbird; and K in lentil cv. CDC Greenstar (Table 3.1). No significant differences ($P > 0.05$) were observed between the grand means of the elemental values of the abaxial and adaxial surfaces of pulses except for Ca.

In comparison to Canadian Desi-type chickpea, CDC Consul abaxial (1.8 ± 0.7%) and adaxial (2.2 ± 1.0%) surfaces, the Australian Desi-type chickpea genotypes were reported to have Ca levels in the range of 0.32 to 0.43 g/kg in the 29% pearling flour fraction of the outer periphery of the cotyledons (Wood et al., 2014b). Since intra-epidermal crystals are known to be calcium carbonate and calcium oxalate (Yashvanth et al., 2013), the presence of an epicuticular wax layer on both abaxial and adaxial cotyledon surfaces is further supported by the presence of Ca according to EDS findings. However, it was not possible to establish a significant correlation between the differences in surface Ca% to the presence/absence of grainy crystals, mainly due to the complex nature of the epicuticular wax film.

Apart from these key elements, N, Na, P and Cl were also detected as minor elements on a few of the abaxial and adaxial surfaces studied. N was present mainly on the adaxial surface of chickpea cv. CDC Consul (1.5 ± 0.4%), chickpea cv. CDC Orion (1.4 ± 0.3%), field pea cv. Alaska (5.8 ± 1.7%), field pea cv. Carneval (2.1 ± 3.6%), faba bean cv. Snowbird (3.1 ± 1.8%), and lentil cv. CDC Greenstar (6.5 ± 1.6%) as well as on the abaxial surface of chickpea cv. CDC Orion (1.2 ± 0.7%), field pea cv. Carneval (0.8 ± 0.7%) and faba bean cv. Snowbird (2.2 ± 0.9%).

Table 3.1. Elemental composition of chickpea, faba bean, field pea and lentil cotyledon abaxial and adaxial surfaces.

Pulse	Cultivar	Surface	Mass Concentration (%)				
			C	O	K	Ca	Mg
Chickpea	Consul	Abaxial	61.94 ± 2.24	32.38 ± 1.17	2.26 ± 0.46	1.77 ± 0.71	1.21 ± 0.42
		Adaxial	62.13 ± 1.83	31.01 ± 1.09	1.50 ± 0.94	2.17 ± 0.99	1.73 ± 0.42
Chickpea	Orion	Abaxial	62.45 ± 2.76	32.09 ± 4.41	1.50 ± 0.53	2.14 ± 0.24[a]	1.03 ± 0.15
		Adaxial	62.29 ± 1.83	35.06 ± 1.77	0.30 ± 0.19	0.94 ± 0.14[b]	1.05 ± 0.16
Field pea	Alaska	Abaxial	63.54 ± 0.47[a]	30.79 ± 1.41	3.53 ± 0.89	1.04 ± 0.39	1.10 ± 0.26
		Adaxial	55.00 ± 1.35[b]	32.26 ± 3.91	3.41 ± 0.67	0.46 ± 0.40	0.87 ± 0.40
Field pea	Carneval	Abaxial	63.06 ± 1.61	29.35 ± 1.40	3.04 ± 1.44	1.82 ± 0.67	1.22 ± 0.13
		Adaxial	60.02 ± 0.88	34.68 ± 5.09	1.19 ± 0.26	1.11 ± 0.36	0.94 ± 0.31
Faba bean	Athena	Abaxial	57.44 ± 1.33	36.80 ± 0.81[b]	3.49 ± 0.45[a]	1.20 ± 0.41	0.49 ± 0.11[b]
		Adaxial	56.95 ± 0.41	39.89 ± 0.62[a]	0.81 ± 0.03[b]	0.95 ± 0.07	1.40 ± 0.18[a]
Faba bean	Snowbird	Abaxial	52.29 ± 0.95	37.05 ± 0.33	2.49 ± 0.43	3.81 ± 0.27[a]	2.16 ± 0.14[a]
		Adaxial	54.86 ± 1.30	37.19 ± 1.29	2.62 ± 0.70	0.84 ± 0.24[b]	1.39 ± 0.34[b]
Lentil	Greenstar	Abaxial	60.84 ± 1.77	30.98 ± 1.05	1.65 ± 0.28[a]	2.25 ± 1.46	1.37 ± 0.55
		Adaxial	61.96 ± 0.51	28.82 ± 1.45	0.68 ± 0.18[b]	0.49 ± 0.20	0.61 ± 0.14
Lentil	Maxim	Abaxial	58.45 ± 2.54	37.36 ± 2.92	1.02 ± 0.28	1.65 ± 0.46	1.52 ± 0.40
		Adaxial	59.42 ± 0.74	36.53 ± 0.66	0.89 ± 0.15	1.17 ± 0.20	1.99 ± 0.32
Mean		Abaxial	60.00 ± 3.55	33.35 ± 2.56	2.37 ± 0.88	1.96 ± 0.80[a]	1.26 ± 0.44
		Adaxial	59.08 ± 2.91	34.43 ± 3.36	1.42 ± 1.00	1.02 ± 0.50[b]	1.25 ± 0.43
Grand mean			**59.5 ± 3.3**	**33.9 ± 3.2**	**1.9 ± 1.0**	**1.5 ± 0.8**	**1.3 ± 0.4**

[a,b]Means (n = 3) ± SD followed by different superscripts in each column for abaxial and adaxial surfaces of each pulse type are significantly different ($P < 0.05$).

Sodium was detected on the abaxial surface of chickpea cv. CDC Consul (0.4 ± 0.1%) and faba bean cv. Athena (0.6 ± 0.1%) and the adaxial surface of chickpea cv. CDC Orion (0.1 ± 0.0%) and field pea cv. Alaska (0.5 ± 0.5%). Phosphorus was present on the adaxial surface of field pea cv. Alaska (2.3 ± 0.4%) and abaxial surface of lentil cv. CDC Greenstar (2.9 ± 1.0%). Chlorine was found only on the abaxial surface of the chickpea cv. CDC Consul. It is also noteworthy that in the present study, no harmful heavy metals were detected on the cotyledon abaxial or adaxial surfaces.

The presence of K, P, Mg, Ca and Na as the key minerals in faba bean flour reported by Multari et al. (2016) is comparable to the surface elemental composition of faba beans determined in this study, except for P, which could be associated with phytic acid. Faba bean Athena is a high-tannin cultivar in contrast to Snowbird, which is a low-tannin cultivar. Tannins have been confirmed to be present mainly in the testa of faba beans in the form of condensed tannins and are known to be associated with the dehulling efficiency of seeds (Martin et al., 1991; Mwasaru et al., 1988). Condensed tannins present in the cotyledon can bind to proteins minimizing their bioavailability (Multari et al., 2015; Wang et al., 2010). The presence of tannins could have interfered with the detection of N on Athena cotyledon abaxial and adaxial surfaces by significantly minimising the amount of N detected on the subsurface through protein-tannin complex formation. This is not only true for faba bean. Tannins are also present in chickpeas (Wang et al., 2010), field peas (Wang et al., 1998) and lentils (Neves, & Lourenço,1988) and could be associated with the absence of N in both lentil cultivars and field pea cv. Carneval based on the EDS results of the present study. Similarly, N was detected on both the abaxial (0.8 ± 0.8%) and adaxial (1.4 ± 0.3%) surfaces of chickpea hard-to-mill Kabuli type cv. CDC Orion. This was analogous to the presence of protein in the abaxial and adaxial

surfaces of Australian Kabuli-type chickpea cv. Bumper was confirmed by compositional analysis (protein content 24.86%: N= 3.98%) of the 29% pearling flour fraction of the outer periphery of the cotyledons (Wood et al., 2014a, 2014d). Canadian Desi-type chickpea cv. CDC Consul contained N only on the abaxial surface (0.4 ± 0.1%), which could be related to its easy-to-mill nature. However, the presence of a protein-filled outer epidermis has been confirmed on both the abaxial and adaxial surfaces of the Australian Desi-type, chickpea cv. Amethyst by light microscopy analysis (Wood et al., 2011,).

Detection of N on both abaxial (2.2 ± 0.9%) and adaxial (3.1 ± 1.8%) cotyledon surfaces of faba bean cv. Snowbird validates the presence of a proteinaceous substance in the epicuticular wax film, which is known to be associated with hardness (Scanlon et al., 2018). This proteinaceous substance could be associated with lectins commonly found in faba beans. Lectins can also be found in chickpeas and other pulses (Multari et al., 2015), which could be associated with Mg and Ca for the interlocking mechanism at abaxial and adaxial surfaces (Wood et al., 2014b). The interlocking mechanism is associated with the dehulling/milling properties of the beans. On the other hand, epicuticular waxes are known to include fatty acids, primary alcohols, alkyl esters, aldehydes, alkanes, cinnamyl alcohol esters, and alkylresorcinols (Zhao et al., 2019) and are defined as esters of long-chain fatty acids and primary alcohols.

Based on the overall comparisons (Table 3.1), it can be concluded that both the abaxial and adaxial surfaces of the two cultivars of chickpea, faba bean, field pea, and lentil investigated consist of cuticle layers of similar key elemental composition but with a high percentage of carbon (59.5 ± 3.3%) and oxygen (33.9 ± 3.2%) confirming the prevalence of organic molecules comparable to the EDS results of *Leucas aspera*, L. stems (Yashvanth et al., 2013). C and O

alone accounted for 87.3% to 97.4% of the relative elemental concentrations, while N could have exhibited weaker signals.

EDS elemental analysis comes with its limitations. Its elemental detection is not influenced by the chemical nature of the elements, but rather by the inter-elemental overlaps. Elements with an atomic number larger than 10 and with a minimal elemental concentration of 10 ppm are detectable (Pivovarova & Andrews, 2013; Scimeca et al. 2018). Fig. 3.5 presents the EDS spectrum of faba bean cv. Athena abaxial surface as a histogram plot of mid-energy (1-10 keV) X-rays expressed in counts per second. The peak appearing at 2.2 keV corresponding to Au, reflects the gold coating of the samples. Two different peaks for the elements K and Ca were observed probably due to multiple relaxation pathways giving multiple emission lines. Heavier elements have more orbitals, and therefore more lines giving rise to different X-ray energy levels, such as k-alpha, and k-beta (Pivovarova & Andrews, 2013).

Single-shelled hydrogen was not detectable since it has no core electrons but valence electrons, which participate in chemical bonding. These signals from H can overlap with the signals from the excitation of valence electrons of other exterior atoms present (Stojilovic, 2012). Furthermore, the X-ray take-off angle was not constant due to the undulating cell topographies. Therefore, the mass concentration (%) values are not absolute but relative; thus, the elemental analysis results should be considered semi-quantitative. However, since these EDS results were used for key elemental comparisons of abaxial and adaxial cotyledon surfaces, these limitations did not interfere with the interpretations.

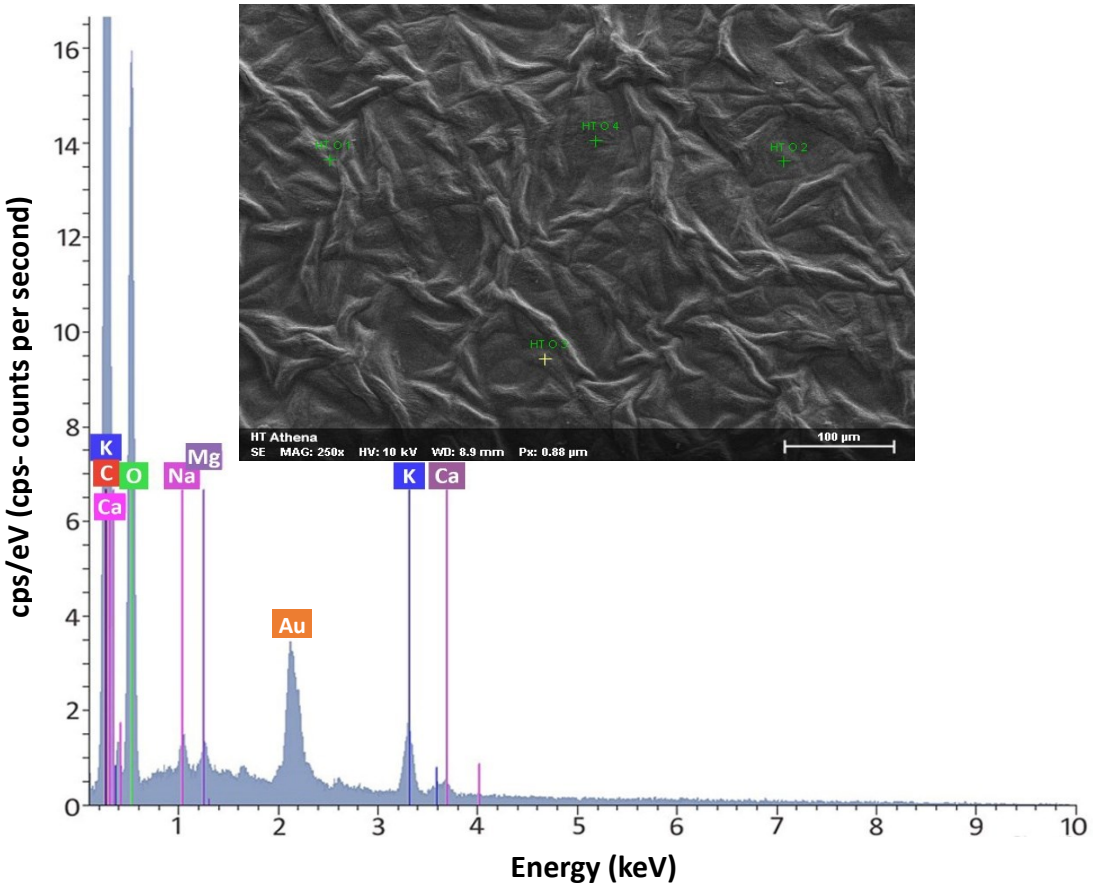

Fig. 3.5. EDS spectrum of faba bean Athena abaxial surface.

The dietary fibre present in pulses contains a complex mix of heterogenous substances such as cellulose, hemicellulose, pectin, lignin and gums (Vasić et al., 2009) and these adhesive gums are complex carbohydrates, which can include salts of K, Mg or Ca as detected on all the abaxial and adaxial pulse cotyledon surfaces tested in this study (Table 3.1). Moser (1998) used cellulase and pectinase enzymes to digest the faba bean cotyledon surface compounds and identified a cutinized cell wall and lignin, which are complex carbohydrates insoluble in water. Although not capable of identifying or quantifying the cuticular wax profiles, the EDS elemental analysis was useful to fill the gap in the understanding of the cuticular surface elemental chemistry of the pulses evaluated. In summary, epicuticular wax films can be

claimed to be a complex layer of different bio-macromolecules (e.g. complex carbohydrates, cutins and proteins) embedded with micro-molecules (e.g. calcium oxalate or calcium carbonate), which could be correlated to the higher standard deviations observed in the elemental compositions.

3.4. CONCLUSIONS

SEM's and HIM's revelation of exomorphic microstructural features and elemental information of epicuticular wax projections facilitated the understanding of similarities/differences between the abaxial and adaxial cotyledon surfaces of different pulses. These findings confirm that the adaxial surface of all the cultivars of chickpea, faba bean, field pea and lentil cotyledons examined were cemented by an epicuticular wax film similar to the abaxial surface. However, the morphological characteristics based on the shape of the epidermal cells, amount of projection and micromorphology of the epicuticular wax films differed substantially depending on the species, cultivar (genotype) and location (abaxial or adaxial). These differences can influence their milling behaviours in terms of the ease of pearling or grinding, involving the separation of cotyledon-seed coat and cotyledon-cotyledon interfaces, which ultimately impacts the energy requirements.

This is the first report on the semi-quantitative surface elemental composition of chickpea, field pea, faba bean and lentil abaxial and adaxial topographies. Both the abaxial and adaxial surfaces of all eight cultivars consisted of cuticle layers of a similar key elemental composition: C>O>K>Mg>Ca alongside the minor elements N, Na, P and Cl. However, the elemental mass concentrations significantly varied ($P < 0.05$) between the abaxial and adaxial surfaces of field pea Alaska (C%), chickpea Orion (Ca%), faba bean Athena (O, K and Mg%),

faba bean Snowbird (Ca and Mg%) and lentil Greenstar (K%). No significant variations ($P > 0.05$) were observed in the grand mean elemental composition of pulse abaxial and adaxial surfaces, except for Ca. These compositional variations were also dependent on the species, cultivars (genotypes) and the location of the topographical surface. The ease of milling or pearling of pulses could be directly associated with the architecture and micromorphology of the abaxial and adaxial surfaces and their chemical composition. The findings would be of great value to understanding pulse dehulling, splitting and grinding characteristics as well as to interpreting the compositional differences observed in the pearled flour fractions, which can ultimately enhance process design, targeting value-added pulse ingredients from different pulse flour fractionation approaches.

CHAPTER 4: Potential of sequential pearling to explore macronutrient distribution across faba beans (*Vicia faba* L.) for chemical-free hybrid fractionation[3]

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CHAPTER 4

4.1. INTRODUCTION

Affiliated with the declaration of 2016 as the 'International Year of Pulses' by the sixty-eighth session of the United Nations General Assembly, value-added utilization of beans, lentils, peas and other edible dry leguminous seeds has grown recently around the world (Joshi & Rao, 2017). Faba bean (*Vicia faba* L.), also known as fava bean or broad bean is a symbiotic nitrogen-fixing legume (Allen, 2013). It is a good source of lysine-rich proteins (~22-38%, N×6.25) and contains a broad range of essential amino acids that are complementary to cereal proteins (Mayer-Labba, et al., 2021; Millar et al., 2019; Multari et al., 2016), and thus can enhance the poor protein quality of cereal-based staple foods (Mattila et al., 2018).

Although produced on a large scale in many parts of the world, from a dietary standpoint, it is underutilized as a food ingredient due to the presence of several anti-nutritional factors described in Chapter 2 (Section 2.2.2). The levels of these negative factors can be minimized by different fractionation approaches. Although conventional dry fractionation methods are a sustainable alternative to energy-intensive wet fractionation methods, they can only lead to relatively modest enrichment in protein content and are not efficient in removing these negative factors (Schutyser et al., 2015; Schutyser & van der Goot, 2011). The formation of the problematic amino acid derivative lysinoalanine (LAL) has been linked to alkali extraction and a lesser extent to thermal treatment (Gould & MacGregor, 1977). Levels of LAL present in alkali-treated soy protein isolates had a negative correlation to net protein utilisation and nephrotoxic reactions in rats (Gould & MacGregor, 1977). Therefore, wet processing

methods free of alkali and thermal treatments should be explored to cater to the consumer demand for clean-label food ingredients.

Hybrid processing employing a combination of dry and aqueous fractionation has gained attention to enhance protein purity with the expense of less water and energy as compared to wet fractionation alone (Schutyser et al., 2015). The optimization of protein fractionation warrants the understanding of the distribution patterns of the macro-components such as starch, protein and dietary fibre within the bean. The concept of general-purpose tangential abrasive dehuller (TAD), which employs abrasive action has been previously used to study the dehulling properties of cereals (Oomah et al., 1981; Sahay, 1990) and legumes (Oomah et al., 2010; Opoku et al., 2003; Reicher et al., 1984; Sahay, 1990). Unlike dehulling, which is specific to the removal of the inedible hull, pearling is the gradual removal of grain/kernel tissues from the surface towards the interior by abrasive action (Yeung & Vasanthan, 2001). Pearling has been investigated to develop functional food ingredients based on the degree of component distribution in barley (Blandino et al., 2015; Liu et al., 2009; Yeung & Vasanthan, 2001) and wheat kernels (Hemery et al., 2007). Pearling has also been used to remove the saponin-rich outer layers of quinoa (Mattila et al., 2018); to compare the chemical distribution of dehulled whole and split chickpeas (Wood et al., 2014a, 2014b); and to understand the protein, lipid and fatty acid distribution in dehulled whole peas (Kosson et al., 1994).

Understanding the distribution of macronutrients in the tissues of pulse grains would be beneficial for optimizing the level of dehulling/pearling for protein isolation. However, there are no studies to date that report the macro-component distribution pattern of faba beans or the applicability of sequential pearling to understand its nutrient distribution. Further work is also

warranted to produce different fractions by pearling for use as the feed material for wet fractionation of proteins. Therefore, the objectives of this study were: a) to determine the distribution of the nutrients (ash, dietary fibre, protein, and starch) in faba beans using sequential pearling, b) to apply the macronutrient distribution patterns to produce protein-enriched fractions, and c) to develop a hybrid fractionation approach to isolate proteins by subsequent aqueous extraction of the protein-enriched fractions from pearling. Meeting these objectives is valuable for sustainable process development, targeting protein isolation from faba beans and value-added utilisation of the co-products.

4.2. MATERIALS AND METHODS

4.2.1. Materials

High-tannin faba bean cultivar, Athena (HT) originated from "91020" × "Quatro" and low-tannin faba bean cultivar, Snowbird (LT) originated from "Alfred B" × "8103" were provided by W.A. Grain and Pulse Solutions, Innisfail, AB, Canada. All the reagents were analytical reagent grade except for acetonitrile, which was HPLC gradient grade (Fisher Scientific, Waltham, MA, USA). The analytical standards (purity ≥ 95%), raffinose, stachyose and verbascose, and all the enzymatic assay kits were supplied by Megazyme International Ltd. (Bray, Wicklow, Ireland). Gallic acid and tannic acid standards (purity ≥ 95%) and Folin Ciocalteu's reagent were acquired from Sigma Chemical Co. (St. Louis, MO, USA). Milli-Q water was obtained from a water purification system (Millipore Corp., Bedford, MA, USA).

4.2.2. Sample preparation

4.2.2.1. Abrasive dehulling and sequential pearling

Whole faba bean cultivars HT and LT (0.5 kg) were dehulled for 30 s in six trials through a prototype model tangential abrasive dehuller (TAD) developed by the former Agricultural Value-Added Engineering Centre (AVEC) of Bio-industrial Opportunities Section, Alberta Agriculture, Forestry and Rural Economic Development, Edmonton, AB, Canada. The feed size was optimized based on the dehulling efficiency of TAD, which had an integral milling chamber with eight vertically aligned abrasive disks mounted on a common drive shaft driven by a 0.6 kW motor. Following dehulling, two batches were pooled, and the groats (dehulled grains) were manually separated from the hulls and the by-products using sieves (0.85 mm and 2.0 mm). The groats were further subjected to batchwise sequential pearling in 9 cycles, 30 s each, monitored by time control, ultimately resulting in 10 different pearling flour fractions and residual pearls. At the end of each cycle, the pearling flour fractions were passed through a 0.5 mm sieve and separated from the pearls and by-products (0.85 mm and 2.0 mm sieves). The motor frequency (100 Hz) and rotational speed (~2900 rpm) were kept constant for all three trials.

4.2.2.2. Single-step pearling

Whole faba bean cultivars HT and LT (0.5 kg) were dehulled for 30 s in six trials through a TAD based on sequential pearling results. Following the separation of beans from the hulls by aspiration, the groats were pearled for 270 s (corresponding to cycles 2-10 above) in six trials, two of which were pooled forming three batches. All the pearling flour fractions

were passed through a 0.5 mm sieve and the by-products were separated by sieving (0.85 mm and 2.00 mm sieves).

4.2.2.3. Manual dehulling

Both HT and LT unprocessed beans were conditioned (1:3, w:w) with Milli- Q water at room temperature (23 ± 1°C) for 2 h, drained and manually dehulled following the protocol of Chapter 3 (Section 3.2.2). The beans were dried for 3 h at 30 °C in a forced-air oven (Fisher Scientific Isotemp 750G Oven, Missouri City, TX, USA).

4.2.2.4. Grinding

The manually dehulled faba beans and the pearls coming from sequential and single-step pearling treatments were ground at 8000 rpm in an ultra-centrifugal mill (ZM 200, Retsch mill, Hann, Germany) equipped with a 0.5 mm ring sieve. All the flour fractions (≤ 0.5 mm) were sealed in air-tight heavy-duty plastic containers and stored at 23 ± 3 °C until analysis.

4.2.3. Hybrid fractionation

The specific conditions of the hybrid fractionation protocol of faba bean are depicted in Fig. 4.1. Briefly, HT/LT faba bean single-step pearling flour fraction was homogenised (Polytron, PTMR 2100, Kinematica AG, Malters, Switzerland) with water at a solvent/feed (S/F) ratio of 2 (v/w) ratio followed by overhead stirring (Heidolph RZR 2021, Schwabach, Germany). After repeating these steps, the wet sieved (WS Taylor's Sieve Shaker, RX-812-CAN, Mentor, OH, USA) retentates were combined to produce the dietary fibre fraction. The filtrate resulting from wet sieving was centrifuged (Beckman Coulter, Avanthi SER JSE10K11,

Indianapolis, IN, USA) and the residue was subjected to further steps to recover the starch fraction. The supernatant from step 8 was treated with cold water following the method of Murray et al. (1979) with minor modifications.

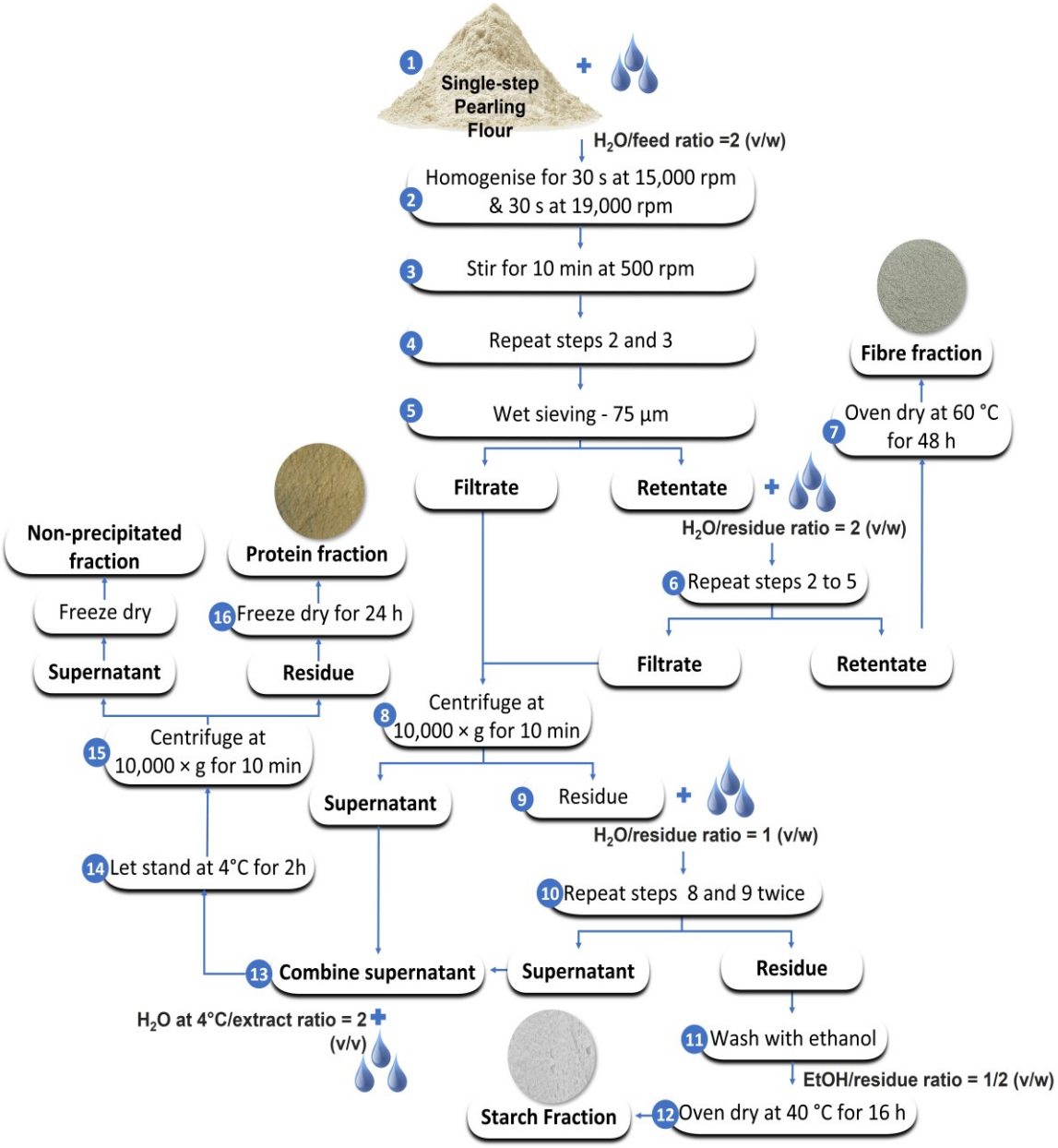


Fig. 4.1. Flow chart for the aqueous fractionation of faba bean to isolate micellar proteins, starch isolates and dietary fibre concentrates.

It is hypothesized that precipitation of aqueous fractionated proteins was achieved by the protein micellization concept. The protein and non-precipitated fractions were freeze-dried (Labconco Freezone 18, Kansas, MO, USA) whereas the starch and dietary fibre co-products were oven-dried (Fisher Scientific Isotemp 750G, Missouri City, TX, USA). All the fractions obtained according to the method depicted in Fig. 4.1 were ground (<0.5 mm) and stored at 23 ± 3 °C until further analysis.

4.2.4. Characterisation

The sequential pearling fractions were characterised by mass balance, proximate composition, total dietary fibre (TDF) and total starch (TS) contents, and morphology. Single-step pearling fractions were assessed for the above characteristics in addition to resistant starch (RS), non-resistant/solubilised starch (SS), total raffinose family oligosaccharides (RFOs) and their components (raffinose, stachyose and verbascose), as well as total polyphenol (TPP) and total tannin (TT) contents in comparison to the whole and manually dehulled bean flours. Hulls were assessed for moisture, ash, protein, TPP and TT contents. The fractions of the hybrid scheme (Fig. 4.1) were profiled for mass balance and nitrogen, protein, total starch and dietary fibre on a dry matter basis. The protein fractions were also assessed for their morphology and protein secondary structural changes.

4.2.4.1. Morphological characterisation

The morphological characteristics of flour fractions were examined under high vacuum using a variable pressure field emission scanning electron microscope (VP-FESEM, Carl Zeiss

Sigma 300, Oberkochen, BW, Germany) after gold coating as described previously in Chapter 3 (Section 3.2.3).

4.2.4.2. Chemical characterisation

4.2.4.2.1. Determination of proximate composition

Dry matter content (%) was determined in a forced-air oven (Fisher Scientific Isotemp 750G, Missouri City, TX, USA) by loss in mass at 105 ± 1 °C for 24 h (Altuntaş & Yildiz, 2007). Ash content was determined according to AOAC 923.03 method (AOAC, 2000) using a muffle furnace (Thermolyne Corporation F-A1730, Dubuque, IA, USA) at 550 ± 1 °C for 16-18 h. Protein content was determined based on apparent nitrogen (N)% according to the Dumas principle using an elemental analyser (Leco TruSpec- FP-428, Mississauga, ON, Canada), which was calibrated with ethylenediaminetetraacetic acid (EDTA) and rye flour. Crude protein content (%) was calculated based on the protein conversion factor of 6.25 as proposed by the AOAC 999.23 method (AOAC, 2000) and compared to the use of 5.85 (Murray et al., 1979) for protein isolates. The same elemental analyser was used to analyse the carbon (C)% content.

Crude fat content was determined gravimetrically following the extraction of fat from ~0.500±0.010 g of sample into 10 mL hexane in pre-weighed 16 mm x 125 mm screw cap culture tubes with polytetrafluoroethylene (Teflon) lined caps in a shaking water bath (Jeio Tech BS-06/11/21/31, Seoul, South Korea) for 16 h (25±1 °C, 100 rpm). They were vortexed at 10,000 rpm for 15 s and centrifuged (Eppendorf AG 5810, Hamburg, Germany) for 10 min at 1900 ×g. Using a Pasteur pipette, hexane was transferred to another set of pre-weighed culture tubes. Complete transfer of hexane was not necessary since the amount of hexane

transferred was considered in the calculations. Hexane was removed under a stream of nitrogen in a water bath set at 50-60 °C (Yoshida et al., 2008). It is assumed that crude fat is evenly distributed in the hexane layer, thus the proportion of the hexane transferred is equivalent to the proportion of the sample represented by the extracted crude fat. Crude fat content can be calculated based on Eqs. (4.1) and (4.2).

$$Corrected\ sample\ weight\ (g) = \frac{Hexane\ transferred\ weight\ (g)}{Intital\ hexane\ weight\ (g)} \times Sample\ weight\ (g) \quad (4.1)$$

$$Crude\ fat\ (\%) = \frac{Crude\ fat\ weight\ (g)}{Corrected\ sample\ weight\ (g)} \times 100 \quad (4.2)$$

4.2.4.2.2. Determination of carbohydrate components

The contents of soluble dietary fibre (SDF), insoluble dietary fibre (IDF) and total dietary fibre (TDF=IDF+SDF) were analysed using the Megazyme 2017 K-TDFR assay method (Megazyme International Ltd., Bray, Wicklow, Ireland). Samples (0.5000±0.010 g) were treated with 10 mL ethanesulfonic acid-tris (hydroxymethyl) aminomethane (MES-TRIS) buffer (pH 8.2) and the rest of the procedure was kept the same.

Total starch content was measured according to the Megazyme 2017 K-TSTA assay procedure (Megazyme International Ltd., Bray, Wicklow, Ireland). Due to the presence of resistant starch, flour samples were pre-treated with dimethyl sulfoxide following the addition of aqueous ethanol (80% v/v) and analysed according to the adaptation of the AOAC 996.11 method with spectrophotometric (Jenway 6300, Essex, England, UK) assessment at 510 nm. Resistant and non-resistant starch contents were determined colourimetrically according to the Megazyme 2019 K-RSTAR assay protocol (Megazyme International Ltd., Bray, Wicklow, Ireland). The TS content was also expressed as the sum of RS and SS contents.

Total galactosyl-sucrose oligosaccharides, raffinose, stachyose and verbascose were determined using the Megazyme 2018 K-RAFGL assay procedure (Megazyme International Ltd., Bray, Wicklow, Ireland). α-Galactosidase hydrolyses the RFOs to D-galactose and sucrose. Subsequently, invertase hydrolyses sucrose to D-glucose and D-fructose. D-Glucose content was determined as described above for starch. RFOs contents were expressed on a molar basis as verbascose equivalent.

The RFOs were extracted and analysed for their components, raffinose, stachyose and verbascose according to Landry et al. (2016) with minor modifications. Briefly, 200 mg samples were shaken with 4 mL of EtOH (50%, v/v) for 1 h (Burrell Wrist Action Shaker, 75-765 BT, Pittsburgh, PA, USA) at a high setting (10). The supernatant was collected after centrifugation at 3000 ×g for 10 min (Eppendorf AG 5810, Hamburg, Germany). The pellet was washed with 2.5 mL EtOH (50%, v/v) twice, shaken for 5 min and centrifuged for 5 min. All the supernatants were combined and diluted to 10 mL with EtOH (50%, v/v). An aliquot (2.0 mL) of the dilution (supernatant: EtOH (90%, v/v), 1:1) was stored at -20 °C for 1 h and centrifuged at 10,000 ×g for 10 min (Eppendorf accuSpin Micro, Osterode, Germany) to precipitate the proteins. The extracts were vacuum-dried (Eppendorf™ Vacufuge™ Concentrator 022820001, Schwabach, Germany) at 45 °C under V-AL mode for 1 h and dissolved in 1 mL Milli-Q water for high-performance liquid chromatographic analysis (Shimadzu 20A HPLC, Canby, OR, USA) of raffinose, stachyose and verbascose and free sucrose and D-glucose contents concurrently.

The HPLC instrument consisted of a solvent delivery system, degasser, temperature-controlled column oven and autosampler, and was coupled to an evaporative light-scattering detector (Altech, ELSD 3300HP, Deerfield, IL, USA). The separation of the analytes was

carried out on a Supelcosil^{TM} LC-NH_2 (250 × 4.6 mm, 5 µm) resin-based column (Sigma-Aldrich Co. LLC, Bellefonte, PA, USA). The elution was performed at 30 °C with mobile phases A (0.05% formic acid) and B (0.05% formic acid in acetonitrile) with a 1.5 mL/min flow rate. The linear gradient was with 95% mobile phase B for 4 min, then 40% for 4.5 min and 95% until the end of 12.1 min. The injection volume was 20 µL and quantification and assignment of peaks were based on the peak areas and retention times (Appendix A: Fig. A.1) of the standard (raffinose, stachyose and verbascose) curves plotted with concentrations between 10 and 500 µg/mL and internal standard lactose. Individual reference response factors were used to quantify the individual RFOs. The ELSD temperature was set at 50 ºC, the gas flow was maintained at 1.8 mL/min and the gain was 1. The linearity of analysis was assessed based on standard curves at concentrations between 50 and 120% of the working range in sample concentrations. The minimal detection limit across analytes was between 50 and 60 µg/mL.

4.2.4.2.3. Determination of protein secondary structure conformational changes

Flour samples in their dry form were evaluated for molecular orders and secondary structural changes of the protein molecules by Attenuated Total Reflection-Fourier Transform Infrared Spectroscopy (ATR-FTIR, Alpha, Bruker, Berlin, Germany). ATR-FTIR spectra were collected at a resolution of 4 cm^{-1} by accumulating 64 scans to obtain average analytical results and enhance the signal-to-noise ratio in the range of 4000-400 cm^{-1}. Transmittance data were converted to absorbance and ATR correction was employed in OMNIC 9.2.86 (Thermo Fisher Scientific Inc., Dubuque, IA, USA). Protein secondary structural changes were identified by analysing the amide I region (1700-1600 cm^{-1}).

The second derivative spectra of the amide I region were obtained through Origin 2021b software (Origin Lab Corporation, Northampton, MA, USA) to identify any protein secondary structural changes resulting from the hybrid fractionation. As a first step, baseline correction was performed by selecting the Quick Peak option of Gadgets. Then, the Mathematics/Differentiate option of Analysis was used to generate the second derivative spectra, following the Savitzky-Golay smoothing function with a polynomial order of 2 and points of window of 7. The secondary structure-related peaks in the amide I region were compared to the literature (Wang et al., 2013; Yang et al., 2018). Peak fitting was performed by selecting the Gaussian Peak function of the Multi Peak Fit option of Analysis.

4.2.4.2.4. Determination of total polyphenol and total tannin contents

Total polyphenol content (TPP) was determined following the ISO 14502-1 method (ISO, 2005) with few modifications. Flour samples (0.2500 ± 0.0100 g) were extracted with 2.50 mL of 70% methanol (v/v) in a water bath at 70 ºC. The samples were vortexed at the beginning of 20 min and every 5 min. The extracts were cooled to room temperature and centrifuged at 1095 ×g for 10 min. The flour residue was re-extracted following the above steps and the supernatants were combined and topped up to 5.00 mL with 70% methanol and dilutions were done with distilled water as needed.

Aliquots (250 µL) of gallic acid standard solutions (5 to 200 µg/mL) and diluted sample extracts were allowed to react with 1.00 mL of 7.5% (w/v) sodium carbonate solution within 3 to 8 min following the addition of 1.25 mL of 10% (v/v) Folin-Ciocalteu's phenol reagent. These tubes were vortexed and allowed to stand at room temperature for 60 min and the absorbance was measured at 765 nm. The concentration of total polyphenolics in the liquid

extracts was expressed as milligrams of gallic acid equivalent (GAE) per gram of sample dry weight.

An aliquot (1.75 mL) of the sample extract was mixed with 125 µL of 10% Folin's reagent and 250 µL of saturated Na_2CO_3 (7.5%, w/v) solution to analyse the total tannin (TT) content (Kyamuhangire et al., 2006). The mixture was vortexed for 15 s, incubated for 30 min and spectrometric absorbance was read at 700 nm. The concentration of tannins in the liquid extracts was expressed as milligrams of tannic acid equivalent (TAE) per gram of sample dry weight based on the tannic acid standard calibration curve (5 to 500 µg/mL).

4.2.5. Statistical analysis

All the results are expressed on a dry weight basis (db) except for Table 4.1. All the sequential pearling results expressed as mean ± SD were based on duplicate analyses of three independent samples (n=6) except for TDF results (n=3). Single-step pearling and hybrid fractionation results are based on three independent samples (n=3). Significant differences ($P < 0.05$) between samples were tested by two-way analysis of variance (ANOVA) followed by Duncan's multiple range test. Any comparison between two sets of data was tested at a 95% confidence level according to the two-sample t-test. Additionally, to understand the correlation between different analysed parameters with the level of pearling, Pearson's correlation analysis was performed ($P < 0.05$). All these statistical analyses were carried out using SAS Studio University Version for Windows (SAS Institute Inc., Cary, NC, USA).

4.3. RESULTS AND DISCUSSION

4.3.1. Macronutrient distribution of sequential pearling fractions

The external layers of the dehulled grains (groats) removed during abrasive pearling are known as pearling flour, whereas the remainder is pearled grain referred to as pearl (Fig. 4.2). It is important to note that during pearling of pulses, some get pearled as whole beans while some can split and get pearled as split beans. According to the surface morphological and elemental analyses of the faba bean cultivars LT and HT (Chapter 3, Section 3.3), the split surface (adaxial) of the bean was cemented by an epicuticular wax film with a key elemental composition similar to that of their outer cotyledon surface (abaxial). Therefore, the splitting of the beans during the pearling process was disregarded and the whole and split beans were not separated.

The results presented in Table 4.1 express the yield of different seed fractions resulting from sequential pearling. The pearling flour removal rates on average were higher for HT in comparison to LT during each cycle, although HT was difficult to split. The total pearling flour yields from the 2^{nd} to 10^{th} cycle were 23.4 ± 0.4% for LT and 30.5 ± 3.8% for HT. The remaining pearls after 270 s of pearling (LT: 49.3% and HT: 43.3%) account for 56.4 ± 3.6% and 50.3 ± 4.1% of the cotyledon, respectively. In contrast, 5 min of abrasive pearling of whole/split chickpeas has only resulted in 59-65% pearls relative to the initial seed weight (Wood et al., 2014a, 2014d). Differences in the level of dehulling/pearling cannot be solely attributed to the pearling time, but rather to the type of dehuller/pearler used and the genotypic variations of the grain (Reichert et al., 1984). Genotypic differences in the dehulling/pearling behaviour have been previously reported for Desi and Kabuli-type chickpea cultivars (Wood et al., 2014a, 2014d).

Fig. 4.2. Faba bean pearling process and the fractions obtained.

Dehulled cotyledons were pearled for 270 s to obtain the single-step pearling flour fraction, which encompasses the sequential pearling flour fractions 2-10.

Table 4.1. Mass balance (wt.%) of faba bean sequential pearling fractions relative to the starting raw material

Sample	Step	Level of Pearling (%)	Pearling Flour (%)	Pearls (%)	Hulls (%)	By-products (%)
Snowbird	1	0-2	1.45 ± 0.13	82.07 ± 1.47	12.19 ± 1.09	3.43 ± 0.20
(LT)	2	2-5	2.46 ± 0.21	76.06 ± 2.88	0.63 ± 0.14	1.93 ± 0.57
	3	5-9	2.75 ± 0.44	71.41 ± 3.83		1.02 ± 0.27
	4	9-12	2.81 ± 0.07	68.30 ± 3.05		0.60 ± 0.12
	5	12-15	2.72 ± 0.15	64.76 ± 3.39		0.43 ± 0.08
	6	15-18	2.64 ± 0.03	61.94 ± 2.82		0.30 ± 0.06
	7	18-21	2.67 ± 0.17	58.52 ± 3.23		0.22 ± 0.04
	8	21-24	2.65 ± 0.05	55.19 ± 3.71		0.11 ± 0.05
	9	24-27	2.61 ± 0.24	52.56 ± 3.28		0.10 ± 0.04
	10	27-30	2.36 ± 0.06	49.30 ± 3.80		0.08 ± 0.03
Total	1-10	0-30	24.70 ± 0.42		12.73 ± 1.26	8.22 ± 1.20
Athena	1	0-2	1.69 ± 0.16	79.94 ± 1.42	13.84 ± 0.55	3.17 ± 0.17
(HT)	2	2-7	3.73 ± 0.69	73.64 ± 2.72	0.28 ± 0.13	1.71 ± 0.57
	3	7-12	3.80 ± 0.93	68.84 ± 3.50		1.05 ± 0.48
	4	12-17	3.70 ± 0.64	64.83 ± 3.49		0.42 ± 0.11
	5	17-21	3.51 ± 0.42	60.96 ± 3.58		0.30 ± 0.04
	6	21-25	3.38 ± 0.34	57.35 ± 3.50		0.26 ± 0.06
	7	25-29	3.30 ± 0.33	53.46 ± 3.92		0.25 ± 0.04
	8	29-32	2.87 ± 0.10	50.37 ± 3.51		0.20 ± 0.04
	9	32-36	3.27 ± 0.58	46.98 ± 3.57		0.18 ± 0.02
	10	36-39	2.95 ± 0.19	43.28 ± 3.69		0.19 ± 0.03
Total	1-10	0-39	32.20 ± 3.84		14.11 ± 0.45	7.74 ± 1.06

Mean ± SD (n = 3)

Step 1: dehulling for 30 s, Steps 2-10: sequential pearling for 30 s per cycle using TAD.

The varietal differences in the present study could be associated with seed hardness, their endurance to splitting (Reichert et al., 1984; Scanlon et al., 2018; Schutyser et al., 2015), composition and interactions at their abaxial (cotyledon-seed coat interface) and adaxial (cotyledon-cotyledon interface) surfaces (Chapter 3, Section 3.3). The high tannin cv. Athena had higher ($P < 0.05$) hull content as compared to the low tannin cv. Snowbird according to both mechanical dehulling (Table 4.1) and manual dehulling (HT: 14.4% and LT: 12.3%) results. Vacuum cleaning/dusting of the pearling equipment after each pearling step to minimize contamination between cycles resulted in an average loss of 4.3 ± 0.1% for LT and 3.8 ± 0.2% for HT in addition to the by-products mainly consisting of broken groats (chips) and hulls. To minimise the loss of cotyledon fragments during mechanical dehulling in the TAD, manually dehulled beans were used to represent the cotyledon fraction.

The schematic drawing in Fig. 4.2 depicts the degree of pearling being explored as a function of time (30 s per cycle), and the SEM images (Fig. 4.3a1-b5) correspond to their morphology, revealing the distribution pattern of macronutrients (dietary fibre, protein and starch). Pearls of LT (Fig. 4.3a5) and HT (Fig. 4.3b5) have more starch granules that are detached from the protein matrix and cell wall material as compared to the rest of the pearling flour fractions (Fig. 4.3a1-a4 and Fig. 4.3b1-b4). The pearls were milled (≤0.5 mm sieve) prior to SEM analysis, whereas the pearling flour fractions (≤0.5 mm) were directly obtained by the abrasive action of the TAD. Despite the mechanical differences, both ultra-centrifugal milling and abrasive pearling steps could reduce adhesion between starch granules and small protein bodies (Saldanha do Carmo et al., 2020), leading to their detachment from each other and other large cellular fibrous components (Fig. 4.3).

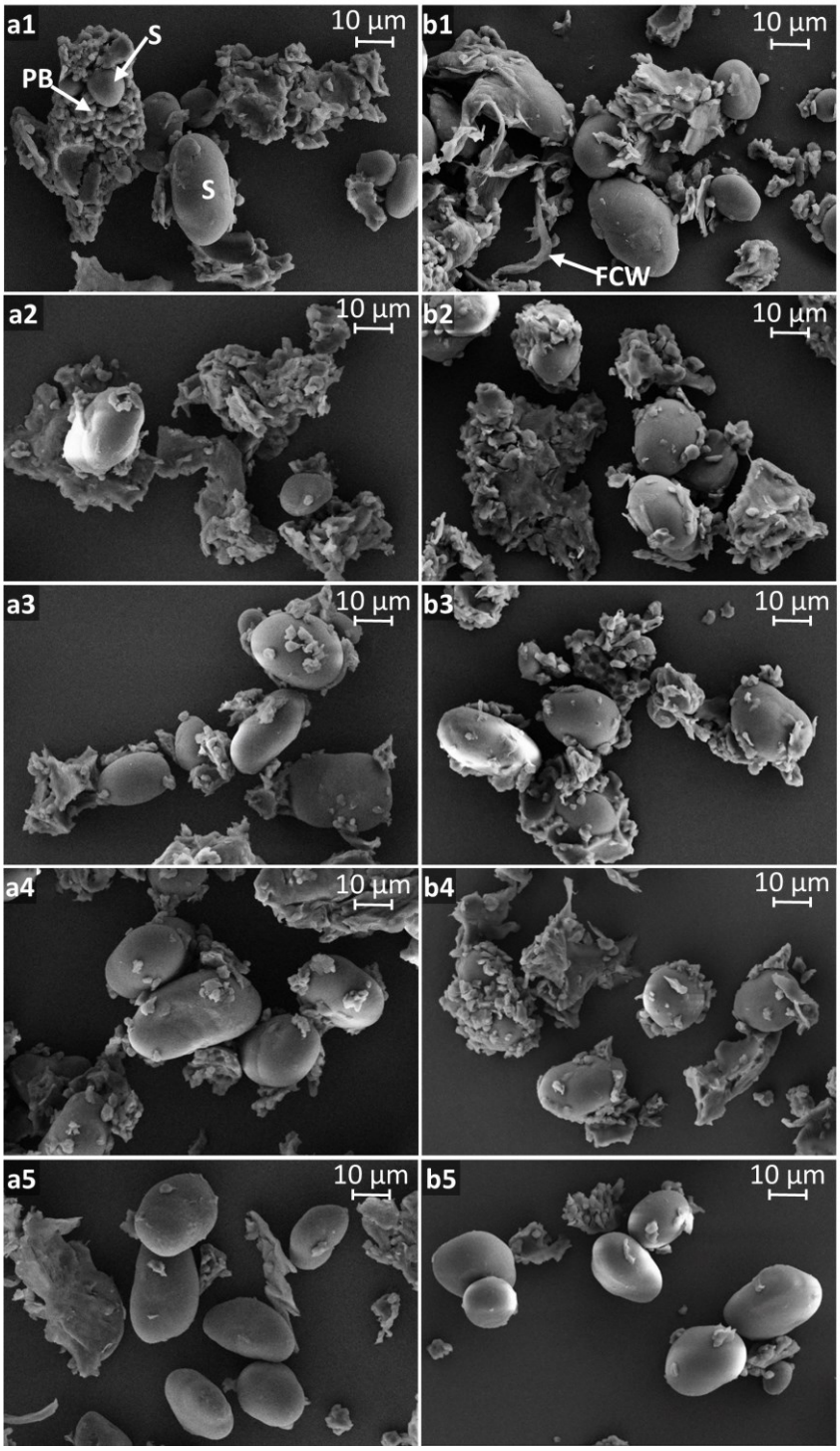
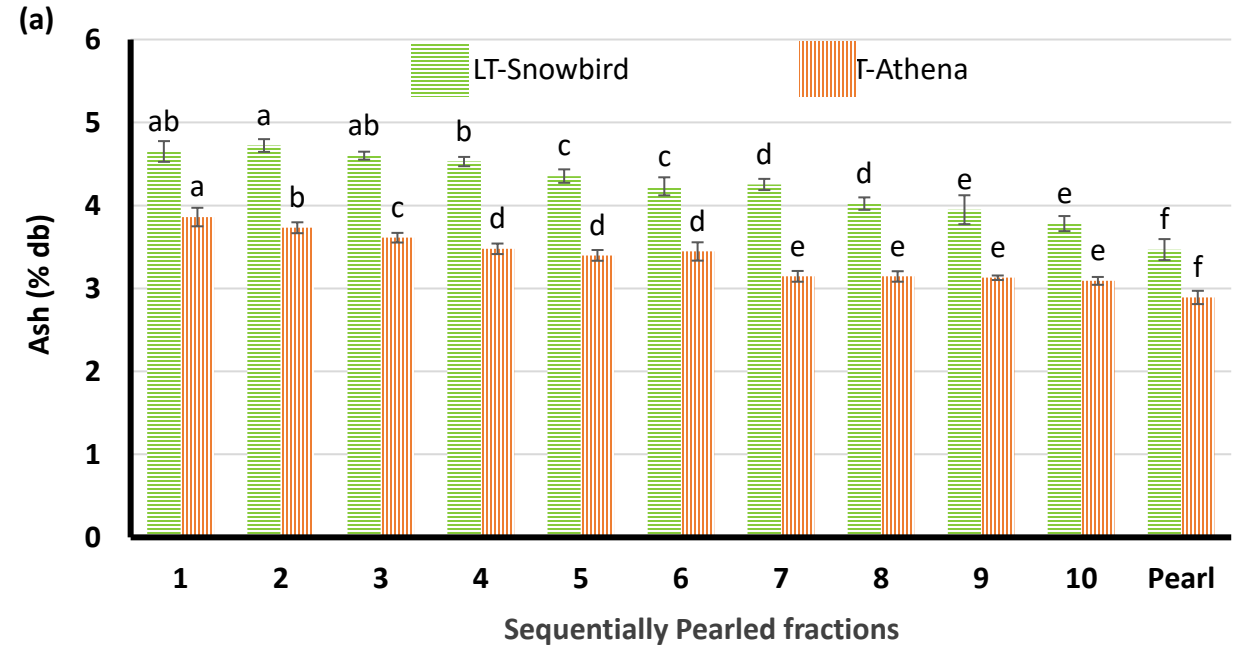


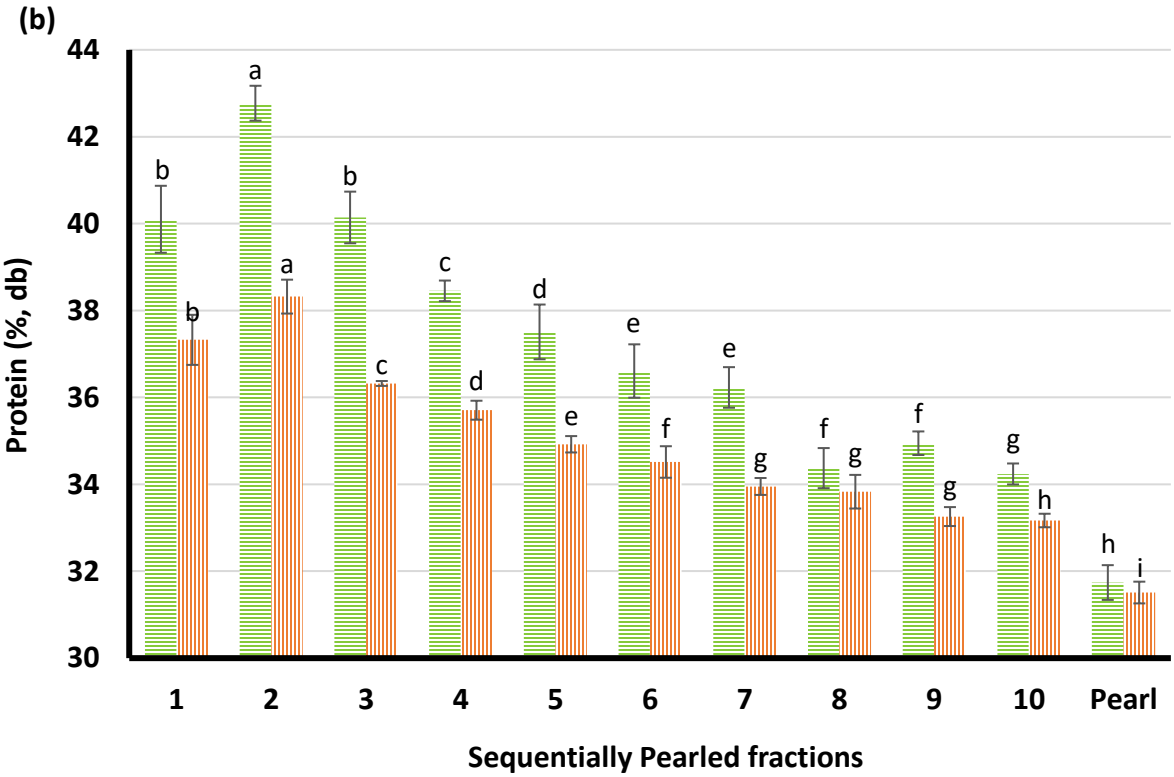
Fig. 4.3. SEM images of faba bean sequential pearling fractions (≤ 0.5 mm).

Morphological characteristics of (a) Snowbird and (b) Athena with (1) 2nd, (2) 4th, (3) 6th and (4) 8th pearling flour fractions and (5) pearls as shown in Fig. 4.2. Starch granules (S) embedded in a protein matrix (P) with protein bodies (PB) surrounded by a fibrous cell wall material (FCW). (b-c: ×1000 magnification).

The first sequential pearling flour fraction produced by the dehulling step had significantly lower ($P < 0.05$) protein (LT: 40.1 ± 0.8% and HT: 37.3 ± 0.6%) and starch (LT: 24.5 ± 0.4% and HT: 24.0 ± 0.9%), but higher TDF (LT: 30.0 ± 0.7% and HT: 29.6 ± 0.9%) contents as compared to the second pearling flour fractions (Fig. 4.4). These values cannot be solely attributable to the cotyledon external layer composition, since the first layer was contaminated with the hull. According to Karataş et al. (2017), faba bean hulls are abundant in dietary fibre (82.3%), but deficient in ash (4.4%), protein (5.0%) and starch (0.9%). Both LT and HT hulls contained lower protein (4.7 ± 0.1 and 3.9 ± 0.1, respectively) and ash (3.5 ± 0.2 and 3.4 ± 0.2%, respectively) contents as compared to the cotyledon. Dehulling before dry fractionation of faba beans is recommended since hulls may interfere with the separation efficiency by adhering to both protein matrix and starch granules.

The second pearling fraction can be considered the first pure layer of the cotyledon. Faba cotyledon ash content (Fig. 4.4a) reduced steadily from 4.7 ± 0.1 to 3.5 ± 0.1% for LT and 3.7 ± 0.1 to 2.9 ± 0.1% for HT from the 2nd pearling fraction towards the core. This progressive decrease could be due to mineral components being mainly deposited in the outermost fractions of the kernel (Blandino et al., 2015; Hemery et al., 2007). More deposits on the outer layer could also be related to heavy metal (cadmium) contamination in most external fractions, as reported for wheat by Sovrani et al. (2012). However, LT and HT cotyledon surface elemental composition analyses reported previously in Chapter 3 (Section 3.3.2) confirmed the absence of heavy metals, disproving their influence on the distribution pattern.


(a)
LT-Snowbird
T-Athena
Ash (% db)
0
1
2
3
4
5
6
ab a ab b c c d d e e e e f f
1 2 3 4 5 6 7 8 9 10 Pearl
Sequentially Pearled fractions


(b)
Protein (%, db)
30
32
34
36
38
40
42
44
b b a a b c c d d e e f e g f g f g g h h i
1 2 3 4 5 6 7 8 9 10 Pearl
Sequentially Pearled fractions

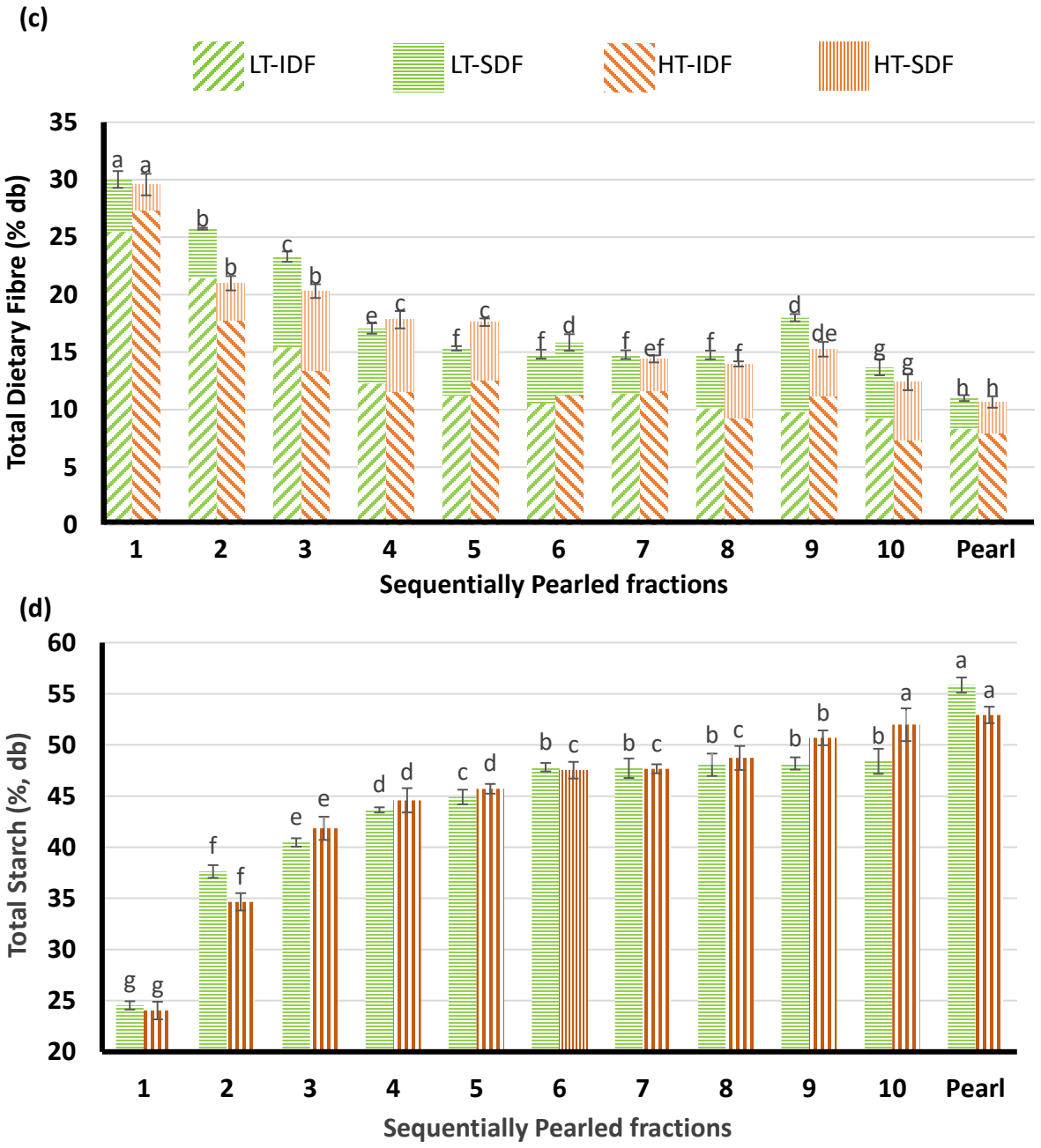


Fig. 4.4. Macronutrient distribution of faba bean pearled fractions: Snowbird (LT) and Athena (HT) faba bean cultivars for (a) ash; (b) protein; (c) total dietary fibre; and (d) total starch contents (%) on a dry weight basis.

Total dietary fibre (TDF) = insoluble dietary fibre (IDF) + soluble dietary fibre (SDF). Pearl: 43-45% of the cotyledon remaining after 270 s of sequential pearling.

[a-i]Mean (n = 6) ± SD values within a particular cultivar with different letters are significantly different ($P < 0.05$) according to Duncan's multiple range test.

The first pearling flour fraction of HT (3.9 ± 0.1%) had a significantly lower ($P < 0.05$) ash content as compared to LT (4.6 ± 0.1%) resembling the trend in the rest of the fractions. This is plausibly due to the high affinity of tannins present in the cv. Athena towards minerals (Multari et al., 2015). Faba cotyledon protein content (Fig. 4.4b) decreased from 42.8 ± 0.4 to 34.2 ± 0.2% for LT from 38.3 ± 0.4 to 33.2 ± 0.2% for HT in the 2-10 pearling flour fractions. The protein content of the remaining pearls (LT: 31.7 ± 0.4%, HT: 31.5 ± 0.2%) was significantly lower ($P < 0.05$) than the rest of the pearling flour fractions. Except for fractions 6 and 7, and 8 and 9 of LT and 7, 8 and 9 of HT, the rest had significant differences ($P < 0.05$) in their protein contents, which was of a gradually decreasing trend across the adjacent layers. On the contrary, higher protein contents were reported in the 10-40% intermediate fraction of barley (Blandino et al., 2015) and 10-15% intermediate fraction of common wheat (Sovrani et al., 2012) compared to the bran layer.

Total dietary fibre content (Fig. 4.4c) of faba pearling flour fractions (2-10) ranged from 25.7 ± 0.1% to 13.7 ± 0.7% for LT and 21.0 ± 0.6% to 12.4 ± 0.7% for HT, which progressively reduced till the 8th fraction for both LT and HT. The 9th fraction had a significantly higher ($P < 0.05$) TDF content (18.0 ± 0.3 and 15.3 ± 0.7 %, respectively) as compared to the 8th fraction (14.8 ± 0.4 and 13.9 ± 0.2%, respectively) for both LT and HT and then declined towards the core. Although barley TDF content had a similar trend, barley β-glucan was reported to be in a greater concentration in the inner 40-100% fraction (Blandino et al., 2015). The TDF distribution profile was reflective of the IDF contents that were higher than the corresponding SDF contents. Although any delay in washing the IDF residues with 95% ethanol and acetone can overstate IDF values leading to an underrepresentation of SDF

values according to the Megazyme 2017 K-TDFR assay protocol, it should not affect the TDF results.

The decreasing contents of protein, ash and TDF in pearling flours were offset by starch contents (Fig. 4.4d), which increased from the 2nd pearling fraction towards the core for both LT (37.6 ± 0.6 to 56.1 ± 1.2%) and HT (34.7 ± 0.9 to 52.9 ± 0.8%). It is also evident from the SEM micrographs (Fig. 4.3) as well as Pearson's correlation analysis (Appendix A: Fig. A.2) that the presence of starch granules gradually increased with the level of pearling while protein and dietary fibre became less apparent. Comparative analyses of the outer periphery and core of chickpeas (Otto et al., 1997; Wood et al., 2014a, 2014b) and pea (Otto et al., 1997) have shown a similar trend. Kosson et al. (1994) also reported higher concentrations of protein and lipids in the outer periphery of peas, which gradually reduced towards the core.

Pearson's correlation analysis showed a strong positive dependency ($r = 0.9$, $P < 0.0001$) between the protein and ash contents (Appendix A: Fig. A.2.a1-a2), and protein and TDF contents (Appendix A: Fig. A.2.b1-b2) with the level of pearling regardless of the cultivar. On the contrary, a strong negative correlation ($r = -0.9$, $P < 0.0001$) between the protein and starch contents was observed (Appendix A: Fig. A.2.c1-c2). It can be concluded that the macro-nutrient distribution pattern observed in this study is specific to pulses rather than cereals coming from the architectural differences of the kernels. These results demonstrate that regardless of the cultivar, by nature, ash, protein and dietary fibre were concentrated in the external layers of faba beans and gradually reduced towards the inner fractions while starch content showed an inverse trend.

4.3.2. Macronutrient composition of single-step pearling fractions

The single-step pearling flour fractions (LT: 55% and HT: 57%) representing a collective fraction of layers 2 to 10 (Fig. 4.2) were produced by taking advantage of the above data on macronutrient distribution. These fractions had a significantly higher ($P < 0.05$) protein content as compared to whole beans (Table 4.2). According to the proximate composition analysis results, single-step pearling flour fractions of both LT and HT also had significantly higher ($P < 0.05$) ash and crude fat contents, as compared to the whole bean. In contrast, the protein and fat contents of the pearls were substantially lower ($P < 0.05$) than the outer periphery, similar to the findings of Otto et al. (1997). The remaining pearls were significantly ($P < 0.05$) richer in starch, as compared to their corresponding whole bean flours regardless of the cultivar (Table 4.2). The total starch results based on both Megazyme 2017 K-TSTA and Megazyme 2019 K-RSTAR (sum of RS and SS) assays were comparable. The remaining starch-rich pearls were 37.4% (HT) to 39.6% (LT) of the raw material by mass accounting for 43.4% and 45.4% of the cotyledon, respectively. These starch-enriched pearls show potential for food applications, for example, as thickeners in soup mixes. The single-step pearling flour yields were higher, and the losses were lower for both LT and HT as compared to the sequential pearling flour outputs.

The crude protein content (N×6.25) of German faba bean cultivars (Makkar et al., 1999) was in the range of 26-30%, whereas a Swedish study (Mayer-Labba et al., 2021) reported it to be in the range of 30-38%. Griffiths and Lawes (1978) reported a protein range of 22-38% for 33 different faba bean varieties and other studies reported 29-30% protein content (Güzel & Sayar, 2012; Karataş et al., 2017; Schutyser & van der Goot, 2011; Sosulski & Youngs, 1979). These findings illustrate the large variability in the composition of faba bean varieties

and lines of diverse origin, grown in different locations around the world. The whole and dehulled faba bean cv. Snowbird has been previously reported to contain 25.85% and 26.27% protein, respectively (Cho et al., 2019). However, these findings cannot be compared to the results of the present study since these results are on a wet weight basis and the protein conversion factor is unspecified.

Table 4.2. Proximate composition, carbohydrate components, total polyphenol and total tannin contents of different faba bean cvs. Snowbird (LT) and Athena (HT) fractions (% dry weight basis).

Component (wt.%)	Snowbird (LT) Fractions			
	Whole	**Dehulled**	**PF**	**Pearl**
Ash	3.68 ± 0.14^{c}	3.98 ± 0.16^{ab}	4.22 ± 0.08^{a}	3.82 ± 0.04^{bc}
Fat	2.50 ± 0.05^{b}	2.18 ± 0.11^{c}	3.06 ± 0.13^{a}	1.71 ± 0.08^{d}
Protein	30.51 ± 0.39^{d}	34.06 ± 0.29^{b}	39.07 ± 0.06^{a}	31.57 ± 0.29^{c}
IDF	10.16 ± 0.44^{a}	9.40 ± 0.69^{b}	9.95 ± 0.73^{b}	8.53 ± 0.23^{c}
SDF	3.18 ± 0.20^{a}	1.42 ± 0.33^{b}	2.89 ± 0.71^{a}	2.50 ± 0.40^{a}
TDF	13.34 ± 0.63^{a}	10.82 ± 0.58^{b}	12.84 ± 0.05^{a}	11.03 ± 0.19^{b}
RS	8.37 ± 0.22^{a}	8.34 ± 0.22^{a}	6.76 ± 0.50^{c}	7.48 ± 0.23^{b}
SS	37.86 ± 0.70^{c}	41.01 ± 0.30^{b}	37.50 ± 0.29^{c}	45.22 ± 0.76^{a}
TS†	46.23 ± 0.50^{c}	49.35 ± 0.37^{b}	44.26 ± 0.27^{d}	52.70 ± 0.53^{a}
TS*	46.23 ± 0.25^{c}	49.03 ± 0.37^{b}	44.18 ± 0.11^{d}	53.72 ± 0.16^{a}
Sucrose	2.070 ± 0.087^{b}	2.369 ± 0.384^{b}	3.081 ± 0.164^{a}	2.202 ± 0.210^{b}
Raffinose	0.139 ± 0.045^{a}	0.136 ± 0.011^{a}	0.148 ± 0.024^{a}	0.127 ± 0.017^{a}
Stachyose	0.768 ± 0.016^{c}	1.016 ± 0.073^{b}	1.377 ± 0.046^{a}	1.292 ± 0.127^{a}
Verbascose	2.492 ± 0.146^{a}	1.752 ± 0.203^{b}	2.077 ± 0.0262^{b}	1.924 ± 0.188^{b}
HPLC RFO	3.399 ± 0.195^{a}	2.904 ± 0.250^{b}	3.603 ± 0.260^{a}	3.343 ± 0.152^{a}
Spec RFO	2.733 ± 0.233^{a}	2.689 ± 0.203^{a}	2.989 ± 0.148^{a}	2.735 ± 0.185^{a}
TPP (mg GAE/g)	3.740 ± 0.298^{bc}	4.049 ± 0.152^{b}	5.339 ± 0.126^{a}	3.286 ± 0.806^{c}
TT (mg TAE/g)	0.920 ± 0.543^{a}	0.512 ± 0.356^{a}	0.938 ± 0.341^{a}	0.971 ± 0.277^{a}

Table 4.2. (continued)

Component (wt.%)	Athena (HT) Fractions			
	Whole	Dehulled	PF	Pearl
Ash	3.63 ± 0.37^{b}	3.49 ± 0.10^{b}	4.13 ± 0.04^{a}	3.25 ± 0.06^{b}
Fat	3.00 ± 0.06^{b}	2.50 ± 0.07^{c}	3.68 ± 0.15^{a}	2.49 ± 0.08^{c}
Protein	29.63 ± 0.20^{c}	34.08 ± 0.15^{b}	37.37 ± 0.03^{a}	31.43 ± 0.02^{c}
IDF	21.68 ± 0.93^{a}	9.67 ± 0.60^{b}	9.25 ± 0.36^{b}	7.68 ± 0.18^{c}
SDF	5.21 ± 0.40^{a}	0.88 ± 0.36^{c}	2.34 ± 0.28^{b}	1.99 ± 0.69^{c}
TDF	26.89 ± 0.56^{a}	10.55 ± 0.47^{bc}	11.58 ± 0.14^{b}	9.67 ± 0.85^{c}
RS	4.06 ± 0.40^{c}	4.61 ± 0.35^{b}	4.27 ± 0.03^{bc}	5.55 ± 0.11^{a}
SS	42.42 ± 0.90^{b}	41.73 ± 0.63^{b}	37.38 ± 0.46^{c}	46.55 ± 0.48^{a}
TS†	46.48 ± 0.80^{b}	46.34 ± 0.30^{b}	41.66 ± 0.43^{c}	52.10 ± 0.58^{a}
TS*	46.45 ± 0.88^{b}	46.73 ± 0.10^{b}	41.45 ± 0.20^{c}	51.71 ± 0.66^{a}
Sucrose	2.349 ± 0.188^{b}	2.963 ± 0.537^{ab}	3.472 ± 0.152^{a}	2.490 ± 0.543^{b}
Raffinose	0.146 ± 0.013^{b}	0.166 ± 0.005^{a}	0.163 ± 0.005^{ab}	0.122 ± 0.012^{c}
Stachyose	0.835 ± 0.017^{b}	1.237 ± 0.033^{a}	1.262 ± 0.110^{a}	0.822 ± 0.026^{b}
Verbascose	1.925 ± 0.115^{ab}	1.651 ± 0.110^{b}	2.169 ± 0.286^{a}	1.843 ± 0.078^{ab}
HPLC RFO	2.905 ± 0.138^{b}	3.054 ± 0.078^{b}	3.594 ± 0.374^{a}	2.787 ± 0.057^{b}
Spec RFO	2.813 ± 0.411^{a}	2.219 ± 0.034^{c}	2.248 ± 0.041^{bc}	2.349 ± 0.186^{ab}
TPP (mg GAE/g)	5.412 ± 0.668^{a}	4.087 ± 0.311^{b}	6.044 ± 0.259^{a}	2.859 ± 0.113^{c}
TT (mg TAE/g)	10.782 ± 0.725^{a}	2.018 ± 0.139^{b}	3.057 ± 0.139^{b}	2.075 ± 0.372^{b}

$^{a\text{-}d}$Mean (n = 3) ± SD values within a row for a particular cultivar followed by different letters are significantly different ($P < 0.05$) according to Duncan's multiple range test. n = 6 for TPP and TT.

Whole: whole bean flour, Dehulled: manually dehulled bean flour, PF: pearling flour (LT: 55% and HT: 57% pearling flour of the cotyledon from single-step pearling), Pearl: 43–45% of the cotyledon remaining after single-step pearling.
IDF: insoluble dietary fibre, SDF: soluble dietary fibre, TDF: total dietary fibre (IDF+SDF), RS: resistant starch, SS: solubilised starch, TS†: total starch (RS+SS), TS*: total starch assessed *via* Megazyme 2017 K-TSTA assay protocol.
HPLC RFO: sum of raffinose family oligosaccharides, raffinose, stachyose and verbascose, Spec RFO: analysed spectrophotometrically by Megazyme 2018 K-RAFGL assay procedure.
TPP: total polyphenol content (mg gallic acid equivalent/g sample) and TT: total tannin content (mg tannic acid equivalent/g sample).

The differences between the whole and dehulled bean protein contents (Table 4.2) of the two faba bean cvs, Snowbird and Athena investigated in this study were most probably due to the fibrous nature (lower protein contents) of the hulls. The key difference between the two faba bean cultivars is stemming from their tannin contents. However, a considerable portion of tannins was localised in the hulls of HT (7.3 ± 0.8 mg TAE/g) as opposed to LT (0.1 ± 0.0 mg TAE/g).

Higher tannin contents have been reported in coloured seed coats than those of light-coloured ones (Patterson et al., 2017), as visually apparent in Fig. 4.2. Removing the seed coat and splitting the cotyledon are essential processing steps that can lead to reduced cooking times and anti-nutrient levels, critical for the value-added utilization of pulses (Saldanha do Carmo et al., 2020). According to two-sample t-test results, HT whole bean flour had a significantly higher ($P < 0.05$) total polyphenol content as compared to LT whole bean flour. TPP contents of LT and HT hulls were 2.6 ± 0.2 and 46.6 ± 1. 5mg GAE/g, respectively. Condensed tannins are reported to be localised mainly in the outer anatomical layer (seed coat) of faba beans, which is intended as part of their defence mechanism against pathogens (Kosinska et al., 2011). Due to their special affinity to proteins, they can form a tannin-protein complex and precipitate the proteins. Condensed tannins can also form complexes with starch and impede their digestibility, as well as bind to minerals and restrict their bioavailability (Multari et al., 2015). Although tannins are frequently considered anti-nutritional factors (Multari et al., 2015), they can also reduce the risk of many diseases due to their antioxidant, antimicrobial, antiviral and anti-inflammatory properties (Ignat et al., 2011).

TPP contents of LT and HT whole beans were similar to those reported by Baginsky et al. (2013) and Barakat et al. (2017) using methanol as the extraction solvent. The ethanol extracts of Barakat et al. (2017) revealed the presence of 23 phenolic components, mainly ellagic acid, pyrogallol, e-vanillic acid and catechin. However, large variability in the TPP content has been observed, depending on the seed variety, ranging from 2.8 to 5.0 mg GAE/g (Mayer-Labba et al., 2021; Millar et al., 2019), which are comparable to the results of the present study (Table 4.2). According to Turco et al. (2016), faba bean cotyledons have more TPP than their respective hulls and whole seeds. This was true for LT in this study; however, HT had more TPP and TT concentrated in the hulls. Lattanzio et al. (2005) also confirmed that seed coats of cowpea had most of the tannins localised between the outer integument and the aleurone layer. The bioactive constituents barely present in the protein isolates (van der Goot et al., 2016) are present in the less-refined pearled fractions (Fig. 4.3), indicating that these components are eliminated during protein isolation. It is noteworthy that HT faba bean cultivars can be better utilised for food applications after pearling, as the TT content was significantly ($P < 0.05$) reduced in the pearling flour fraction (3.1 ± 0.1 mg TAE/g) as compared to the whole beans (10.8 ± 0.7 mg TAE/g). Unlike HT, the TPP content of LT was significantly ($P < 0.05$) higher in the pearling flour fractions as compared to the whole bean. TPP content of HT pearling flour was unaffected by the pearling step probably because the removal of tannins must have overshadowed the change in the TPP content.

The total dietary fibre (Table 4.2) content of HT whole bean flour was significantly ($P < 0.05$) higher than that of the pearling flour fraction, unlike LT. Whole faba bean TDF values from this study were comparable to the findings (12.8-16.6% and 12.2-13.5%, respectively) of Mayer-Labba et al. (2021) and Singh et al. (2014). No substantial differences ($P > 0.05$) were

observed in the IDF contents of dehulled and pearling flour fractions of both cultivars. Except for the LT dehulled flour, the rest of the LT fractions were not significantly ($P > 0.05$) different in their SDF contents.

HPLC analysis results (Table 4.2) confirmed the presence of the galactosyl-sucrose oligosaccharides, verbascose followed by stachyose and raffinose. The total RFOs quantified *via* the Megazyme spectrophotometric assay showed a similar trend to that of the of RFOs quantified *via* the HPLC method. Single-step pearling flour of LT and HT had significantly higher ($P < 0.05$) sucrose contents as compared to the rest of the fractions. Raffinose contents of different LT fractions were not significantly different ($P > 0.05$). HT pearls had significantly lower ($P < 0.05$) raffinose content as opposed to the rest of the fractions. LT whole bean flour had substantially higher ($P < 0.05$) verbascose content as compared to the other fractions, whereas there was no difference in the verbascose contents of HT fractions. Mayer-Labba et al. (2021) reported a positive relationship between seed size in different faba bean cultivars and their corresponding RFO contents. Raffinose, stachyose and verbascose results based on their findings ranged from 1.1 to 3.9, 4.4 to 13.7 and 8.0 to15.0 g/kg (db), which are comparable to the findings of this study.

Based on various studies, RFOs contain α-galactoside-glucose non-reducing bonds and are not readily digested because of the lack of α-galactosidase enzyme in the human digestive system, which can hydrolyse the α-1,6-galactosidic linkage. Therefore, these oligosaccharides tend to accumulate in the lower intestine, undergo anaerobic fermentation by bacteria (Multari et al., 2015), and cause abdominal discomfort (flatulence) in both humans and ruminants. However, other studies have also proven the health-beneficial properties of these oligosaccharides, pertaining to the improvement of gut health (Švejstil et al., 2015).

In the production of pearled barley, the grains are typically pearled to detach the hull and outer bran layers, resulting in a 30-40% (w/w) pearling flour as a by-product that is mainly used as animal feed (Blandino et al., 2015; Jadhav et al., 1998). In this study, applying a similar pearling approach to faba beans resulted in two value-added products: 1) a 55-57% pearling flour fraction enriched in protein and dietary fibre, and 2) 43-45% pearls elevated in starch with potential for various food applications. Nearly 62-63% of the dehulled bean protein is concentrated in the pearling flour, whereas nearly 49% of the starch is concentrated in the pearls. Even though a pearling level of 55-57% determined in this study is higher than the typical levels adopted by the industry, the pearling flour with a higher protein content obtained at such a level of pearling would be a viable feed material for subsequent wet processing for protein isolation.

4.3.3. Hybrid fractionation

4.3.3.1. Extractability of protein, starch and dietary fibre

A hybrid fractionation protocol involving dry and aqueous processing steps to isolate faba bean proteins is outlined in Fig. 4.1 and additional steps have been carried out to recover the starch and dietary fibre fractions as co-products. For commercial purposes, a clarification step (centrifugation) can be used instead of wet sieving to remove the insoluble particulate matter (usually cellular debris and starch granules) from protein, which will reduce the number of steps (Fig. 4.1, Steps 5 and 6) in the proposed method. However, wet sieving is advantageous for commercial purposes if the starch and dietary fibre fractions are to be recovered as co-products. This method is scalable with unit operations employed at an industrial scale and is less resource-intensive with the proposed smaller solvent-to-feed (S/F) ratio of 2:1 (v/w) × 2 as opposed to the larger 10:1 × 2 ratio commonly used in the industry and further scale-up

analysis is recommended. In this technique, regardless of the cultivar, the use of "single-step pearling flour" as a raw material resulted in high-purity protein isolates (Table 4.3). These results were comparable to the findings of Arntfield et al. (1985), who also claimed 98.5% removal of faba bean vicine and convicine by micellization of proteins.

Table 4.3. Chemical composition (%) of faba bean hybrid fractions on a dry weight basis.

Fraction	Component	Snowbird (LT)	Athena (HT)
Protein	Carbon	55.67 ± 0.05	55.86 ± 0.16
	Nitrogen	16.10 ± 0.01	16.34 ± 0.00
	Protein (N×6.25)	100.60 ± 0.09	02.11 ± 0.01
	Protein (N×5.85)	94.16 ± 0.11	95.57 ± 0.01
	Starch	5.66 ± 0.09[a]	2.69 ± 0.40[b]
Starch	Carbon	48.01 ± 0.06	49.27 ± 0.06
	Nitrogen	0.45 ± 0.01	0.68 ± 0.04
	Starch	9.22 ± 1.31[b]	91.79 ± 1.44[a]
Dietary Fibre	Carbon	50.10 ± 0.11	48.32 ± 0.05
	Nitrogen	5.78 ± 0.03[a]	4.45 ± 0.01[b]
	Starch	30.81 ± 0.51	33.35 ± 0.32
	TDF	29.91 ± 0.29	29.74 ± 1.74
Intermediate fraction	Carbon	52.60 ± 0.60	50.10 ± 0.10
	Nitrogen	5.05 ± 0.06	5.70 ± 0.01
	Starch	55.08 ± 1.28	53.47 ± 0.23
Non-precipitated fraction	Carbon	46.12 ± 0.06	46.10 ± 0.11
	Nitrogen	9.42 ± 0.03[a]	8.09 ± 0.01[b]
	Protein (N×6.25)	58.90 ± 0.16[a]	50.54 ± 0.06[b]
	Protein (N×5.85)	55.13 ± 0.21[a]	47.31 ± 0.09[b]
	Starch	2.50 ± 0.05[b]	2.69 ± 0.40[a]

[a-b]Mean (n = 3) ± SD values within a row for a particular category followed by different letters are significantly different ($P < 0.05$) according to two-sample t-test results. TDF: total dietary fibre.

The protein fraction resulted from Step 16 of Fig. 4.1. The intermediate fraction is the layer between the protein supernatant and starch residue after centrifugation (Fig. 4.1, Step 8). The non-precipitated fraction is the supernatant resulting from Step 15 of Fig. 4.1.

It is important to note that when assessing the protein content of foodstuff, the nitrogen content is multiplied by a factor of 6.25, a historical nitrogen-to-protein conversion factor, assuming the nitrogen content of proteins to be 16%. Although widely accepted, using N×6.25 has resulted in protein contents above 100% for plant protein isolates based on dry matter

(Mariotti et al., 2008), which was also true in the present study. Calculating the protein content of food using a constant conversion factor of 6.25 expresses the nitrogen content in a different form. Although a protein conversion factor specific to faba bean proteins has not been previously established, the protein conversion factor of 5.85 from literature was also used (Table 4.3) to compare these findings to that of Arntfield et al. (1985) and Murray et al. (1979).

The yields of LT and HT protein isolates were 20 and 22%; starch isolates were 32 and 26%; dietary fibre concentrates were 8 and 7%; and the non-precipitated fractions were 20% and 25%, respectively, based on the single-step pearling flour used as the feed for the wet fractionation. The 12% intermediate layer was a by-product of this process (obtained in Step 8, Fig. 4.1), resulting from the inability to separate protein, which was insoluble in water and had a similar shape/density to that of small starch granules. Using S/F of 2 to dilute the protein extracts (Step 13 of Fig. 4.1) resulted in 42.5% protein recovery according to Arntfield et al. (1985). Although the recovery of proteins resulting from the micellization steps was ~52-61%, the non-precipitated fraction resulted in ~30-34% protein recovery. Therefore, the total recovery of protein from this aqueous fractionation approach should be ~82-95%, which is the amount of protein recovered from step 13 of Fig. 4.1, as the initial total protein content of the feed is derived from the sum of both protein and non-protein nitrogen content. The strong protein-protein interactions during protein micellization exclude much non-proteinaceous material according to Murray et al. (1979). Therefore, the nitrogen content of the precipitated protein fraction could mainly account for the true protein content.

Water was used instead of salt solution to take advantage of the naturally abundant minerals (Multari et al., 2016) to extract the salt-soluble globulins (Multari et al., 2015) from the single-step pearling flour fraction of faba beans (~4% ash). In this process (Fig. 4.1),

following the salting-in process of faba bean proteins, where at low salt concentrations the ions act to stabilise protein-water interactions and increase protein solubility, the addition of water at 4 °C would drastically reduce the ionic strength of the medium (Murray et al., 1979) and cause the proteins to go through a sequence of dissociation steps to adapt to the new environment. These steps would then lead to the formation of protein micelles resulting from the accumulation of amphiphilic proteins, which grow in size and number until precipitation occurs (Murray et al., 1979). According to the SEM images of the freeze-dried LT (Fig. 4.5a1) and HT (Fig. 4.5b1) protein isolates, finely globular structures appear to be lumped into relatively uniform coarse aggregates. High magnification images of LT (Fig. 4.5a2-3) and HT (Fig. 4.5b2-3) revealed the protein micelles to be part of a dense network of relatively uniform aggregates of smaller spherical sub-structures.

4.3.3.2. Protein secondary structure conformational changes

The main FTIR spectral characteristics of the faba bean (Fig. 4.6a) were comparable to the findings of Wang et al. (2013). Both faba beans dehulled flour and micellar protein isolates of the cvs. LT and HT exhibited a strong band at ~3280 cm^{-1}, attributed to the stretching vibrations of O-H and N-H, whereas the band at ~2929 cm^{-1} corresponds to the asymmetric stretching vibration of C-H in CH_2 groups of carbohydrates, lipids or proteins (Johnson et al., 2020). The secondary structure of proteins could be better understood by analysing the amide I band where the C=O stretch has a predominant role ascribed to the band at ~1634 cm^{-1}. The band at ~1523 cm^{-1} is due to C-N stretching and N-H bending vibration of the amide II region, which could vary due to the level of H-bonding (Johnson et al., 2020). Both the bands at ~1455 and 1391 cm^{-1} mainly correlate to the bending vibrations of CH_2 and CH_3, respectively. The peak at ~1237 cm^{-1} can be assigned to the vibrations of the amide III band.

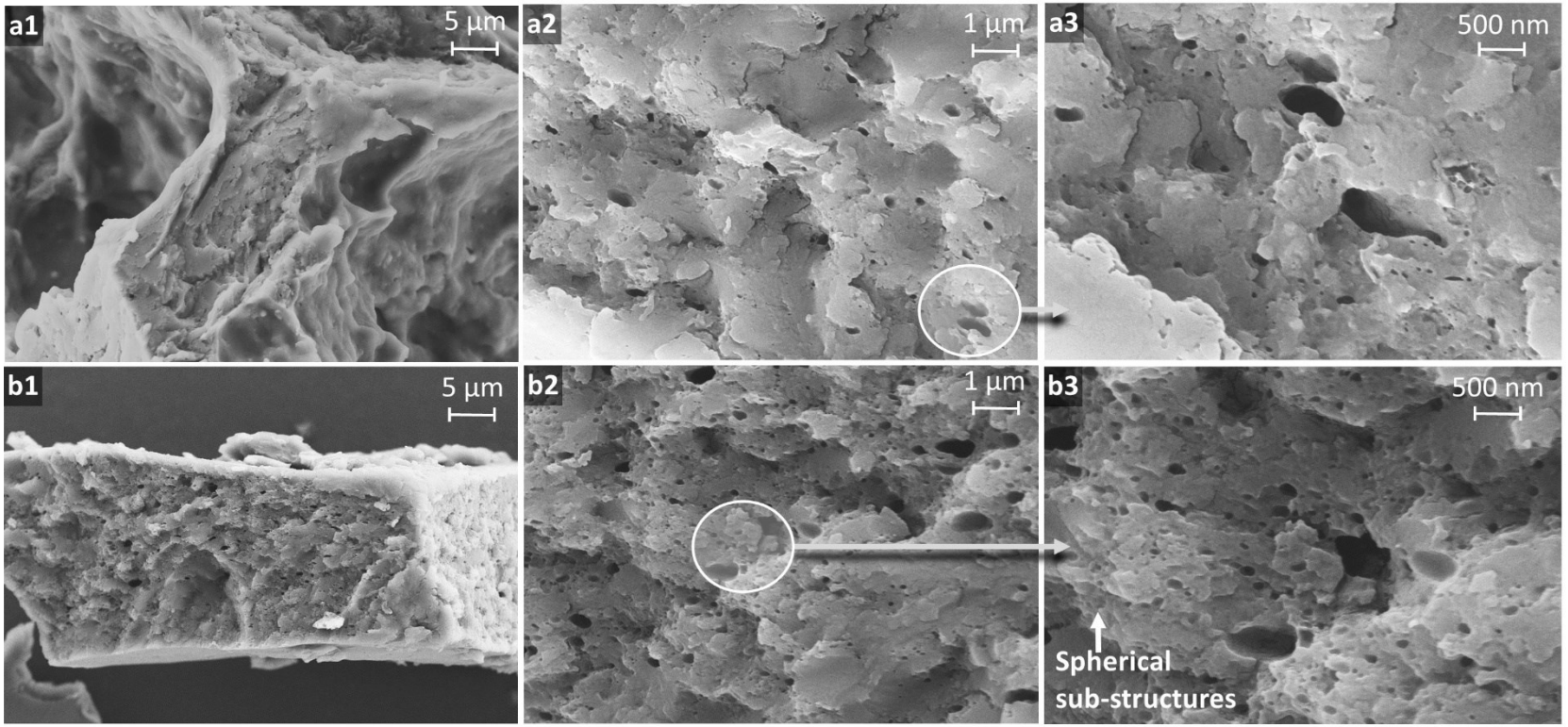
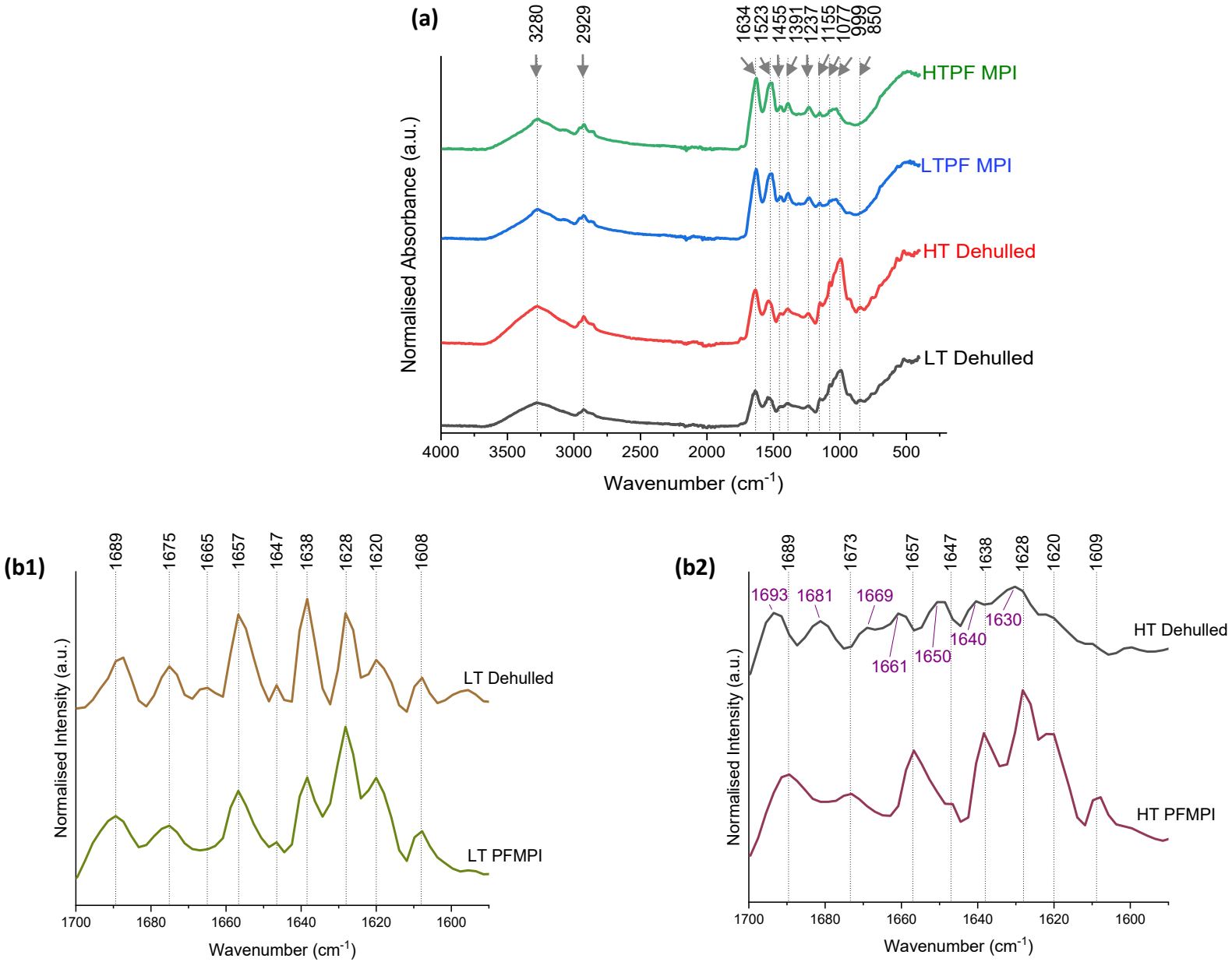


Fig. 4.5. SEM micrographs of faba bean hybrid-fractionated protein isolates (≤ 0.5 mm).

Morphological characteristics of micellar protein isolates obtained from faba bean cvs. (a) Snowbird, and (b) Athena (1:×2000, 2:×10,000, 3:×20,000 magnification).


(a)
3280
2929
1634
1523
1455
1391
1237
1155
1077
999
850
HTPF MPI
LTPF MPI
HT Dehulled
LT Dehulled
Normalised Absorbance (a.u.)
4000
3500
3000
2500
2000
1500
1000
500
Wavenumber (cm-1)
(b1)
1689
1675
1665
1657
1647
1638
1628
1620
1608
LT Dehulled
LT PFMPI
Normalised Intensity (a.u.)
1700
1680
1660
1640
1620
1600
Wavenumber (cm-1)
(b2)
1689
1673
1657
1647
1638
1628
1620
1609
1693
1681
1669
1661
1650
1640
1630
HT Dehulled
HT PFMPI
Normalised Intensity (a.u.)
1700
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Wavenumber (cm-1)

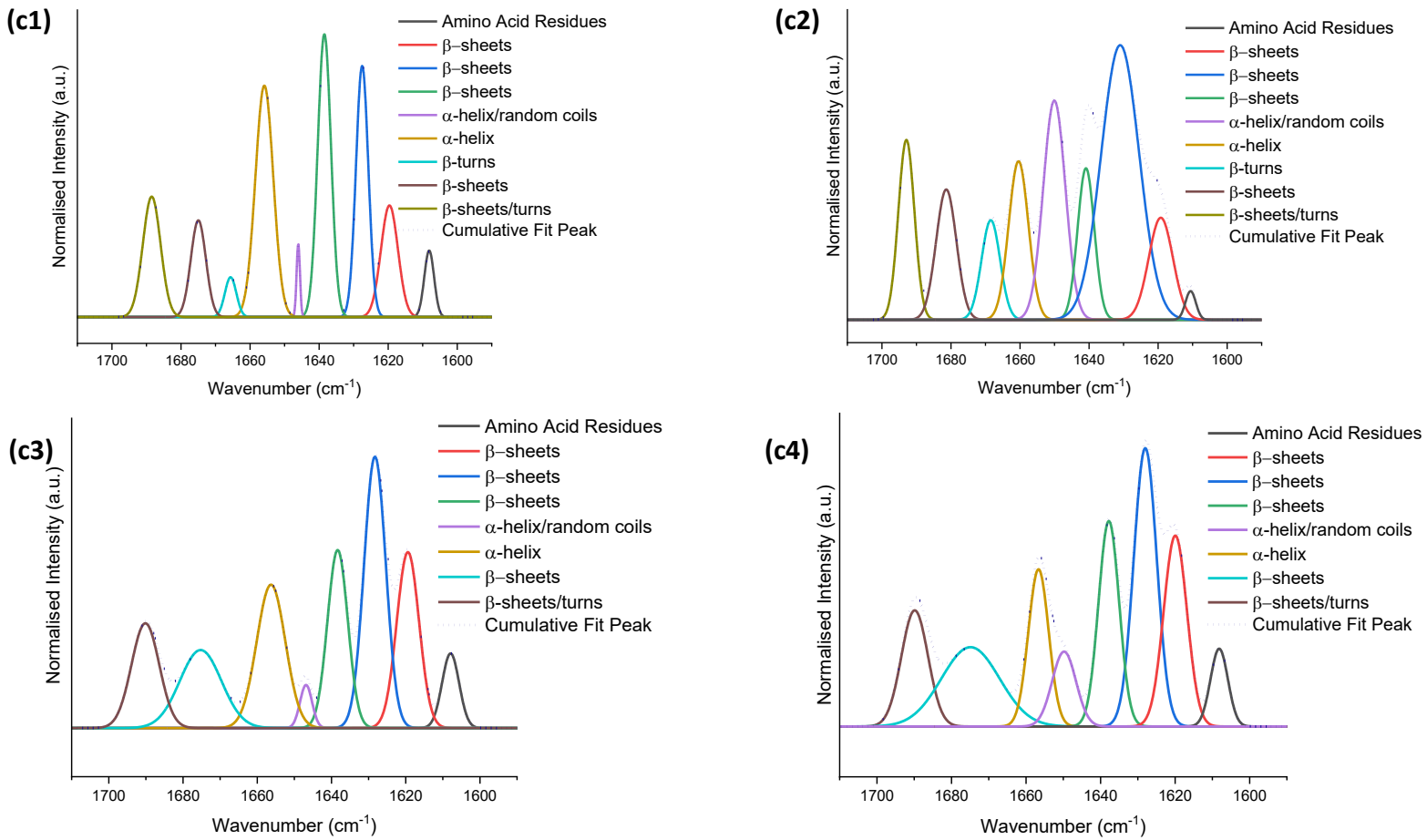


Fig. 4.6. Secondary structural changes of faba bean Snowbird (LT) and Athena (HT) hybrid fractionated protein isolates as compared to dehulled bean flour.

FTIR spectra (400-4000 cm^{-1}) of faba bean dehulled flours and protein isolates obtained by hybrid fractionation corresponding to (a) the specific variation of selected functional groups; (b) second derivative spectra of amide I vibrational region (1600-1700 cm^{-1}) showing changes in protein secondary structure of LT (b1) and HT (b2); (c) curve fitted amide I spectra of LT; and (d) curve fitted amide I spectra of HT, dehulled bean flour (c1, d1) and protein isolate (c2, d2). Micellar protein isolates from single-step pearling flour of Snowbird (LTPF MPI) and Athena (HTPF MPI) were from Step 16 of Fig. 4.1.

The spectral bands at ~1155, 1077 and 999 cm^{-1} correspond to C-OH stretching vibrations of carbohydrates, and the weak peak at ~850 cm^{-1} can be assigned to α-glycosidic bonds (Johnson et al., 2020; Wang et al., 2013). FTIR spectra of the amide I region provide key insights into protein conformational/secondary structural changes during processing. According to Fig. 4.6b1, the amide I region of dehulled LT flour mainly consisted of 9 bands at ~1608 cm^{-1} (vibrations of amino acid residues), ~1620 cm^{-1} (β-sheets), ~1628 cm^{-1} (β-sheets), ~1638 cm^{-1} (β-sheets), ~1647 cm^{-1} (α-helix/random coils), ~1657 cm^{-1} (α-helix), ~1665 cm^{-1} (β-turns), ~1675 cm^{-1} (β-sheets) and ~1689 cm^{-1} (β-sheets/turns) comparable to the findings of Yang et al. (2018). Although both LT and HT dehulled bean flour showed 9 bands in the amide I region, their corresponding protein isolates only had 8 bands (Fig. 4.6b-d), due to the absence of β-turns relating to the bands at ~1665 cm^{-1} for LT and ~1669 cm^{-1} for HT. The relative peak intensities of β-sheets have increased at the expense of α-helix and β-turns. The β-turns frequently occur at the surfaces of globular proteins. As a consequence, turns are primarily composed of hydrophilic residues. The β-sheet structures are amphiphilic with hydrophilic groups exposed to water and hydrophobic moieties hidden in the core of the globular proteins to avoid water. The stable arrangement of β-sheets leads to the micellization of the proteins (Murray et al., 1979). This could be the reason for the overall increase in the peak intensities of β-sheets in the micellar protein isolates. However, all the secondary structures of faba proteins were still maintained except for β-turns.

Unlike the second derivative spectra of LT dehulled flour and HT protein isolates, HT dehulled flour had overall lower peak intensities. The peaks showing up at ~1630, 1640, 1650, 1661, 1669, 1681 and 1693 cm^{-1} must have shifted from 1628, 1638, 1647, 1657, 1665, 1673 and 1689 cm^{-1}, respectively, highlighting the potential interactions between tannins and protein

secondary structures (Fig. 4.6c1-2). However, both LT and HT protein isolates shared similar IR spectral characteristics (Fig. 4.6b1-2). possibly resulting from the major reduction of tannins during the protein isolation steps. Tannins are of higher molecular weight and can vary greatly in their structure and composition. Although their affinity towards proteins is higher as there are many bonding points with carbonyl groups, tannin-protein complexes are generally unstable as the hydrogen bonds continually break and re-form with pH (Kosińska et al., 2011; Rodríguez-Espinosa et al., 2019). Based on these findings, it may be concluded that the hybrid-fractionation process led to the reduction of tannins in the protein isolates and minimal protein conformational changes or secondary structural changes. The large β-sheets are the dominant secondary structure of faba bean proteins followed by α-helix in their dry form, which is also true for their aqueous solutions (Yang et al., 2018).

4.4. CONCLUSIONS

Composition analysis and SEM imaging results of this study demonstrated that ash, dietary fibre and protein bodies were concentrated more in the exterior layers of faba beans and gradually decreased towards the inner layers, which was offset by the inverse trend of starch content regardless of the variety. The protein content was positively correlated ($r = 0.9$, $P < 0.0001$) to the ash, and TDF contents, whereas a negative correlation between the protein and starch ($r = -0.9$, $P < 0.0001$) was observed in both cultivars with the level of pearling. Taking advantage of the component distribution pattern, a 55-57% pearling flour capturing 62-63% of the cotyledon protein was produced alongside the 43-45% pearls, concentrated in starch with the potential for various food applications.

The chemical-free hybrid fractionation of faba bean was not only effective in isolating the protein (> 94% purity) but also in isolating the starch and in concentrating the dietary fibre. Native structures of proteins were preserved in the protein isolates according to FTIR results. It is noteworthy that the underutilised high tannin faba bean cvs. can be better utilized by pearling, which can significantly reduce the tannin content while preserving the total polyphenol content. This outcome suggested that the inclusion of gradual pearling as an upstream processing step in faba bean wet fractionation has the potential to generate native high-purity protein ingredients for the food industry.

CHAPTER 5: Potential of air-currents-assisted particle separation (ACAPS) technology for hybrid fractionation of clean-label faba bean (*Vicia faba* L.) protein[4]

[4]A version of this chapter has been published: Jeganathan, B., Gao, J., Temelli, F. & Vasanthan, T. (2023). Potential of air-currents-assisted particle separation (ACAPS) technology for hybrid fractionation of clean-label faba bean (*Vicia faba* L.) protein. *Journal of Food Engineering, 339*, 111265. https://doi.org/10.1016/j.jfoodeng.2022.111265.

5.1. INTRODUCTION

Increasing demand for planet-friendly protein sources is inevitable with the projected global population growth. Legumes, in particular, will be coveted due to their numerous nutritional benefits, cost-effectiveness, environmental sustainability and wide availability across different regions (Boland et al., 2013; Kumar et al., 2022). Among the legumes, soybean, chickpea, field pea, lentil and lupin have been extensively explored for the utilisation of their proteins as food ingredients (Boukid, 2021; Jarpa-Parra, 2018; Kumar et al., 2022). In addition, there is a need to exploit the underutilised legumes to supplement the global protein demand. One such legume is faba bean (*Vicia faba* L.), also known as fava bean, a nitrogen-fixing bioresource with a high protein content (Multari et al., 2015).

Commercial wet fractionation methods employing the pH-shift method for concentrating pulse protein are more energy and resource-intensive (Schutyser & van der Goot, 2011) as compared to dry fractionation techniques employing pin milling followed by air classification (Schutyser et al., 2015). While pin milling or fine grinding can disintegrate seed constituents into fine particles, extreme fine milling can lead to starch damage, resulting in fragments of starch granules similar in size to that of protein bodies, making their separation from protein more challenging (Schutyser et al., 2015). On the other hand, coarse milling can lead to large aggregates of protein bodies, starch granules and cell wall components that can make fractionation more demanding (Pelgrom et al., 2013; Saldanha do Carmo et al., 2020). Although air classification can separate these cell components based on their physical properties, such as particle size, shape and density (Pelgrom et al., 2015), the relative impurities of both the starch and protein fractions may interfere with their product applications (Vasanthan & Bhatty, 1995). However, these protein fractions contain important bioactive compounds at

higher levels compared to the protein fractions obtained via wet separation techniques (Saldanha do Carmo et al., 2020). For food applications where protein purity is deemed pivotal, wet fractionation methods play a key role (Schutyser et al., 2015). Amino acid derivative lysinoalanine (LAL) formation has been linked to alkali extraction (Gould & MacGregor, 1977) of soy proteins, which are known for their negative association with the net protein utilisation and nephrotoxic reactions in rats (Gould & MacGregor, 1977). Also, the particular wet fractionation conditions employed for protein isolation can have a profound impact on the compositional and physicochemical characteristics of the fractions obtained and the magnitude to which these characteristics are affected. Any robust changes in these characteristics will ultimately influence protein functionality (Jarpa-Parra, 2018). Hybrid processes involving successive dry and wet separation methods have proven to be more economically viable compared to aqueous fractionation alone (Schutyser et al., 2015). Thus, it is necessary to explore other economically viable dry fractionation techniques as upstream processing steps to separate and concentrate the major faba bean components for successive protein isolation.

The relationship between particle size and inherent physicochemical properties and the resulting functional characteristics of grain flour remains unclear. However, the particle size of the flour is a decisive parameter in terms of final product quality (Ahmed et al., 2016). Even in air classification, the cut-off point plays a crucial role, referring to the particle size at which a particle has a 50% chance to move either to the coarse or the fine fraction (Cloutt et al., 1987; Pelgrom et al., 2013). Therefore, it is of utmost importance to understand the particle size-based distribution of the cell components in faba beans following milling to better utilise them in food applications.

Air-currents-assisted particle separation (ACAPS) is a low-cost patented technology that involves the application of vacuum and high-pressure air pulsing to generate air currents in a high-throughput sieving apparatus (Vasanthan, 2017). These dynamic air currents have enhanced the concentration of barley β-glucan in the coarse fraction retained on a sieve for various food applications while starch gets concentrated in the fine fraction, with the potential to be transformed into bioethanol (Lu et al., 2020). The applicability of ACAPS technology in pulse fractionation is still not fully explored. Therefore, the objectives of the present study were: 1) to establish the particle size-based distribution of faba bean macronutrients (dietary fibre, protein and starch) based on sieve separation, and 2) to determine the changes in the composition of ACAPS-treated faba bean flour fractions for subsequent aqueous fractionation to achieve high purity protein isolates.

5.2. MATERIALS AND METHODS

5.2.1. Materials

Canadian high-tannin faba bean cv. Athena (HT) and low-tannin faba bean cv. Snowbird (LT) were obtained from W.A. Grain and Pulse Solutions, Innisfail, AB, Canada. All the chemicals and reagents were of analytical grade and were purchased from Sigma Chemical Co. (St. Louis, MO, USA) unless stated otherwise.

5.2.2. Sample preparation

5.2.2.1. Abrasive dehulling, pearling and grinding

LT and HT faba bean whole grain samples were dehulled and pearled in a Tangential Abrasive Dehuller (TAD) developed by the Bio-industrial Opportunities Section, Alberta

Agriculture, Forestry and Rural Economic Development (Edmonton, AB, Canada) in 500 g batches according to the method previously described in Chapter 4 (Section 4.2.2.1). Following 30 s of abrasive dehulling, two batches from the six trials were pooled and the dehulled beans (groats) were separated from the hulls and by-products by aspiration. The groats were then processed as follows: a) pearled in the same equipment for 270 s to generate single-step pearling flour (Chapter 4, Section 4.2.2.2) or b) ground at 8,000 rpm in an ultra-centrifugal mill (ZM 200, Retsch mill, Hann, Germany) equipped with a 500 μm ring sieve based on preliminary findings to generate dehulled bean flour. All the flour fractions, subjected to pearling and milling, were sealed in plastic containers and stored at 23 ± 3 °C until further analysis.

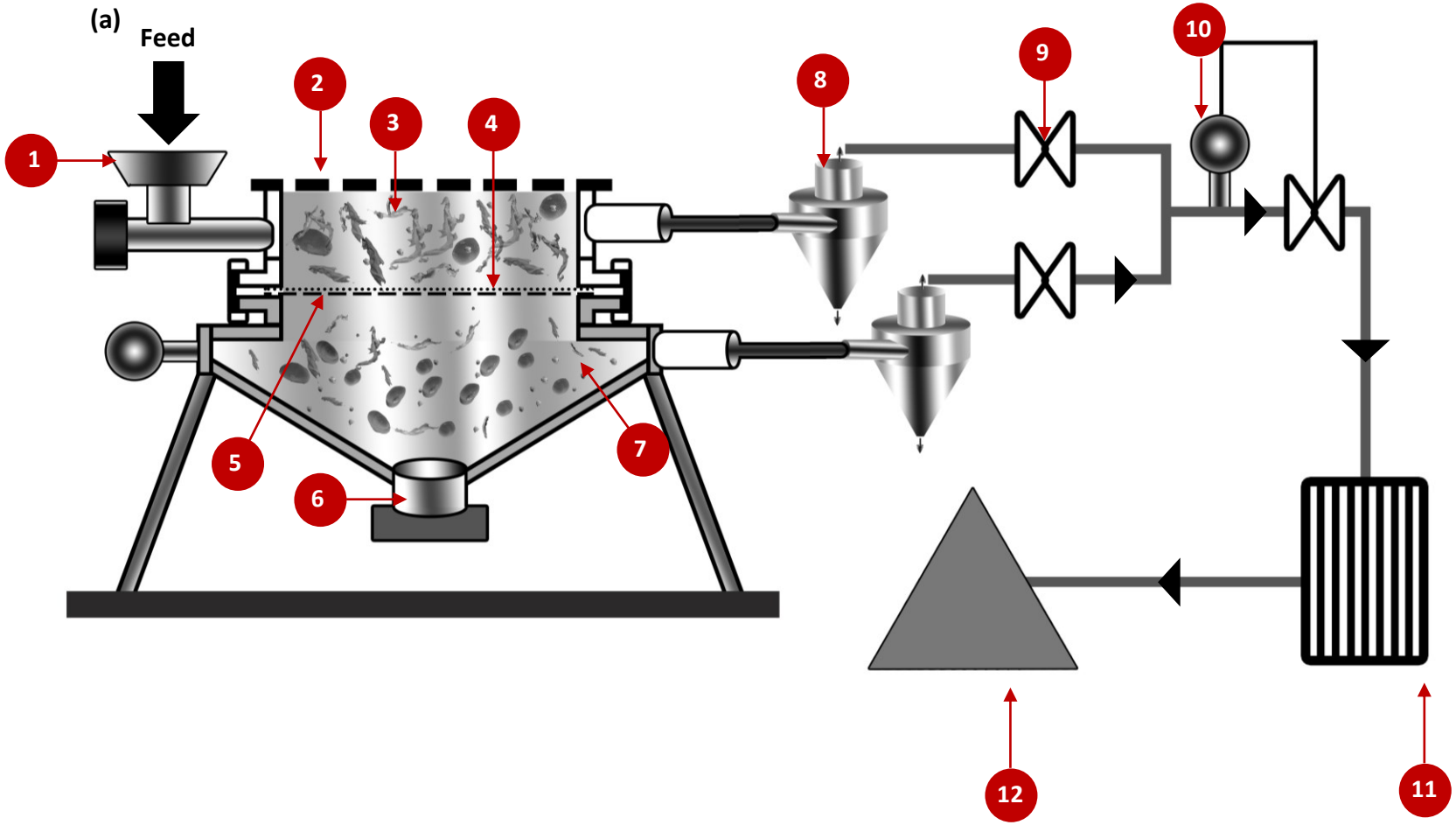
5.2.2.2. Traditional sieve analysis of faba bean flours

The particle size distributions of both LT and HT faba bean flours (75.000 ± 0.010 g) produced by Retsch mill grinding were analysed by sieve separation using the WS Taylor's Sieve Shaker (RX-812-CAN, Mentor, OH, USA). Based on the yield data and their consistency from a preliminary trial, the sieve sizes 250, 150 and 75 μm were selected. These sieves were stacked on top of each other from the lowest to highest sieve size and the samples were sieved for 10 min, followed by manual tapping for 10 s after 5 min of sieving. This experiment was performed for the triplicate samples obtained by dehulling, pearling and milling (Section 5.2.2.1) to determine the yield, composition and recovery of the protein, starch and dietary fibre of the sieve-separated fractions.

5.2.2.3. ACAPS of faba bean flours

The apparatus used in the ACAPS process (Fig. 5.1a) is comprised of top and bottom chambers separated by a sieve. The milled grain particles fed to the hopper are drawn into the top chamber by vacuum. Within the top chamber, dynamic air currents are created through the inlet ports of the top chamber cover when the system is under vacuum. High-pressure air pulsing under the vacuum fluidizes the grain particles and allows the fine particles to pass through a micron-sized sieve. The coarse fibrous fraction remains on the sieve in the top chamber. The cyclone unit attached to the top chamber is used to collect the coarse fraction whereas the second cyclone attached to the bottom chamber is used to collect the fine fraction through the dispensing opening at the apex of the cone.

A lab-scale version (Fig. 5.1b) of the ACAPS unit was used to separate the coarse and fine fractions of LT and HT dehulled and single-step pearling flours. The sample (75.0 g) was fed directly into the top chamber by placing a funnel through the middle opening of the top chamber cover while connected to the vacuum unit (Power: 3.7285 kJ/s). It was confirmed through preliminary trials that 2 min was required to complete particle separation. The coarse particles remaining on top of the sieve (either 63 μm, 75 μm, 125 μm, 150 μm, or 250 μm) in the top chamber and the fine particles passing through the openings of the sieve and entering the bottom chamber were collected and stored in plastic containers at 23 ± 3 °C for compositional analysis.



1: hopper; 2: top chamber cover with openings for air currents; 3: coarse fraction of grains in the top chamber; 4: sieve (<100 µm); 5: metal screen sieve bed; 6: bottom chamber port for removal of fine fraction, cleaning and maintenance, 7: fine fraction of grains in the bottom chamber; 8: cyclone separator; 9: safety valve; 10: pressure gauge, 11: particulate filter; 12: vacuum generator.

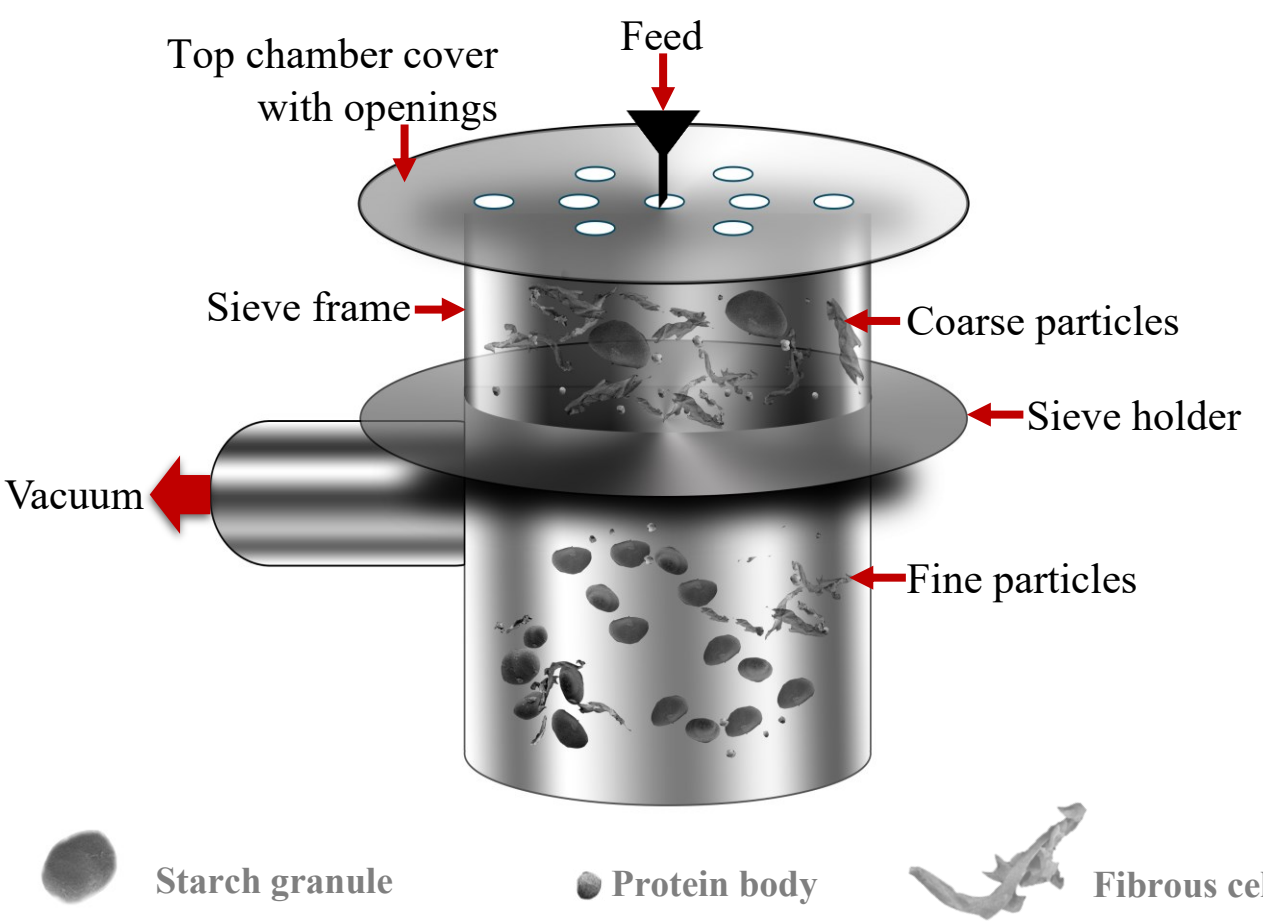


Fig. 5.1. Schematic overview of the distribution of the macro-components, starch, protein and fibre during the air-currents-assisted particle separation (ACAPS) process.

(a) patented air-currents-assisted particle separation (ACAPS) apparatus, and (b) a simplified lab-scale version of the same (adapted and modified from Vasanthan (2017)).

5.2.2.4. Hybrid fractionation of coarse and fine flour fractions from ACAPS

The hybrid fractionation procedure was carried out by subjecting both the ACAPS-treated (250 µm) coarse and fine fractions of faba bean cvs. LT and HT to aqueous fractionation employing micellization according to Fig. 5.2, following the method described in Chapter 4 (Section 4.2.3.). Both the coarse and fine fractions resulting from ACAPS were subjected to aqueous fractionation in order to investigate the potential of these fractions in hybrid fractionation. Briefly, the multi-step extraction was carried out with the help of a homogenizer (Polytron, PTMR 2100, Kinematica AG, Malters, Switzerland) at a solvent/feed (S/F) ratio of 2 (v/w) for 1 min followed by overhead stirring at 500 rpm (Heidolph RZR 2021, Schwabach, Germany) for 10 min, which was repeated twice.

The dietary fibre retentate was separated by wet sieving (WS Taylor's Sieve Shaker, RX-812-CAN, Mentor, OH, USA) with a 75 µm sieve and the centrifugation (Beckman Coulter, Avanthi SER JSE10K11, Indianapolis, IN, US) of the filtrate to separate the protein and starch fractions. Coldwater at 4 °C was introduced to the combined supernatant at 1:2, (v/v) extract: H_2O ratio and allowed to stand at 4 °C for 2 h. Following centrifugation, the residue (protein fraction), as well as the supernatant (non-precipitated fraction), were freeze-dried. The retentate (starch fraction) was ethanol washed at a 1:1, feed:absolute ethanol ratio and oven-dried at 40 °C for 16 h, whereas the wet sieved dietary fibre retentate was oven-dried at 60 °C for 48 h. Both the freeze-dried protein, non-precipitated fraction and oven-dried dietary fibre fractions were ground in the Retsch mill (500 µm, 8000 rpm) and were stored at 23 ± 3 °C in plastic containers until further use.

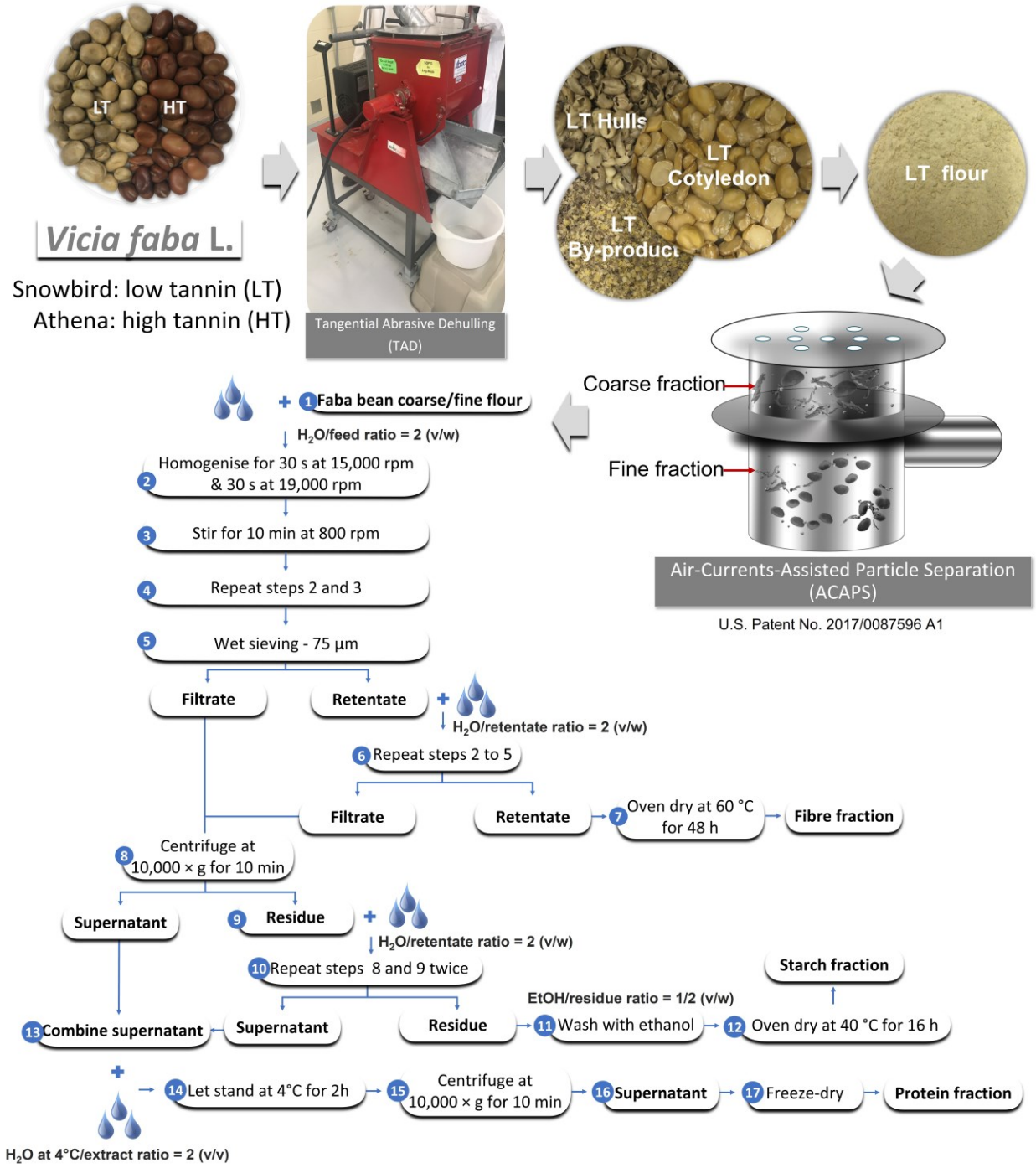


Fig. 5.2. Schematic overview of the air-currents-assisted particle separation (ACAPS) of faba beans followed by subsequent aqueous fractionation to isolate micellar proteins.

5.2.3. Characterisation

5.2.3.1. Morphological characterisation

The morphological characteristics of flour fractions were examined under high vacuum using a variable pressure field emission scanning electron microscope (VP-FESEM, Carl Zeiss Sigma 300, Oberkochen, BW, Germany) after gold coating as described previously in Chapter 3 (Section 3.2.3).

5.2.3.2. Particle size distribution analysis

Particle size distribution of faba bean dehulled flour was analysed by feeding ~2-3 g of sample into a Malvern Mastersizer 3000 laser diffraction particle size analyser, equipped with an aero dry dispersion unit (Malvern Instruments Ltd., Malvern, Worcestershire, UK). Data were collected and analysed using Malvern software (Version 3.10, Malvern Instruments Ltd, Malvern, UK). Particle size was defined in terms of volume percentiles: 10^{th} (Dv[10]), median (Dv[50]) and 90^{th} percentile (Dv[90]), volume-weighted mean particle diameter (D[4,3]), and surface-area weighted mean particle diameter (D[3,2]).

5.2.3.3. Compositional analysis and recovery

Dry matter content (%) was determined by loss in mass at 105 ± 1 °C for 24 h. Protein content was determined in an elemental analyser (Leco TruSpec- FP-428, Mississauga, ON, Canada) based on apparent nitrogen (N)% content according to the Dumas principle (N × 6.25, AOAC, 2000). Total starch content was measured according to Megazyme 2017 K-TSTA assay procedure (Megazyme International Ltd., Bray, Wicklow, Ireland, UK) based on the adaptation of the AOAC 996.11 method. The contents of soluble dietary fibre (SDF), insoluble

dietary fibre (IDF) and total dietary fibre (TDF=SDF+IDF) were analysed using the Megazyme 2017 K217 TDFR assay method (Megazyme International Ltd., Bray, Wicklow, Ireland) following the modifications mentioned in Chapter 4 (Section 4.2.4). Yield and recovery (for protein, starch and total dietary fibre) values were calculated based on Eqs. (5.1) and (5.2), respectively.

$$Yield\ (\%\ db) = \frac{Mass\ of\ the\ corresponding\ output,\ \ db\ (g)}{Mass\ of\ the\ feed,\ \ db\ (g)} \times 100 \quad (5.1)$$

$$Protein\ Recovery\ (\%\ db) = \frac{Yield\ (\%\ db) \times Protein\ content\ of\ the\ output\ (\%\ db)}{Protein\ content\ of\ the\ feed\ (\%\ db)} \times 100 \quad (5.2)$$

5.2.3.4. Attenuated Total Reflection-Fourier Transform Infrared Spectroscopy (ATR-FTIR)

Flour samples in their dry form were evaluated for molecular orders and protein secondary structural changes by ATR-FTIR (Alpha, Bruker, Berlin, Germany) following the method previously described in Chapter 4 (Section 4.2.4.2.3). The impact of the fractionation method on the protein secondary structures was analysed by the second derivative method followed by peak fitting through Origin 2021b software (Origin Lab Corporation, Northampton, MA, USA). Baseline correction was performed in the Quick Peak option of Gadgets by selecting the straight-line mode and subtracting the baseline.

5.2.4. Statistical analysis

Sieve analysis, ACAPS separation and aqueous extraction were carried out in triplicate. Analytical determinations of the samples were carried out in duplicate (n=6) except for TDF analysis (n=3) and the hybrid-fractionated extracts. Mean ± standard deviation (SD) values were expressed on a dry weight basis (db). Analysis of variance (two-way ANOVA) was

performed, and the means were separated based on Duncan's multiple range test ($P < 0.05$) at a 95% confidence level. Paired comparisons between two sets of data were tested at a 95% confidence level according to the two-sample t-test. Additionally, to understand the correlation between the different macronutrients with the particle size distribution, Pearson's correlation analysis was performed ($P < 0.05$). Statistical analyses were carried out using SAS Studio University Version for Windows (SAS Institute Inc., Cary, NC, USA).

5.3. RESULTS AND DISCUSSION

5.3.1. Milling

The milling behaviour of faba beans is correlated to their micromorphological characteristics and elemental composition at the cotyledon-seed coat and cotyledon-cotyledon interface as discussed in Chapter 3 (Section 3.3). In addition, proteins and dietary fibre are more concentrated in the outer layers of faba beans, which was offset by the starch content, based on the findings of sequential pearling as discussed in Chapter 4 (Section 4.3.1). This pattern of macro component distribution could also influence the milling characteristics of faba beans since the dehulling efficiency of red lentils was negatively correlated with the crude protein and dietary fibre contents, while positively correlated with the starch content (Wang, 2008). Accordingly, higher protein and dietary levels in the faba bean cotyledon external layers could be negatively correlated to their milling characteristics. Dehulling/milling behaviour of the grain plays a crucial role apart from the milling parameters, such as grinding force, rotor speed and sieve size on the particle size distribution of the flour as well as the distribution of macro components in different fractions based on the particle size. While coarse milling can be more demanding than fine milling as a result of substantial protein, starch and other cellular

component aggregation (Pelgrom et al., 2013), extreme fine milling can result in portions of starch granules similar in size to that of protein bodies, making their separation more challenging. Based on the preliminary trials with different sieves (250, 500 and 1000 μm) and grinding speeds (6000, 8000 and 10000 rpm), screen size was identified as a more substantial determinant of the release of protein during the grinding step as compared to the rotor speed. The protein content of the flour was significantly greater ($P < 0.05$) and the heat production was minimal when grinding was performed with a 500 μm sieve at 8,000 rpm and was selected as the optimum condition for grinding.

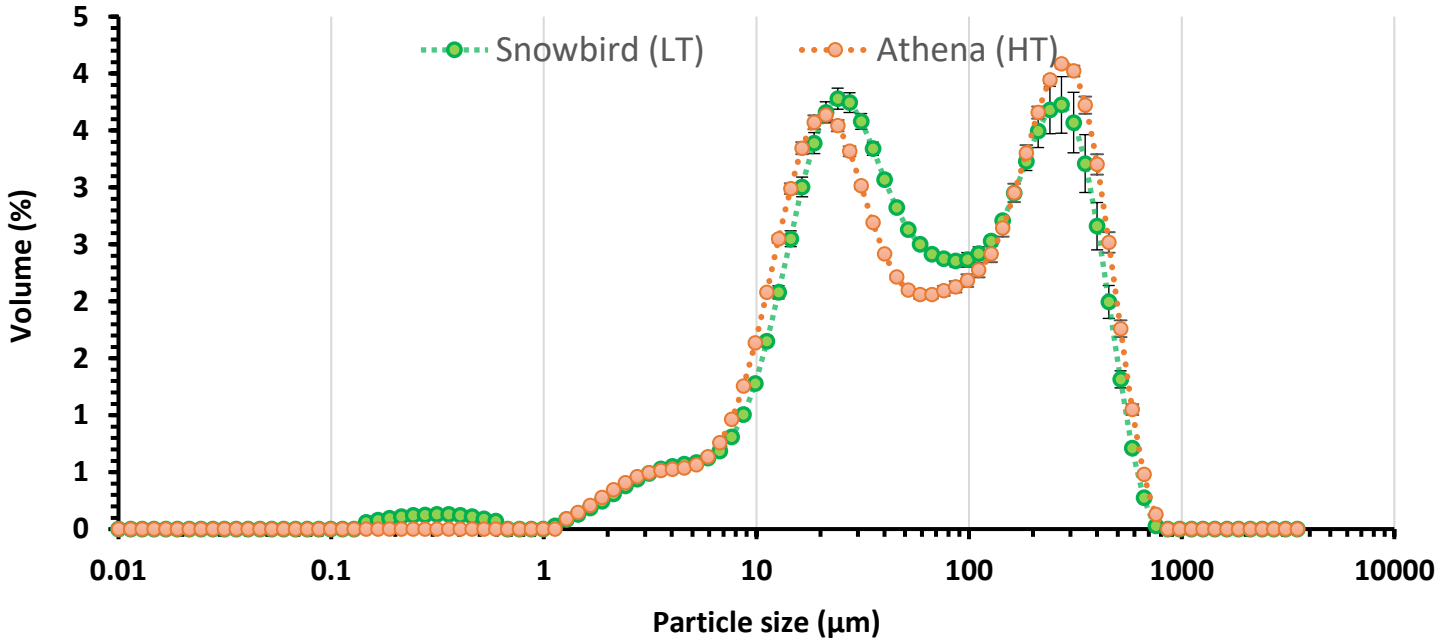
In the ultra-centrifugal mill, size reduction takes place by impact and shearing effects between the rotor and the fixed ring screen/sieve. In the rotor, the centrifugal acceleration throws the feed grain outward with energy where the grain will be crushed on impact by the wedge-shaped rotor teeth moving at a high speed and be ground into a fine powder. This will ensure particularly gentle but fast milling. The feed material will remain in the grinding chamber only for a very short time, which means that the characteristic features of the sample are not altered. TAD was used in this study to remove the seed coats tightly adhering to the beans as well as to pearl the beans. Both abrasive pearling and ultra-centrifugal milling steps could reduce the adhesion between starch granules and small protein bodies, resulting in their separation from each other and other large fibrous components (Saldanha do Carmo et al., 2020).

5.3.2. Macronutrient composition of sieve-separated fractions

Particle size plays a key role in separating the macro components of pulses i.e., starch, protein and dietary fibre (Pelgrom et al., 2015; Schutyser et al., 2015). The most commonly

used techniques in particle size analysis besides image analysis are laser diffraction and sieve analysis. As presented in Fig. 5.3, the volume-weighted mean particle diameter (D[4,3]) of HT (145 μm) was significantly ($P < 0.05$) larger than LT (101 μm). However, the surface-area weighted diameter (D[3,2]) was significantly ($P < 0.05$) larger for LT (29.9 μm) than HT (24.6 μm). For both cultivars, a substantial portion of the particles were larger (Dv[90]: 263 μm for LT and 389 for HT). Only 10% of LT and HT faba bean flour particles were smaller than 15 and 11 μm, respectively. Almost 50% of the faba bean flour particles were either smaller or larger than 50 μm (LT) and 69 μm (HT).

These data suggest that faba beans have a more dispersed particle size distribution and a greater proportion of large particles regardless of the cultivar. This bimodal distribution also proves that the separation of more uniformly distributed coarse and fine fractions is affordable by sieve separation using a ~75 μm sieve. Taken together, this information corroborates the basis for particle size-based separation and separation efficiency. Based on the volume-weighted particle size distribution results illustrated in Fig. 5.3, the faba bean impact milling (500 μm sieve, 8,000 rpm) led to a bimodal distribution of the particles, which confirms that the starch granules were neither damaged nor the fibrous compounds were milled too fine as shown in the SEM images (Fig. 5.4). As depicted in these SEM images, starch granules appeared elliptical, spherical or irregular in shape with a smooth surface and were sized on average at ~6-40 μm. These large starch granules can facilitate the separation of proteins localised in small discrete protein bodies (1-5 μm) during dry fractionation (Saldanha do Carmo et al., 2020; Schutyser et al., 2015).



Sample	Dv[10] (µm)	Dv[50] (µm)	Dv[90] (µm)	D[3,2] (µm)	D[4,3] (µm)
Snowbird	15.00 ± 0.00^{a}	49.57 ± 0.54^{b}	262.67 ± 4.50^{b}	29.90 ± 0.00^{a}	101.13 ± 1.23^{b}
Athena	11.33 ± 0.05^{b}	69.23 ± 1.32^{a}	388.67 ± 3.77^{a}	24.60 ± 0.16^{b}	145.33 ± 1.25^{a}

Fig. 5.3. Particle size distribution of dehulled faba bean flour cvs. Snowbird (LT) and Athena (HT).

$^{a\text{-}b}$Mean ± SD (n = 3) values followed by different letters within a column are significantly different ($P < 0.05$) according to the two-sample t-test. Dv[10]: 10th volume percentile; Dv[50]: 50th volume percentile; Dv[90]: 90th volume percentile, D[4,3]: volume-weighted mean particle diameter; D[3,2]:surface-area weighted mean particle diameter. Dv[10] is the size in microns below which 10% of the sample is represented. Dv[50] is the micron size at which 50% of the sample is either smaller or larger. Dv(90) is the size of the particle below which 90% of the sample fits.

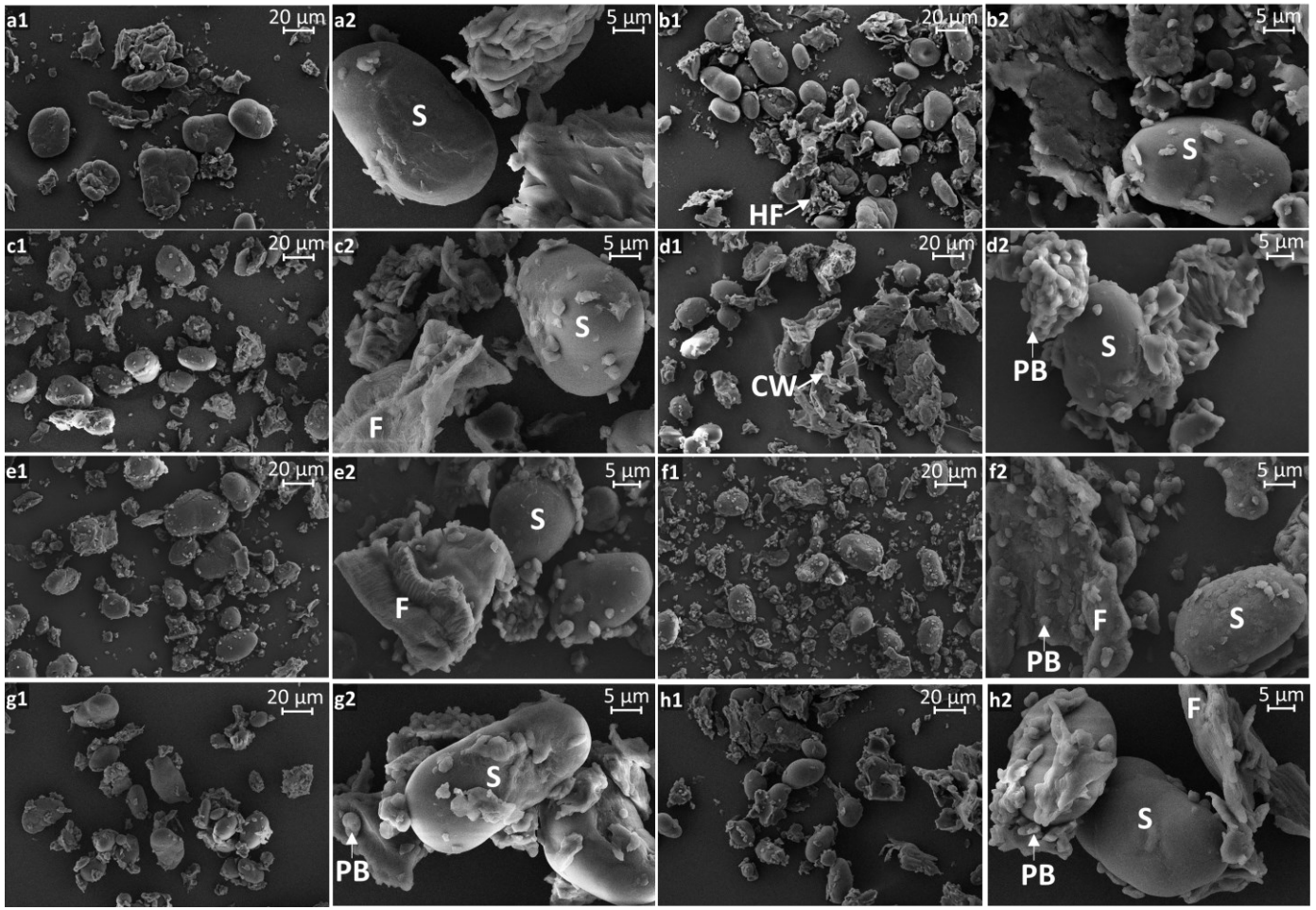
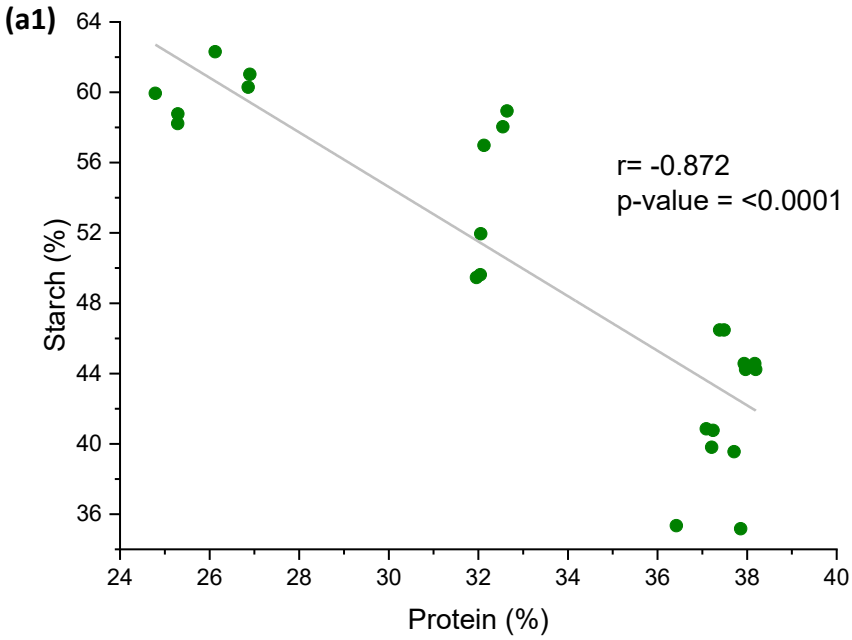


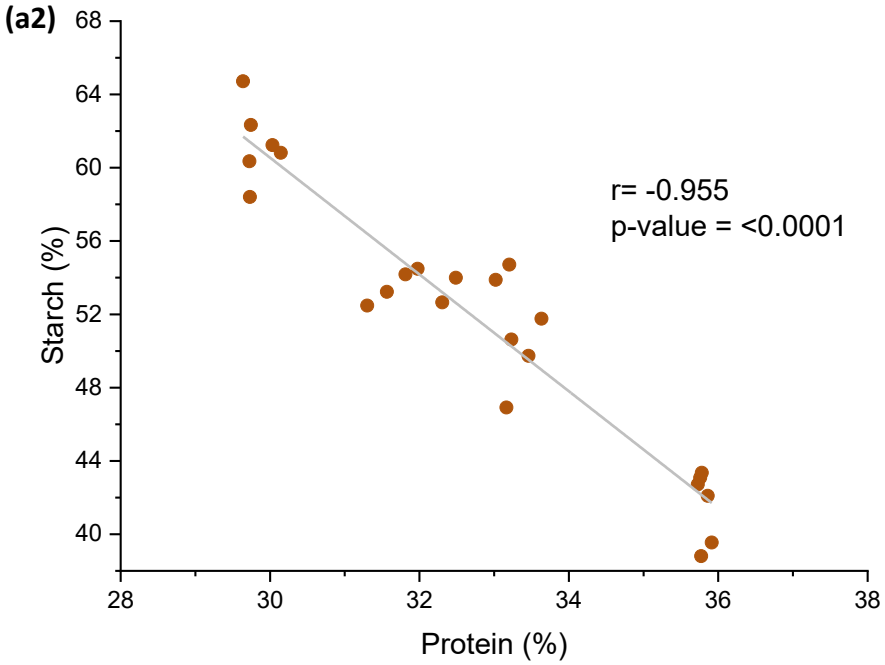
Fig. 5.4. SEM images of faba bean flours: (a,b) whole bean flour, (c-f) dehulled bean flour, and (g,h) coarse fractions of air-currents-assisted particle separation (ACAPS).

Cultivars: Snowbird (LT) (a,c,e,g) and Athena (HT) (b,d,f,h). (1:×500, 2: ×2000 magnification). S: Starch granule; F: fibrous cell wall material; PB: protein body; HF: hull fragments. a-d: < 500 µm (milled), e-f: < 112 µm (fine milled), g-h: 75-500 µm coarse fraction resulting from ACAPS with a 75 µm sieve.

Although architecturally starch granules are larger than the individual protein bodies, the protein bodies usually adhere to the large fibrous fragments, while the starch granules get disintegrated from the protein matrix following dry milling (Fig. 5.4d2). The rubbing action of two materials during impact milling can lead to the exchange of charge, which can lead to contact or frictional charging (Yang et al., 2022). Although the mechanism behind charge transfer remains unclear and is still under investigation, many factors including physical, chemical, electrical and environmental characteristics of the material could impact contact charging (Landauer & Foerst, 2018). This could explain the adherence of protein bodies to other cellular components observed in the SEM images.

This could plausibly explain the strong statistically linear negative relationship ($r = -0.9$, $P < 0.0001$) between the protein and starch contents (Fig. 5.5a1-2) with the increasing particle size regardless of the cultivar. A strong positive correlation ($r = 0.9$, $P < 0.0001$) between the protein and TDF contents was also observed (Fig. 5.5b1-2). It is interesting to note that a similar trend was observed between protein and starch as well as protein and dietary fibre in the different pearling flour fractions as discussed in Chapter 4 (Section 4.3.1.). The aim was to assess the distribution of the macronutrients, protein, starch and dietary fibre in particles of different sizes by traditional sieve analysis. Sieves of 63 and 125 µm sizes were removed from the experiments since no material passed through the 63 µm sieve and only ~1% of the material remained on the 125 µm sieve in the preliminary trial in which the sieves were stacked on top of each other in the ascending order of sieve size. Therefore, 75, 150 and 250 µm sieves were used to further analyse the flour obtained by milling in the Retsch mill using the 500 µm sieve.


(a1)
Starch (%)
Protein (%)
r= -0.872
p-value = <0.0001
64
60
56
52
48
44
40
36
24
26
28
30
32
34
36
38
40


(a2)
Starch (%)
Protein (%)
r= -0.955
p-value = <0.0001
68
64
60
56
52
48
44
40
28
30
32
34
36
38

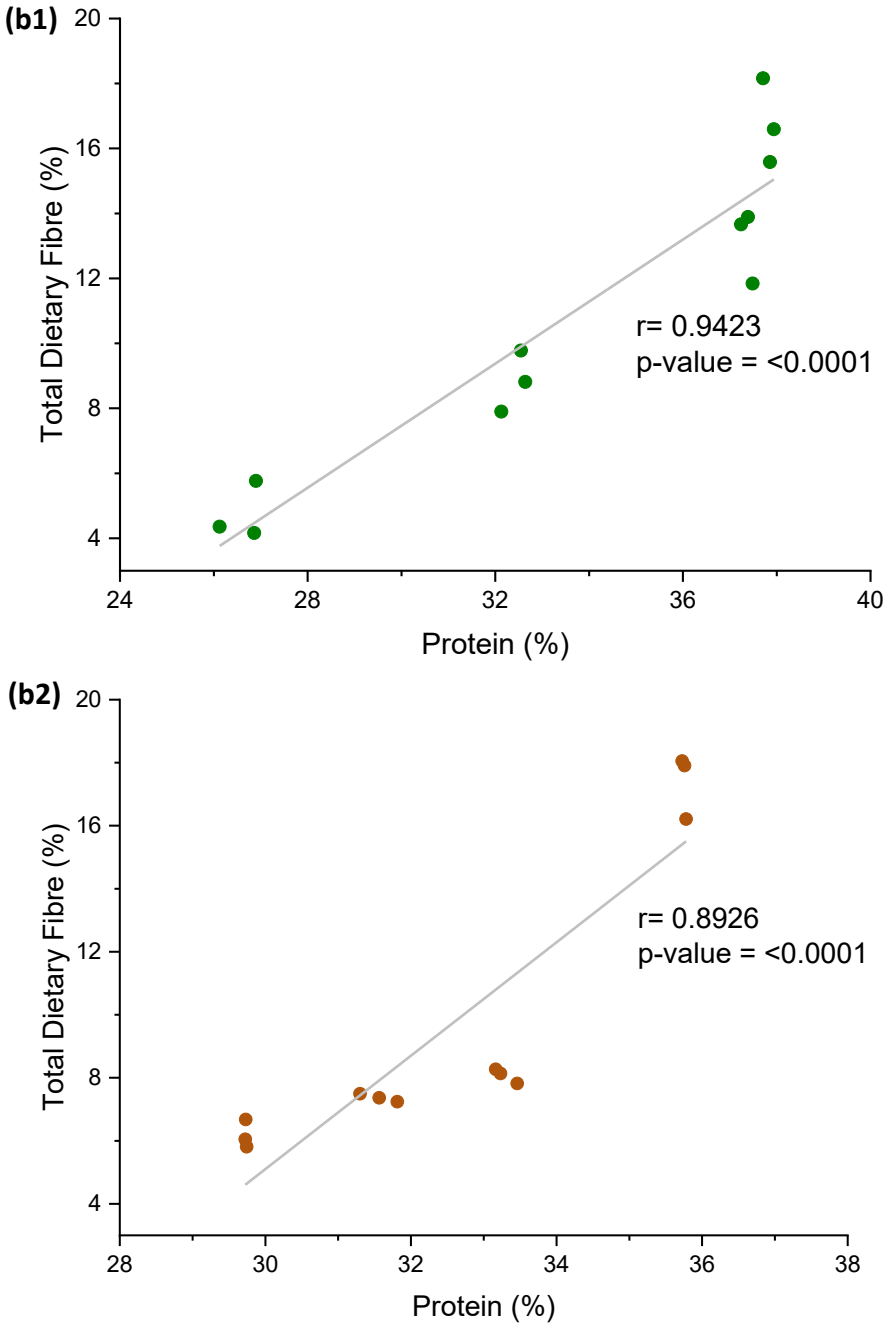


Fig. 5.5. Linear correlation analysis of faba bean (a) protein and starch and, (b) protein and total dietary fibre contents of (1) Snowbird and (2) Athena flour fractions of different particle sizes.

Pearson's correlation coefficient (r) with significance ($P < 0.05$) for the different macronutrients between the different particle size flour fractions (<75 to 500 µm) is given (n = 24 for a and n = 12 for b).

The LT and HT protein contents increased from the fine (<75 µm) to coarse fractions (250-500 µm), bearing a striking resemblance to their TDF contents with significant differences among all the fractions (Table 5.1), except, the protein content of the LT 150-250 µm fraction was not significantly ($P > 0.05$) different from the 250-500 µm fraction. The LT starch content significantly ($P < 0.05$) decreased from that of the fine (75 µm) to coarse (250-500 µm) fraction. The trends of increasing protein and dietary fibre and decreasing starch contents with increasing particle size were observed regardless of the cultivar (Table 5.1). The yield and recovery values given in Table 5.1 were based on the initial compositional analyses of protein (LT: 34.06 ± 0.29% and HT: 34.08 ± 0.15%), starch (LT: 49.53 ± 0.37% and HT: 46.34 ± 0.30%) and dietary fibre (LT: 10.82 ± 0.58% and HT: 10.55 ±0.47%) contents of the dehulled bean flour on a dry matter basis reported previously in Chapter 4 (Table 4.2).

Based on the recovery values, nearly ~84% (LT) and ~92% (HT) of the protein and ~98% of the TDF contents were recovered in the 75-500 µm fraction. However, only ~83% of the starch was recovered in the 75-500 µm fraction of LT as compared to ~92% for HT. Considering that there was a 6-7% loss of feed material in these experiments, normalising the yield to 100% showed that almost ~90% (LT) and ~98% (HT) of the protein, and ~88% (LT) and ~97% (HT) of starch, were recovered in the 75-500 µm fraction, while the TDF recoveries remained almost the same. The yield values of the 150-250 µm fraction were significantly greater for HT in comparison to LT, however, it was *vice versa* with the 75-150 µm fraction. These findings remain complementary to the particle size distribution results.

Table 5.1. Yield (%) of sieve-separated fractions of Snowbird (LT) and Athena (HT) dehulled bean flour using sieves of different sizes and their protein, starch and dietary fibre purity and recovery values (%) on a dry matter basis.

Sample	Particle Size (µm)	Yield %	Purity % (Recovery %)		
			Protein	Starch	Total Dietary Fibre
Snowbird	<75	8.81 ± 0.35^{c}	25.88 ± 0.81^{c} **(6.70 ± 0.22^{c})**	61.21 ± 0.83^{a} **(10.93 ± 0.35^{d})**	4.76 ± 0.88^{d} **(3.88 ± 0.12^{d})**
	75-150	30.68 ± 0.73^{a}	32.23 ± 0.26^{b} **(29.03 ± 0.56^{a})**	57.99 ± 0.80^{b} **(36.06 ± 0.70^{a})**	8.83 ± 0.94^{c} **(25.04 ± 0.48^{c})**
	150-250	30.60 ± 0.79^{a}	37.86 ± 0.31^{a} **(28.95 ± 0.61^{a})**	44.46 ± 0.16^{c} **(27.56 ± 0.58^{b})**	13.33 ± 1.12^{b} **(37.13 ± 0.78^{b})**
	250-500	23.42 ± 0.24^{b}	37.26 ± 0.46^{a} **(26.04 ± 0.21^{b})**	40.48 ± 0.47^{d} **(19.21 ± 0.16^{c})**	16.77 ± 1.30^{a} **(36.30 ± 0.30^{a})**
Athena	<75	2.27 ± 0.29^{d}	29.84 ± 0.19^{d} **(1.99 ± 0.25^{d})**	61.02 ± 0.21^{a} **(2.98 ± 0.38^{d})**	6.18 ± 0.45^{d} **(1.33 ± 0.17^{d})**
	75-150	7.44 ± 0.32^{c}	31.91 ± 0.41^{c} **(6.99 ± 0.30^{c})**	54.22 ± 0.20^{b} **(8.71 ± 0.37^{c})**	7.37 ± 0.12^{c} **(5.20 ± 0.22^{c})**
	150-250	53.02 ± 0.73^{a}	33.29 ± 0.20^{b} **(51.91 ± 0.72^{a})**	49.09 ± 1.58^{c} **(56.17 ± 0.78^{a})**	8.07 ± 0.23^{b} **(40.56 ± 0.56^{b})**
	250-500	31.69 ± 0.70^{b}	35.80 ± 0.06^{a} **(33.37 ± 0.74^{b})**	40.15 ± 1.41^{d} **(27.46 ± 0.61^{b})**	17.39 ± 1.02^{a} **(52.24 ± 1.16^{a})**

$^{a-d}$Mean ± SD (n = 3) values followed by different letters for each macro component of a cultivar within a column are significantly different ($P < 0.05$) according to Duncan's multiple range test. n = 6 for protein purity (%)

Recovery values (%) given within brackets were based on the initial compositional analyses of protein (LT: 34.06 ± 0.29% and HT: 34.08 ± 0.15%), starch (LT: 49.53 ± 0.37% and HT: 46.34 ± 0.30%) and dietary fibre (LT: 10.82 ± 0.58% and HT: 10.55 ±0.47%) contents of the dehulled bean flour on a dry matter basis. Total dietary fibre = insoluble dietary fibre + soluble dietary fibre

5.3.3. Macronutrient composition of ACAPS treated fractions

Taking advantage of the macro component distribution discussed above, different faba bean flour fractions were tested in the lab-scale ACAPS system. The ACAPS technology is a high-throughput sieving apparatus where a vacuum is employed to form air currents to facilitate and enhance the sieving process (Vasanthan, 2017). This separation technique has been previously used for the concentration of barley β-glucan in a time- and cost-efficient manner (Vasanthan, 2017), alongside a starch-rich by-product for bioethanol production (Lu et al., 2020). In the present study, when faba bean flour was subjected to ACAPS with 75 µm sieve openings, only 60% of LT and 58% of HT flour remained on the sieve (Table 5.2) as opposed to the normalised 93% and 98% yields for the 75-500 µm fractions obtained *via* standard sieve analysis (Table 5.1). Therefore, subjecting the samples to ACAPS facilitated the movement of more fine particles into the bottom chamber as compared to traditional sieving, as a result of the air pulsing and vacuum.

The protein content of the coarse fraction (75-500 µm) was significantly higher as compared to the fine fraction (<75 µm) regardless of the variety. When ACAPS separation was performed with a 250 µm sieve, only 21% (LT) and 25% (HT) of the protein and 30% (LT) and 40% (HT) of the TDF remained in the coarse fraction. However, almost 90% of the LT starch, but 72% of the HT starch were recovered in the fine fraction (<250 µm), which was a significant jump as compared to the 46% (LT) and 53% (HT) resulting with a 75 µm sieve (Table 5.2). Subjecting the dehulled bean flour to ACAPS with a 75 µm sieve resulted in a protein and dietary fibre-enriched fraction. In addition, subjecting the single-step pearling flour to ACAPS with a 75 µm sieve resulted in a coarse fraction with 40.89 ± 0.36 and 40.78 ± 0.39% protein content for LT and HT, respectively.

Table 5.2. Yield, purity and recovery values (%) of coarse and fine fractions resulting from air-currents-assisted particle separation (ACAPS) of Snowbird and Athena dehulled faba bean flours on a dry matter basis.

Sample	Particle Size (µm)	Yield %	Purity % (Recovery %)		
			Protein	Starch	Total Dietary Fibre
Snowbird	<75	40.33^{b}	30.02 ± 0.18^{b} **(38.21^{a})**	51.13 ± 0.44^{a} **(45.64^{b})**	9.70 ± 0.61^{c} **(26.87^{b})**
	75-500	59.67^{a}	35.27 ± 0.10^{a} **(61.79^{a})**	42.22 ± 0.30^{b} **(54.36^{a})**	13.27 ± 0.15^{b} **(73.18^{a})**
Athena	<75	41.58^{b}	30.51 ± 0.04^{b} **(40.58^{b})**	51.65 ± 0.86^{a} **(53.33^{a})**	8.62 ± 0.11^{c} **(19.35^{b})**
	75-500	58.42^{a}	34.59 ± 0.14^{a} **(59.42^{a})**	37.02 ± 0.87^{b} **(46.67^{b})**	14.57 ± 0.48^{a} **(80.65^{a})**
Snowbird	<250	81.75^{a}	34.22 ± 0.83^{b} **(82.14^{a})**	54.21 ± 0.32^{a} **(89.81^{a})**	9.57 ± 0.58^{b} **(72.32^{a})**
	250-500	18.25^{b}	38.96 ± 0.33^{a} **(20.88^{b})**	34.99 ± 1.32^{b} **(12.94^{b})**	17.59 ± 0.79^{a} **(29.66^{b})**
Athena	<250	77.43^{a}	33.46± 0.31^{b} **(76.21^{a})**	45.78 ± 1.79^{a} **(71.83^{a})**	10.57 ± 0.14^{b} **(77.61^{a})**
	250-500	22.57^{b}	37.54 ± 0.20^{a} **(24.92^{b})**	37.15 ± 1.53^{b} **(16.99^{b})**	18.71 ± 0.30^{a} **(40.03^{b})**

$^{a-b}$Mean ± SD (n=3) values followed by different letters within a column for a particular category are significantly different ($P < 0.05$) according to the two-sample t-test.

Recovery values (%) given within brackets were based on the initial compositional analyses of protein (LT: 34.06 ± 0.29% and HT: 34.08 ± 0.15%), starch (LT: 49.53 ± 0.37% and HT: 46.34 ± 0.30%) and dietary fibre (LT: 10.82 ± 0.58% and HT: 10.55 ±0.47%) contents of the dehulled bean flour on a dry matter basis.

In the preliminary trials, fine ground (< 112 µm sieve used in Retsch mill) faba bean flour was also subjected to ACAPS with the 75 µm sieve, and only 7% of the LT material and 6% of the HT material passed through the sieve as the fine fraction. As was also the case in standard sieve separation, nothing passed through the 63 µm sieve using ACAPS. However, when the dehulled bean flour was subjected to fine grinding with a 112 µm sieve (Fig. 5.4e1-1f2) more starch granules were detached from the protein matrix and a greater proportion of proteins were disintegrated into discrete protein bodies. However, it is apparent from the SEM images (Fig. 5.4e1-1f2) that the protein bodies are adhering to the large fibrous fragments and starch granules upon fine grinding. This could plausibly explain why the proteins in the fine ground samples subjected to ACAPS with both 63 µm and 75 µm sieves did not pass to the fine fraction.

Preliminary trials done with the pearling flour and finely milled samples did not provide any promising results in terms of yields and recoveries. Therefore, further analyses were not carried out with the pearling flour or fine ground flour. The ACAPS system produces air currents, where a vortexing flow is formed, which are also capable of increasing the charge density of the particles. However, in the tribe-electrostatic separation technique, greater airflow rates have impaired the separation of protein from starch as both protein and starch were found to gain the same polarity (Yang et al., 2022). However, other studies have demonstrated that protein was charged positively, while starch was charged negatively (Landauer & Foerst, 2018). The SEM images (Fig. 5.4g1-h2) of the ACAPS-treated coarse fractions showcase that protein bodies were still attached to the fibrous material and starch granules similar to the fine ground fractions, which could have impeded their separation into the fine fraction. The collective charge build-up during impact milling and ACAPS could have interfered with the separation of the fine particles from

the coarse fraction of the finely milled faba bean flour; however, understanding the changes in charge density of particles during ACAPS requires further research.

5.3.4. Hybrid fractionation

5.3.4.1. Extractability of protein, starch and dietary fibre

Although milling and concomitant sieving/ACAPS can result in fractions enriched in one or more of the macro components, the isolation of proteins for specific functional food applications is deemed essential through wet fractionation. Often at a low salt concentration regime, with an increasing salt concentration, the solubility of legume protein increases referred to as salting-in. Water was used instead of salt solution to take advantage of the minerals (~4% ash) naturally present in faba beans to extract the salt-soluble but water-insoluble globulins. The mineral ions will act to stabilise the protein-water interactions by decreasing the electrostatic energy between the protein molecules and increasing the protein solubility (Ahmed et al., 2016). Following the salting-in process of faba bean proteins, introducing water at 4 °C could have resulted in a sequence of dissociation reactions of the proteins. Different protein molecules interacting with each other could have formed a protein network leading to the accumulation of amphiphilic proteins in size and number until proteins precipitate. This process is known as micellization since it can lead to the formation of protein micelles (Ahmed et al., 2016).

The SEM images of the protein isolates (Fig. 5.6) confirm that the proteins still exhibit the globular structure of the proteins referred to as protein bodies in the native state. Freeze-drying the protein isolates produced from the ACAPS coarse and fine fractions could have extended the globular structure of the micellar proteins two-dimensionally resulting in sheet-like (Fig. 5.6a1 and c1) and rod-shaped (Fig. 5.6b1 and d2) structures, respectively.

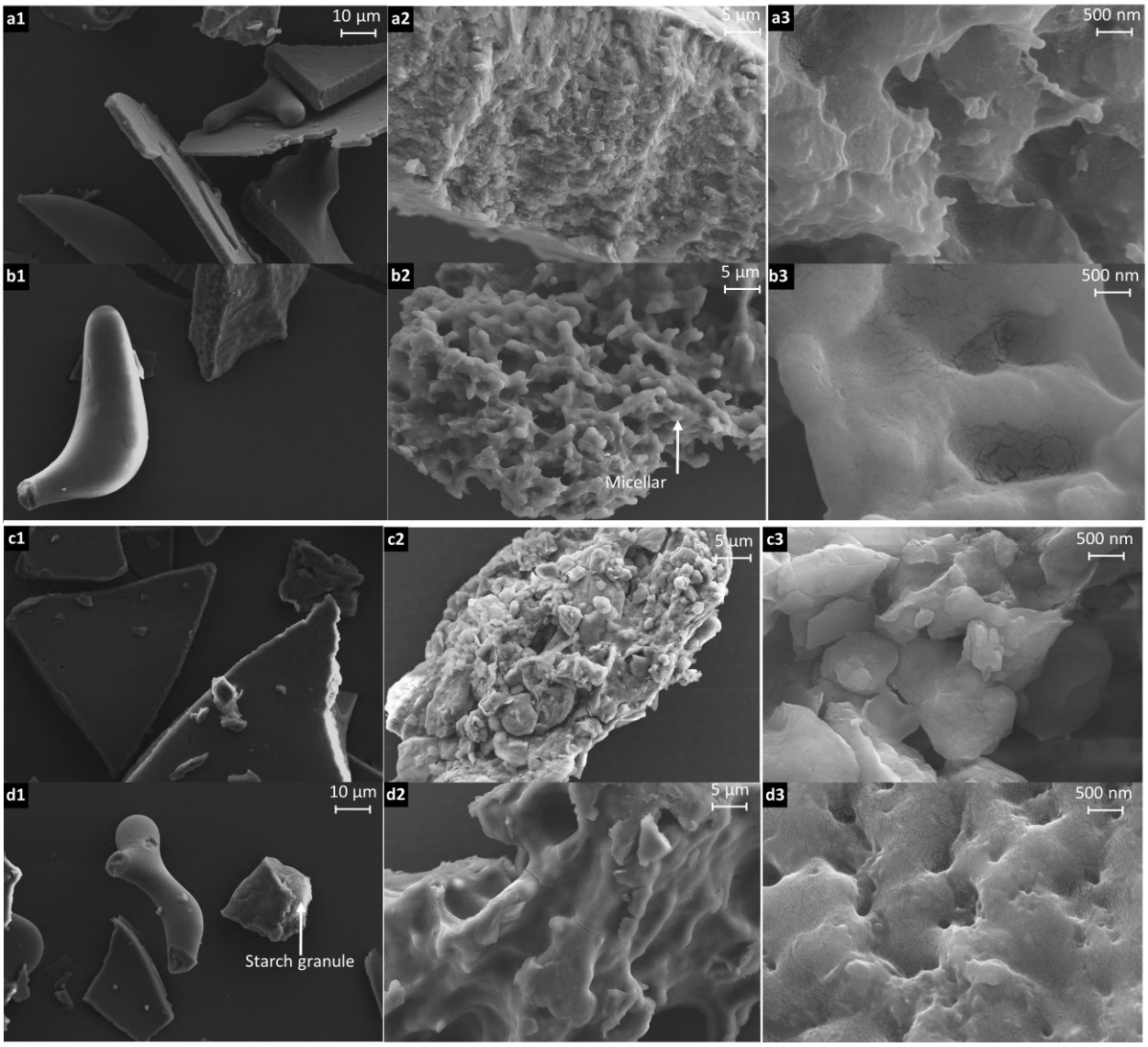


Fig. 5.6. SEM images of faba bean hybrid-fractionated micellar protein isolates.

(a,b) Snowbird (LT) and (c,d) Athena (HT), resulting from the (a,c) coarse and (b,d) fine fractions obtained by air-currents-assisted particle separation (250 µm sieve) subjected to micellization (1:×1000, 2:×2,000 and 3:×20,000 magnification).

The high-resolution images of the protein isolate produced from LT coarse (Fig. 5.6a2-3) and fine (Fig. 5.6b2-3) fractions as well as HT coarse (Fig. 5.6c2-3) and fine (Fig. 5.6d2-3) fractions, revealed the formation of protein micelles as a result of introducing cold water. The LT fine fraction (Fig. 5.6b2) in particular revealed the formation of a dense network of relatively uniform aggregates of smaller spherical protein sub-structures (<200 nm). These results were comparable to the findings discussed in Chapter 4 (Section 4.5) confirming that regardless of the dry fractionation approach or the raw material being used, the micellization step resulted in protein isolates, which exhibited morphologically similar structures.

Hybrid fractionation efficiency was investigated relative to the purities and yields of the respective fractions obtained (Table 5.3). Chemical-free aqueous fractionation of the ACAPS-treated (250 μm) flour fractions resulted in protein isolates, starch isolates and dietary fibre concentrates. Protein yields and recoveries were greater for the HT coarse fraction as compared to LT, which signifies a better protein extractability of the underutilised HT cultivar as compared to the LT cultivar. The protein recovery of the hybrid fractionated LT coarse fraction was 48% whereas it was 54% for the HT coarse fraction. HT. Although the starch isolates of >91% purity were obtained from both the coarse and fine fractions, starch recovery was greater in the HT fine fraction (76%) as compared to the coarse fraction (39%), which was the opposite for LT. These values corroborate that a larger surface area per volume resulting from smaller particles is likely to result in a greater yield, which is in agreement with the particle size distribution results (Fig. 5.3).

Table 5.3. Yield, protein, starch and dietary fibre purity and recovery values (%) of the water extracted outputs of the coarse and fine fractions resulting from ACAPS of dehulled faba bean flours on a dry matter basis.

Fraction	Component	LT-Coarse	LT-Fine	HT-Coarse	HT-Fine
Protein	Yield	19.27 ± 0.04[a]	9.67 ± 0.10[b]	21.33 ± 0.33[a]	5.79 ± 0.10[b]
	Nitrogen	15.64 ± 0.03	14.20 ± 0.06	15.09 ± 0.05	13.96 ± 0.01
	Protein	97.74 ± 0.18[a]	88.77 ± 0.36[b]	94.30 ± 0.29[a]	87.26 ± 0.05[b]
		(48.34 ± 0.06)	(25.08 ± 0.07)	(53.58 ± 0.12)	(15.09 ± 0.01)
	Starch	0.95 ± 0.06[b]	5.59 ± 0.09[a]	2.58 ± 0.05[b]	7.15 ± 0.13[a]
		(0.73 ± 0.05)	(1.10 ± 0.02)	(1.48 ± 0.03)	(0.84 ± 0.02)
Non-precipitated Fraction	Yield	22.77 ± 0.28[a]	19.26 ± 0.08[b]	17.64 ± 0.61[a]	13.25 ± 0.25[b]
	Nitrogen	9.69 ± 0.06	11.36 ± 0.03	10.73 ± 0.00	11.14 ± 0.03
	Protein	60.58 ± 0.35[b]	71.00 ± 0.16[a]	67.07 ± 0.01[b]	69.64 ± 0.19[a]
		(35.40 ± 0.16)	(39.95 ± 0.06)	(31.52 ± 0.50)	(27.62 ± 0.09)
	Starch	0.98 ± 0.01[a]	0.69 ± 0.04[b]	0.89 ± 0.09[a]	0.86 ± 0.02[a]
		(0.89 ± 0.02)	(0.27 ± 0.02)	(0.42 ± 0.04)	(0.23 ± 0.01)
Starch	Yield	20.77 ± 0.68[b]	31.90 ± 0.10[a]	15.46 ± 0.10[b]	40.58 ± 0.10[a]
	Nitrogen	0.26 ± 0.01	0.37 ± 0.01	0.22 ± 0.01	0.48 ± 0.02
	Protein	1.62 ± 0.07[b]	2.30 ± 0.08[a]	1.36 ± 0.04[b]	3.02 ± 0.12[a]
		(0.86 ± 0.02)	(2.15 ± 0.05)	(0.56 ± 0.02)	(3.66 ± 0.11)
	Starch	94.05 ± 0.96[a]	91.44 ± 1.24[b]	94.33 ± 0.65[a]	92.13 ± 1.56[b]
		(78.15 ± 2.50)	(59.11 ± 0.80)	(39.26 ± 0.46)	(75.75 ± 1.28)
Dietary Fibre	Yield	16.86 ± 0.10[a]	10.17± 0.20[b]	18.87 ± 0.61[a]	10.15 ± 0.01[b]
	Nitrogen	4.31 ± 0.01	4.16 ± 0.02	4.82 ± 0.07	3.92 ± 0.08
	Protein	26.93 ± 0.01[a]	25.98 ± 0.15[b]	28.49 ± 0.34[a]	24.51 ± 0.52[b]
		(12.35 ± 0.02)	(7.72 ± 0.03)	(14.33 ± 0.17)	(7.43 ± 0.11)
	Dietary Fibre	53.84 ± 0.57[a]	36.41 ± 0.92[b]	44.81 ± 0.07[a]	35.75 ± 0.17[b]
		(54.66 ± 0.58)	(34.21 ± 0.86)	(45.22 ± 0.07)	(33.53 ± 0.16)
	Starch	17.05 ± 0.23[a]	12.02 ± 0.08[b]	15.47 ± 0.25[a]	12.17 ± 0.13[b]
		(12.18 ± 0.16)	(2.48 ± 0.02)	(7.86 ± 0.13)	(2.50 ± 0.03)
Intermediate Fraction	Yield	4.28 ± 0.01[a]	3.80 ± 0.30[b]	3.89 ± 0.10[a]	3.76 ± 0.55[a]
	Nitrogen	4.20 ± 0.04	5.80 ± 0.02	6.24 ± 0.04	4.07 ± 0.12
	Protein	26.25 ± 0.22[b]	36.22 ± 0.13[a]	26.09 ± 0.27[a]	25.46 ± 0.73[b]
		(2.89 ± 0.02)	(4.02 ± 0.01)	(2.70 ± 0.03)	(2.86 ± 0.06)
	Starch	21.66 ± 1.73[b]	45.69 ± 1.02[a]	38.03 ± 0.92[b]	81.67 ± 0.41[a]
		(3.71 ± 0.30)	(3.52 ± 0.08)	(3.98 ± 0.10)	(6.21 ± 0.03)

[a-b] Mean ± SD (n = 3) values followed by different letters within a row for a particular cultivar are not significantly different ($P > 0.05$) according to the two-sample t-test.

LT: Snowbird, HT: Athena
Coarse: fraction remaining on top of air-currents-assisted particle separation (ACAPS) sieve in the top chamber,
Fine: fraction passing through ACAPS sieve opening and entering the bottom chamber
Recovery values (%) given within brackets were based on the compositional analyses of protein, starch and dietary fibre (Table 5.2) contents of the ACAPS-treated faba bean coarse and fine fractions obtained using a 250 µm sieve on a dry matter basis.

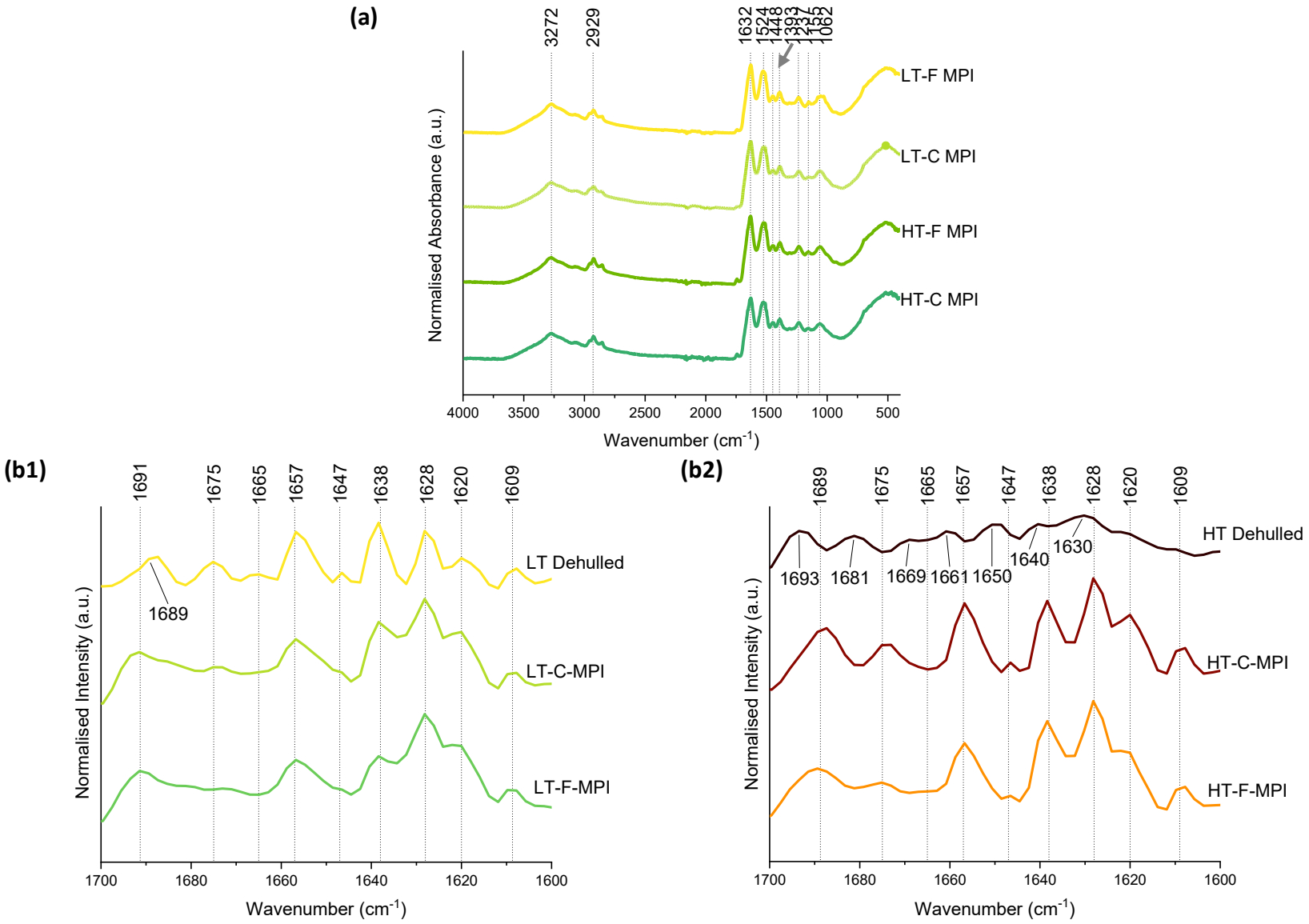
Variations in terms of purity and yield of the protein, starch and dietary fibre fractions resulting from the differences in the coarse and fine fractions obtained by ACAPS treatment were more significant. The coarse fractions resulted in protein fractions of 98% and 94% purity as compared to the fine fractions, which were 89% and 87% for LT and HT, respectively. This could be because smaller starch granules of similar size/shape to that of protein bodies must have passed into the fine fraction, interfering with the separation of protein from starch. Also, the wet extraction of the coarse fraction resulted in dietary fibre concentrates (LT: 54% and HT: 45% purity), which were significantly greater in TDF content than the corresponding fine fractions (36%). The dietary fibre concentrate resulting from the coarse fraction also has a considerable amount of protein (LT: 27% and HT: 28% purity); thus, creating the potential for a fraction enriched in both protein and dietary fibre for food applications. Faba bean is a significant source of nitrogenous anti-nutrients vicine and convicine (Johnson et al., 2020), which are relatively small molecules in comparison to proteins (Khazaei et al., 2019). Therefore, the removal of any remaining vicine and convicine or any non-protein nitrogenous compounds from the protein fractions could be achieved by dialysis (Collier, 1976). The micellar protein isolates are known to be minimal in non-protein nitrogenous compounds due to their shift into the non-precipitated fraction (LT: 61-71% purity and 19-25% yield and HT: 67-70% purity and 13-30% yield), which is comparable to the findings of Arntfield et al. (1985).

Nearly 84% of the protein in the 18% ACAPS-treated coarse fraction was recovered in the protein (48%) and non-precipitated fractions (35%) of LT, whereas 65% of the protein in the 82% fine fraction was recovered in the corresponding protein and non-precipitated fractions. To achieve maximum recovery of the proteins, the ACAPS-treated coarse fraction

can be used in minimally processed food applications, while the fine fraction can be subjected to water extraction followed by micellization.

5.3.4.2. Protein secondary structure conformational changes

FTIR spectroscopy is a robust non-destructive method for detecting a wide range of functional groups and their molecular conformational changes (Wang et al., 2013). The LT and HT micellar protein isolates exhibited FTIR spectral characteristics (Fig. 5.7) similar to the findings discussed in Chapter 4 (Fig. 4.6). The stretching vibrations of O-H and N-H correspond to the strong band at ~3272 cm^{-1} (Fig. 5.7a) and the asymmetric stretching vibration of C-H in CH_2 groups of carbohydrates, lipids or proteins can be ascribed to the band at ~2929 cm^{-1}. The predominant band at ~1632 cm^{-1} appearing in the amide I region can be assigned to the C=O stretch. The C-N stretching and N-H bending vibration of the amide II region, which could vary due to the level of H-bonding can be allocated to the band at ~1524 cm^{-1}. Both the bands at ~1448 and 1393 cm^{-1} can be correlated to the bending vibrations of CH_2 and CH_3, respectively. The peak at ~1237 cm^{-1} can be assigned to the vibrations of the amide III band. Both the spectral bands at ~1155 and 1062 cm^{-1} correspond to the C-OH stretching vibrations of carbohydrates (Johnson et al., 2020; Wang et al., 2013). The amide I, II and III bands of FTIR protein spectra are highly sensitive to changes in protein secondary structures expressed as the dynamics of protein folding/unfolding with the amide I band as the most widely used (Johnson et al., 2020; Wang et al., 2013).


(a)
3272
2929
1632
1524
1448
1393
1335
1062
LT-F MPI
LT-C MPI
HT-F MPI
HT-C MPI
Normalised Absorbance (a.u.)
4000
3500
3000
2500
2000
1500
1000
500
Wavenumber (cm-1)
(b1)
1691
1675
1665
1657
1647
1638
1628
1620
1609
LT Dehulled
1689
LT-C-MPI
LT-F-MPI
Normalised Intensity (a.u.)
1700
1680
1660
1640
1620
1600
Wavenumber (cm-1)
(b2)
1689
1675
1665
1657
1647
1638
1628
1620
1609
HT Dehulled
1693
1681
1669
1661
1650
1640
1630
HT-C-MPI
HT-F-MPI
Normalised Intensity (a.u.)
1700
1680
1660
1640
1620
1600
Wavenumber (cm-1)

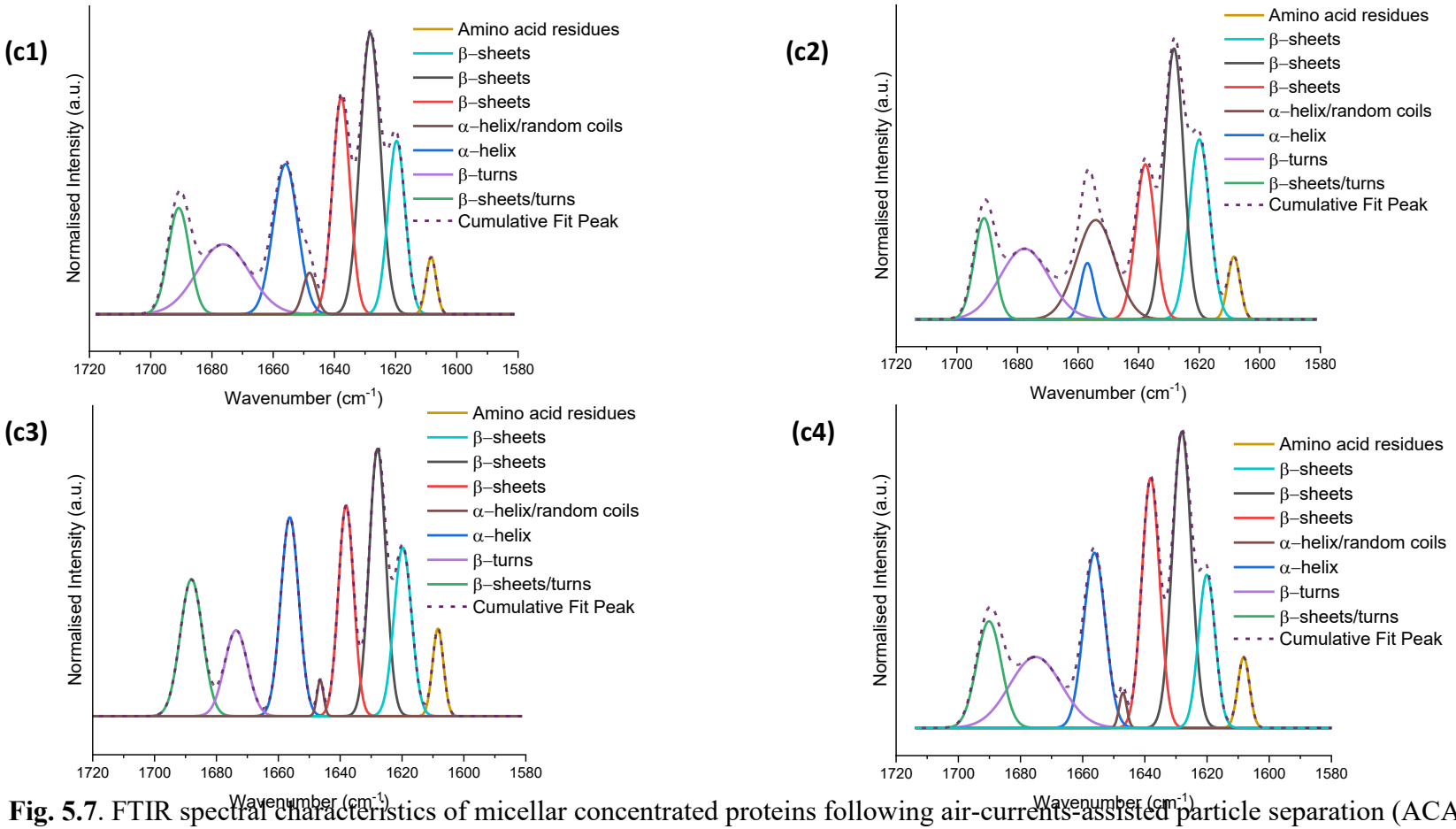


Fig. 5.7. FTIR spectral characteristics of micellar concentrated proteins following air-currents-assisted particle separation (ACAPS of faba bean cvs. Snowbird (LT) and Athena (HT).

(a) FTIR spectra (400-4000 cm^{-1}) of faba bean cvs. Snowbird (LT) and Athena (HT) micellar protein isolates (MPI) obtained from the coarse (C) and fine (F) fractions of ACAPS corresponding to the specific variation of selected functional groups, (b) second derivative spectral peak identification of amide I vibrational region (1600-1700 cm^{-1}) attributed to secondary structures of (b1) Snowbird and (b2) Athena micellar protein isolate in comparison to the dehulled beans, and (c) curve-fitted second derivative peaks of (c1) LT-C-MPI, (c2) LT-F-MPI, (c3) HT-C-MPI and (c4) HT-F-MPI.

According to Fig. 5.7b1 and b2, the amide I region of both LT and HT micellar protein isolates mainly exhibited 8 bands at ~1609 cm^{-1} (amino acid residues), ~1620 cm^{-1}, ~1628 cm^{-1} and ~1638 cm^{-1} (β-sheets), ~1647 cm^{-1} and ~1657 cm^{-1} (α-helix), ~1675 cm^{-1} (β-sheets), and ~1689/1691 cm^{-1} (β-sheets/turns) comparable to the results of Yang et al. (2018). The dehulled faba bean flour of these faba bean cultivars resulted in 9 bands. However, ACAPS followed by micellar concentration of proteins lead to the diminishing of the low-intensity band at ~1665 cm^{-1} corresponding to β-turns, resulting in 8 bands (Fig. 5.7b1-64) similar to the trends observed previously in Chapter 4 (Fig. 4.6). The overall peak intensities of β-sheets (1620-1638 cm^{-1}) are enhanced at the expense of α-helix (1647-1657 cm^{-1}).

The second derivative spectra of HT dehulled bean flour showed lower peak intensities as compared to LT dehulled bean flour, possibly resulting from the potential interactions of tannins and protein secondary structures. Tannins can form complexes with proteins due to their affinity toward the carbonyl group bonding points (Rodríguez-Espinosa et al., 2019). The peaks showing up at ~1693, 1681, 1669, 1661, 1650, 1640 and 1630 cm^{-1} (Fig. 5.7b2) must have shifted from 1689, 1675, 1665, 1657, 1647, 1638 and 1628 cm^{-1}, respectively, emphasizing the major reduction of tannins during the HT protein isolation steps. Spectral characteristics of both LT-C-MPI and LT-F-MPI, as well as HT-C-MPI and HT-F-MPI were identical. Both LT and HT micellar protein isolates shared similar IR spectral characteristics, except for the peak corresponding to β-sheets/turns appearing at 1691 cm^{-1} for LT appearing at 1689 cm^{-1} for HT (Fig. 5.7b1-2). The hybrid-fractionation process led to minimal protein conformational changes or secondary structural changes. Based on these findings, it can be concluded that large β-sheets are the dominant secondary structure of faba bean proteins followed by α-helix and β-turns.

5.4. CONCLUSIONS

The compositional analysis results after milling and sieving faba bean flour showed that protein and dietary fibre content gradually increased with the increasing particle size, which was offset by starch distribution. Subjecting the dehulled bean flour to ACAPS treatment with a 75 μm sieve resulted in value-added coarse fractions enriched in both protein (LT: 62% and HT: 60%, recovery) and dietary fibre (LT: 73% and HT: 81%, recovery). Meanwhile, ACAPS treatment with a 250 μm sieve, resulted in protein-rich coarse fractions (LT: 39% and HT: 38%, N×6.25). Subjecting this coarse fraction to micellization resulted in protein isolates (LT: 97.74±0.18% and HT: 94.30±0.29%), starch isolates (LT: 94% and HT: 94%) and dietary fibre concentrates (LT: 54% and HT: 45%) as co-products.

The hybrid-fractionation approach of this study is a promising method for extracting dietary fibre concentrates alongside the high-purity protein and starch isolates with large yields. The micellization step enabled the further separation of the protein from non-protein nitrogen. The mild wet fractionation process offers the potential to produce fractions similar to conventional wet fractionation while excluding the use of chemicals. The proposed method is advantageous as the water can be recycled and any changes to the physicochemical properties can be minimised, as investigated in parallel studies and presented in later chapters. It was confirmed that ACAPS followed by water-only wet fractionation had minimal impact on protein secondary structure, which means it can be utilized as a native functional food ingredient in food applications. Furthermore, this novel process has the potential to add or skip processing steps depending on the desired final ingredient composition and functionality. The findings highlight that hybrid-fractionation shows promise with regard to other alternatives in the food industry; ultimately

creating new markets for the underutilised high-tannin faba bean cultivars similar to that of competing low-tannin cultivars.

CHAPTER 6: Isolation of clean-label faba bean (*Vicia faba* L.) proteins: a comparative study of mild fractionation methods against traditional technologies[5]

[5]A version of this chapter has been published: Jeganathan, B., Vasanthan, T., & Temelli, F. (2023). Isolation of clean label faba bean (*Vicia faba* L.) proteins: A comparative study of mild fractionation methods against traditional technologies. *Innovative Food Science and Emerging Technologies, 84,* 103285. https://doi.org/10.1016/j.ifset.2023.103285

6.1. INTRODUCTION

One of humanity's greatest current challenges is meeting the ever-increasing global protein demand under limited resources (Mayer Labba et al., 2021). This has created an imperative shift to plant-based alternative products in the Western world, inflating the popularity of pulses as a sustainable source of protein as well as dietary fibre (Boland et al., 2013; Henchion et al., 2017). Faba bean (*Vicia faba* L.) also identified as fava bean is a gluten-free and GMO-free protein source complementary to the essential amino acid deficiencies of cereal-based diets (Acevedo-Pacheco & Serna-Saldívar, 2016). However, the presence of flatulence-causing oligosaccharides enzyme inhibitors, tannins, vicine and convicine can diminish their food value by impeding protein digestibility and mineral bioavailability or causing favism (Landry et al., 2016; Multari et al., 2015). Faba beans can be broadly classified into low-tannin and high-tannin varieties, and the isolation of faba bean protein fractions free of negative compounds from both low- and high-tannin cultivars is warranted for their value-added utilisation as functional food ingredients.

Dry fractionation approaches such as milling and sieving or air classification based on physical properties (i.e., particle size, shape and density) have been known to result in moderate enrichment in protein content while enhancing the bioactive compounds in the resulting protein concentrates (PCs) with more than ~70% protein purity (Saldanha do Carmo et al., 2020; Schutyser et al., 2015). Alternatively, wet fractionation approaches can be used to obtain protein isolates (PIs) with more than 90% protein content. The nature of the raw material or the type of storage proteins present, extraction conditions such as pH, time, temperature, solvent properties and solvent/feed (S/F) ratio can influence the physicochemical characteristics of the proteins and the magnitude to which these characteristics can affect the functional properties of the proteins (Jarpa-

Parra et al., 2014; Jarpa-Parra, 2018; Karaca et al., 2011; Langton et al., 2020; Ruiz-Ruiz et al., 2012).

The pH-shift method is the most widely used wet fractionation method to isolate legume proteins (Karaca et al., 2011; Langton et al., 2020; McCurdy & Knipfel, 1990) based on the impact of pH on protein solubility. The proteins can gain negative charges in alkaline solutions and become more soluble due to electrostatic repulsive forces, which then become minimal at their isoelectric point (pH 4 to 5), leading to their precipitation (Boye et al., 2010). The major drawbacks of the conventional wet fractionation methods are the large consumption of water (~87 kg of H_20/kg protein isolate) and energy (~63 MJ/kg protein) as compared to hybrid fractionation (~35 kg of H_20/kg protein isolate and ~11 MJ/kg protein, respectively), the partial loss of the native functionality of the proteins (Assatory et al., 2019; Berghout et al., 2015; Sun & Arntfield, 2010), and the formation of the undesirable amino acid derivative lysinoalanine (Gould & MacGregor, 1977). Lysinoalanine formed in alkali- and heat-treated soy protein isolates was shown to induce nephrocytomegaly in the pars recta of the proximal tubule in male rats (Karayiannis et al., 1980).

Compared to the conventional pH-shift method, mild fractionation approaches to extract legume proteins are proven to be more resource efficient by using less water and energy and no chemicals (Geerts et al., 2018). Proteins obtained in this manner retain their native properties and exhibit better solubility (Pelgrom et al., 2015). Aqueous salt ($salt_{(aq)}$) extraction is a relatively common method employed to isolate pulse proteins based on the solubility of the major storage proteins (Wang et al., 2010). Nearly 69 to 78% of the total storage proteins of faba beans are salt-soluble globulins, found mainly in the membrane-bound protein bodies (Multari et al., 2015). Consumer demand for clean-label proteins low or free of salts necessitates the use of an additional step such as micellar precipitation (Murray et al., 1979) or membrane filtration techniques such

as dialysis, diafiltration or ultrafiltration following $salt_{(aq)}$ extraction to remove salts while isolating the proteins (Koros et al., 1996).

Previous studies reported in Chapters 4 and 5 showed that hybrid fractionation of clean-label proteins, involving dry processes such as pearling or air-currents-assisted particle separation (ACAPS) followed by water extraction of micellar protein isolates had minimal impact on the conformational characteristics of protein secondary structures and yielded starch isolates and dietary fibre concentrates as value-added co-products. Regardless of the dry fractionation approach used in Chapters 4 and 5, extracting the proteins with water at a significantly low S/F ratio of 2 and introducing water at 4 °C as part of the micellization step to reduce the ionic strength can lead to a sequence of dissociation reactions and accumulation of amphiphilic proteins until they precipitate as protein isolates (Murray et al., 1978). Precipitation of the protein can also be achieved by removing the salts through dialysis resulting in the formation of protein micelles, which grow in size and number (Koros et al., 1996; Sun & Arntfield, 2010).

To further investigate the role of different levels of salts in mild wet fractionation and to optimise hybrid processes based on protein yield, purity and recovery, it is essential to explore $salt_{(aq)}$-based fractionation approaches in comparison to the pH-shift method. Efficient mild wet processing methods free of alkali and thermal treatments should be explored to cater to the consumer demand for clean-label low-salt food ingredients. To valorise faba beans as an alternative protein source low in salt level, it is imperative to understand the extractability of proteins at different salt concentrations, temperatures and low S/F ratios as opposed to the conventional salt-based mild wet fractionation conditions (Chéreau et al., 2016; Schutyser et al., 2015).

However, the literature lacks a detailed comparison of these different technological advances for the value-added fractionation of faba beans. Therefore, the objectives of the present study were to: 1) explore the impact of different $NaCl_{(aq)}$ solution concentrations, S/F ratios and temperatures on faba bean protein extractability; 2) compare different mild extraction methods involving water or $NaCl_{(aq)}$ solution followed by dialysis, ultrafiltration or micellization to concentrate/isolate faba bean proteins, based on composition, yield, purity and recovery of the macronutrients against the pH-shift method; and 3) investigate the impact of selected mild wet fractionation methods on faba bean protein amino acid and molecular weight profiles, total tannins, raffinose family oligosaccharides, *in-vitro* protein digestibility and trypsin units inhibited.

6.2. MATERIALS AND METHODS

6.2.1. Materials

High tannin faba bean cv. Athena (HT) and low-tannin cultivar Snowbird (LT) were obtained from W.A. Grain and Pulse Solutions, Innisfail, AB, Canada. Coomassie (Bradford) protein assay kit, analytical grade hydrochloric acid and formic acid, and HPLC grade methanol, sodium acetate, sodium borate, sodium hydroxide and ultra-pure water were acquired from Fisher (Waltham, MA, USA). Laemmli sample buffer and 4 to 20% gradient precast Mini-PROTEAN gels were obtained from Bio-Rad (Hercules, CA, USA). Amino acid standards mix P/N AAS18 (2500 pmol/µL), o-pthaldiadehyde (OPA), mercaptopropionic acid, Folin Ciocalteu's reagent, trypsin from porcine pancreas: type IX-S lyophilized powder (13000 to 20000 BAEE U/mg protein), α-chymotrypsin from bovine pancreas: type II lyophilized powder (≥ 48 U/mg protein), pepsin from porcine gastric mucosa: lyophilized powder (≥ 2500 U/mg protein), N-α-benzoyl-L-arginine-4-nitroanilide hydrochloride (L-BApNA) of ≥ 99% purity, β-amino-n-butyric acid

(BABA) and ethanolamine (EA) internal standards and the rest of the chemicals and enzymes were acquired from Sigma-Aldrich Co. (Saint Louis, MO, USA). The analytical standards (purity ≥ 95%), raffinose, stachyose and verbascose, and all the enzymatic assay kits were supplied by Megazyme International Ltd. (Bray, Wicklow, Ireland, UK).

6.2.2. Sample preparation

Dehulled faba bean flour of cvs. LT and HT were produced by abrasive dehulling for 30 s in a Tangential Abrasive Dehuller (Bio-industrial Opportunities Section, Alberta Agriculture, Forestry and Rural Economic Development, Edmonton, AB, Canada) according to Chapter 4 (Section 4.2.2.1). The hulls and by-products were aspirated, and the remaining groats were milled at 8,000 rpm with a 500 μm ring sieve in an ultra-centrifugal mill (ZM 200, Retsch mill, Hann, RP, Germany) and stored at 23 ± 2 °C in plastic containers until further use.

6.2.3. Wet fractionation of faba bean flour

Two major solvents were used: demineralised water (W) without and with the addition of salt (S) or both sequentially (WS) for the wet fractionation of dehulled faba bean flour and compared with the conventional pH-shift method employing alkali extraction and acid precipitation (AA).

6.2.3.1. Salt/water extraction

Faba bean dehulled flours were extracted with either water or $NaCl_{(aq)}$ solution (1% (w/v)) or both sequentially (i.e. water in step 1 and 1% $NaCl_{(aq)}$ solution in step 7) at 23 °C or 35 °C as illustrated in Fig. 6.1. The base method of extraction displayed in Fig. 6.1 was derived from the

previously described methods in Chapters 4 (Section 4.2.3) and Chapter 5 (Section 5.2.2). Briefly, 100 g of dehulled faba bean flour was suspended in water/$NaCl_{(aq)}$ solution and homogenized (Polytron, PTMR 2100, Kinematica AG, Malters, Lucerne, Switzerland) at S/F (v/w) ratio of 2 or 3 followed by overhead stirring (Heidolph RZR 2021, Schwabach, BY, Germany) at 23 ± 1 °C or 35 ± 1 °C. The dietary fibre fraction was removed by wet sieving with a 75 µm sieve (WS Taylor's Sieve Shaker, RX-812-CAN, Mentor, OH, USA) and re-extracted with solvent equivalent to the initial amount used in step 1 (Fig. 6.1) followed by oven drying. The filtrate was centrifuged (Beckman Coulter, Avanthi SER JSE10K11, Indianapolis, IN, USA) to separate the protein, starch and the intermediate layer, which was a blend of both protein and starch fractions. The precipitated solids were re-suspended in demineralised water with half the initial solvent amount (step 1) and centrifuged until the dark brown non-soluble protein layer was no longer visible (~twice). The intermediate layer was carefully scraped off from the starch fraction. The isolated starch pellet was re-suspended in 50 mL of absolute ethanol and filtered under vacuum before oven drying (Fisher Scientific Isotemp 750 G, Missouri City, TX, USA).

The supernatants were combined and subjected to different protein isolation techniques. The protein extracts (i.e. combined supernatants) resulting from water extraction were subjected to either acid precipitation (A) at pH 4.5 for LT and pH 5.0 for HT; dialysis (D) with 6-8 kDa, Spectrum SpectralPor regenerated cellulose dialysis tubing of 25.4 mm diameter (Fisher Scientific, Mississauga, ON, Canada) at 4 °C (D) with a distilled water flow rate of 30 mL/min; heat precipitation (H) at 80 °C for 20 min; micellization (M) by introducing cold water at 4 °C to the supernatant at 1:2 (v/v) extract:H_2O ratio and allowing to stand at 4 °C for 2 h following the protocol of Chapter 4 (Section 4.2.3) and Chapter 5 (Section 5.2.2).

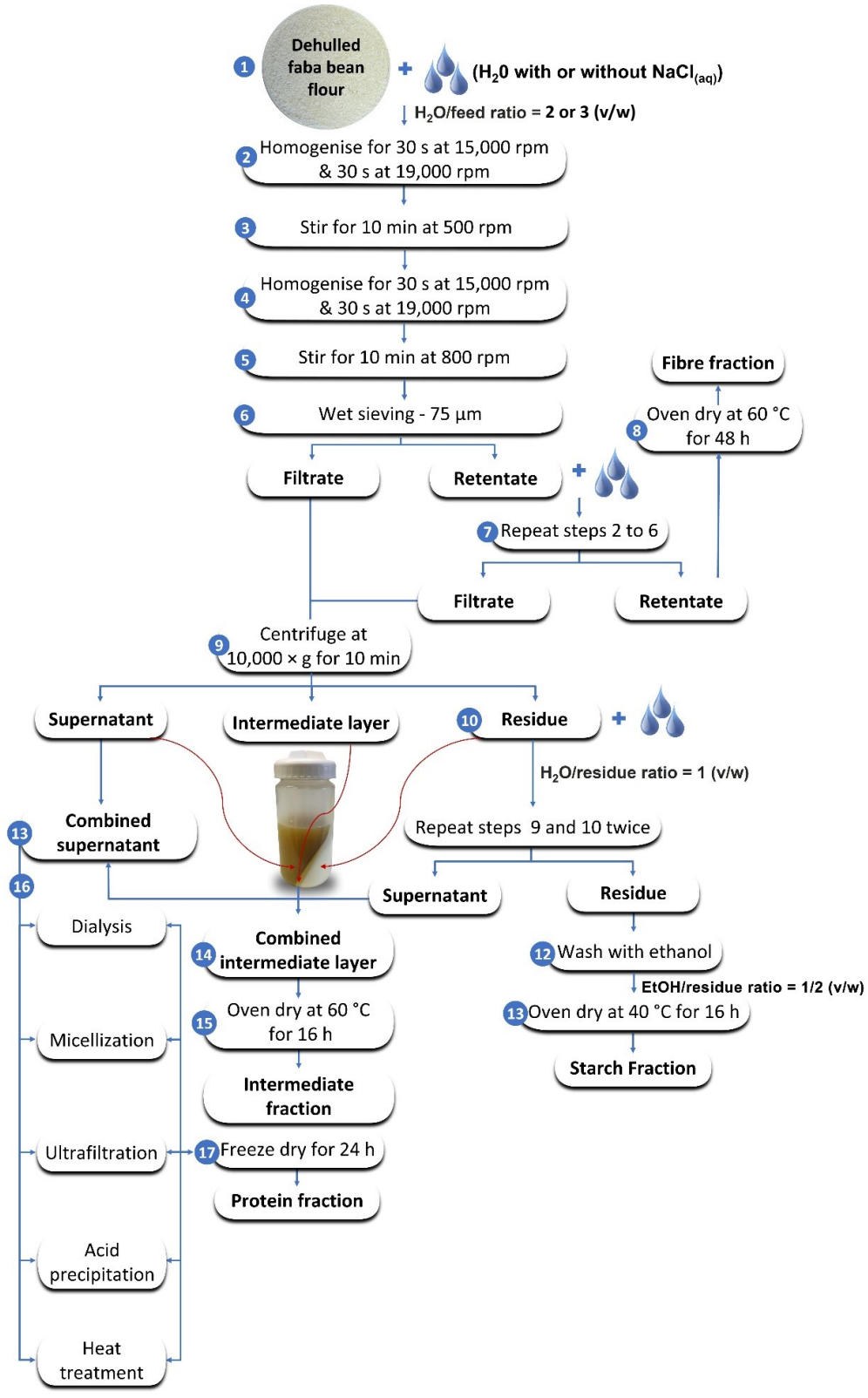


Fig. 6.1. Flow diagram of the different wet fractionation methods for faba beans.

On the other hand, the WS_{23} extracts were subjected to dialysis (1 kDa) or ultra-filtration (U) with a 1 kDa molecular weight cut-off (MWCO) membrane attached to the Cole-Parmer MasterFlex 77521-50 console drive with Easy Load II Head (Thermo Fisher Scientific, Waltham, MA, USA) to isolate the proteins, whereas, the S_{23} extracts were subjected to dialysis. All of the PCs and PIs were freeze-dried (Labconco Freezone 18, Kansas, MO, USA) while uncoagulated, non-precipitated protein fractions from the above steps were oven-dried to recover the proteins.

6.2.3.2. Large-scale extraction

Based on the protein purity, yield and recovery values, the W-D method was selected as the best method. However, since large-scale dialysis is not feasible, ultrafiltration was used instead. The primary extraction steps were carried out in the lab with 1 kg of raw material in two batches. The ultrafiltration and spray drying steps were carried out at Agri-Food Discovery Place, South Campus, University of Alberta to understand the impact of spray drying (Labplant Spray dryer, SS-07A, Keison Products, Chelmsford, Essex, England, UK) and ultrafiltration (Nuetch filter, LJ Star -W.T.Maye, Inc., Knoxville, TN, USA) at larger scale protein extraction. Additionally, residential water was used in place of demineralised water due to the lack of large quantities of demineralised water. As spray drying is a common industry practice, the protein fraction was spray dried under the following conditions: inlet temperature of 200 °C, set temperature of 190 °C, outlet temperature of 65 °C, liquid flow rate of 2 L/h and airflow rate of 4.3 m^3/s. Since the entire process from extraction to spray drying spanned over 2 d, precautionary steps were taken to minimize microbial growth overnight.

6.2.3.3. Alkali extraction and acid precipitation

Isoelectric-precipitated faba bean proteins were obtained by dispersing and homogenising (Polytron, PTMR 2100, Kinematica AG, Malters, Lucerne, Switzerland) dehulled faba bean flour (100 g) in demineralised water at S/F = 3 (v/w), adjusted to pH 10.0 with 1.0 M NaOH, and stirred at 500 rpm for 20 min (Heidolph RZR 2021, Schwabach, BY, Germany) at room temperature (23 ^{o}C). The alkali extract was centrifuged (Beckman Coulter, Avanthi SER JSE10K11, Indianapolis, IN, USA) at 10,000 ×g for 10 min and the supernatant was collected. The pellet was washed with water at S/F = 3 (v/w) and subjected to steps 6 to 8 of Fig. 6.1 to separate the fibre fraction. The filtrate was centrifuged at 10,000 ×g for 10 min and the residue was oven dried to produce the starch fraction. The supernatants were pooled and adjusted to pH 4.0 with 0.1 M HCl. The proteins recovered by centrifugation were stored at −18 °C and freeze-dried. The non-precipitated fraction was rotary evaporated, and oven dried at 60 °C for recovery calculations. All the final fractions were neutralised prior to the drying step. All the flour fractions (≤ 500 μm) were sealed in plastic containers and stored at 23 ± 3 °C until further analysis.

6.2.4. Characterisation

6.2.4.1. Morphological characterisation

Samples were examined under high pressure in a variable pressure field emission scanning electron microscope (VP-FESEM, Zeiss Sigma 300, Oberkochen, BW, Germany) as described previously (Chapter 3, Section 3.2.3).

6.2.4.2. Chemical characterisation

The fractions resulting from wet fractionation (Fig. 6.1) were morphologically assessed and profiled for mass balance, moisture, protein, total dietary fibre and total starch contents on a dry matter basis. The PCs and PIs resulting from the selected methods were characterised for raffinose family oligosaccharides and total tannins to compare with the whole and dehulled bean flours reported previously (Chapter 4, Table 4.2). The selected PCs and PIs were also profiled for their low molecular weight proteins by SDS-PAGE, amino acid composition, *in-vitro* protein digestibility (IVPD), amino acid score (AAS) and *in-vitro* protein digestibility corrected amino acid score (IVPDCAAS). In addition, the faba bean dehulled bean flours were assessed for Osborne's protein solubility-based classification, solubility in NaCl solution and water at different temperatures, and mineral composition.

6.2.4.2.1. Proximate composition analyses

Moisture (%) determination was carried out in a forced-air oven (Fisher Scientific Isotemp 750G, Missouri City, TX, USA) at 105 ± 1 °C for 24 h (Altuntaş & Yildiz, 2007). Protein content was determined according to the Dumas principle (N% × 6.25) of the AOAC 999.23 method (AOAC, 2000) in an elemental analyser (Leco TruSpec- FP-428, Mississauga, ON, Canada). Ethylenediaminetetraacetic acid (EDTA) and rye flour were used as the standards for calibration (Chapter 4, Section 4.2.4.2.1). Crude fat contents of selected PCs and PIs were gravimetrically determined following the extraction of fat using hexane according to Chapter 4 (Section 4.2.4.2.1). The total dietary fibre contents were analysed as a sum of soluble and insoluble dietary fibre in accordance with the Megazyme 2017 K217 TDFR assay method (Megazyme International Ltd., Bray, Wicklow, Ireland) with few modifications as described previously in Chapter 4 (Section 4.2.4.2.2). The total dietary fibre values were corrected for ash and protein contents. Yield and

recovery values of protein, starch and total dietary fibre fractions were calculated based on Eqs. (6.1) and (6.2), respectively.

$$Yield\ (\%\ db) = \frac{Corresponding\ output\ weight,\ \ db\ (g)}{Feed\ weight,db\ (g)} \times 100 \quad (6.1)$$

$$Protein\ Recovery\ (\%\ db) = \frac{Yield\ (\%\ db) \times Protein\ content\ of\ the\ output\ (\%\ db)}{Protein\ content\ of\ the\ feed\ (\%\ db)} \times 100 \quad (6.2)$$

6.2.4.2.2. Mineral analyses

Dehulled faba bean flours were analysed for their mineral composition including B, Na, Mg, Al, P, S, K, Ca, Cr, Mn, Fe, Co, Ni, Cu, Zn, As, Cd and Pb by inductively coupled plasma-optical emission spectroscopy (ICP-OES, Thermo Fisher Corp iCAP 6300 Duo, Cambridge, Cambridgeshire, England, UK). Samples (0.06 to 0.07 g) were digested with nitric acid (5 mL) in a microwave oven (MARS 6, CEM, Matthews, VA, USA) at 185 °C for 10 min. Deionised water (20 mL) was added after cooling them to room temperature. The acid digests were aspirated by a nebulizer into an argon plasma, where they were atomized at temperatures of 5227-7727 °C. An internal standard solution containing yttrium (Y) was used during analysis to correct for matrix effects. Multi-element certified standard reference materials (SRM 1573, National Institute of Standards and Testing, Gaithersburg, MD, USA and Birch leaf standard, Elemental Microanalysis Ltd., Okehampton, Devon, England, UK) were used as external references for the instrument quality control. Continuous Calibration Verification (CCV) checks were performed, and digestion and instrument blanks were also checked.

Faba bean samples (250 mg) were also extracted with 25 mL of Milli-Q water and analysed for chloride content according to method 325.2 of the Environmental Protection Agency (1978). Certified standard solutions purchased from SCP Scientific and Thermo Scientific were used for

calibration, and separate certified solutions were used as external reference and CCV check standards. Measurements of the coloured complexes were made using an automated spectrophotometer (ThermoFisher Gallery Beermaster Plus, Vantaa, Finland).

6.2.4.2.3. Protein solubility-based classification

Osborne's protein solubility-based classification (Osborne, 1924) was conducted according to Appendix B: Fig. B.1 (Peterson & Smith, 1976). Briefly, 50 mg of faba bean flour was homogenised (Mini vortex mixer, Fisher Scientific, Missouri City, TX, USA) with 1 M NaCl, and distilled water in multiple steps. Following homogenisation, the extracts were centrifuged (accuSpin Micro, Fisher Scientific, Osterode, NI, Germany) and the supernatants were combined, subjected to cold water treatment at 4 °C overnight, and centrifuged (accuSpin 400, Fisher Scientific, Osterode, NI, Germany). The supernatant was then subjected to further steps resulting in the albumin fraction. The residue was treated with 0.1N NaOH to obtain the globulin fraction. The residue of the initial centrifugation step was subjected to further treatments to separate the prolamin and glutelin fractions. Two series of the bovine serum standard solutions of varying concentrations (666.7, 533.6, 400.2, 333.5 and 266.8 µg/mL for the first series and 200, 160, 120, 80, 40, and 20 µg/mL for the second series) were prepared. An aliquot, 50 µL of albumin, globulin and glutenin solutions adjusted to 100 µL with distilled water, and 100 µL of prolamin and standard solutions were allowed to react with 1.5 mL Coomassie (Bradford) reagent at room temperature (23 °C), and the absorbance was measured (Jenway 6300, Essex, England) at 595 nm against the reagent blank.

6.2.4.2.4. Effect of $NaCl_{(aq)}$ level, solvent/feed ratio and temperature on protein solubility

The solubility of proteins was determined by dispersing the dehulled faba bean flour of both LT and HT cvs. in $NaCl_{(aq)}$ solutions (S/F = 3, v/w) of varying added NaCl concentrations (0, 0.5, 0.75, 1, 2, 3, 4, 5 and 6%) followed by shaking in a hand shaker (Burrell Wrist Action Shaker, 75-765 BT, Pittsburgh, PA, USA) at high setting of 10 for 1 h. Following centrifugation at 3000 ×g for 20 min at 23 °C, the precipitates were freeze-dried following the removal of the supernatants. The protein contents of the precipitates were determined by LECO elemental analyser (N × 6.25) as indicated in Section 4.2.4.2.1. and the protein solubility was calculated using Eq. (6.3) as the difference from 100 to represent the solubilized fraction.

$$Protein\ solubility\ (\%\ db) = 100 - \left(\frac{Precipitate\ protein(\%\ db) \times Precipitate\ yield\ (\%)}{Initial\ sample\ protein\ (\%\ db\ )}\right) \quad (6.3)$$

The effect of the S/F ratio on faba bean protein solubility was explored in water at S/F = 1, 2, and 3 (v/w) at 23 °C. In addition, the effect of temperature on protein solubility was studied at S/F = 3 (v/w) at 23, 35, 40 and 50 °C.

6.2.4.2.5. Amino acid profile

All the amino acids, except for methionine, cysteine, proline and tryptophan, were quantified in HPLC according to a modified method based on Sedgwick et al. (1991) and AOAC Official Method 982.30 (AOAC, 2005). Briefly, protein isolates (0.0030 ±0.0010 g) were hydrolysed in triplicate with 3.0 mL of 6M HCl at 110 ºC for 24 h in an oven (Fisher Scientific Isotemp 750 G Oven, Missouri City, TX, USA). The tubes were cooled and 200 µL of BABA/EA internal standard solution (25 µmol/mL) and 1 mL HPLC grade water were added and centrifuged (Eppendorf AG 5810, Barkhausenweg, HH, Germany) for 15 min at 3000 ×g. Then, 50 µL of the supernatant was mixed with 50 µL 4.29N NaOH and 400 µL water (pH 9) and transferred to HPLC

vials for analysis. For the quantification of the sulphur-containing amino acids cysteine and methionine, samples were oxidised with performic acid prior to acid hydrolysis according to the AOAC Official Method 985.28 (AOAC, 2005).

Amino acid analysis was carried out using an HPLC system (Agilent Technologies 1200, Santa Clara, CA, USA) equipped with a solvent delivery system, temperature-controlled column oven, autosampler, degasser unit and a fluorescence detector (Excitation: 340 nm, Emission: 450 nm). The compounds were separated on a ZORBAX SB-C18 column (3 x 250 mm, 5-µm) with a guard column made of the same material (Phenomenex Inc., Torrance, CA, USA). Mobile phase A was 0.1 M sodium acetate (pH 7.2):methanol:tetrahydrofuran in a 451:45:1 ratio and mobile phase B was HPLC-grade methanol. Mobile phases A and B were pumped at a flow rate of 0.6 mL/min and the injection volume was 25 µL. For the separation of the analytes, a linear gradient program at 40 °C was used with 20% of mobile phase B from 20 to 39 min, 70% for the next minute and 100% for the last 2.5 min. The amino acid standards were prepared at three levels (150 to 600 nmol/L) to establish the calibration curve. Automated online derivation was carried out with OPA for primary amino acids. Quantification was carried out from the external standard based on relative response factor (RRF) values. Data from both analyses were compiled to have the amino acid profile.

6.2.4.2.6. In-vitro protein digestibility

In-vitro protein digestibility (IVPD (%)) was assessed based on the static enzymatic pH drop method (Hsu et al., 1977), employing a multi-enzyme solution consisting of 1.6 mg trypsin (13000 to 20000 BAEE U/mg protein), 3.1 mg α-chymotrypsin (≥ 48 U/mg protein), 1.3 mg pepsin (≥ 2500 U/mg protein) per mL distilled water. The enzyme cocktail maintained in an ice bath was

added to the faba bean protein suspension (1 mg N/mL) maintained at 37 °C at a 1:10 (v/v) ratio and incubated at 37 °C for 10 min (Alonso et al., 2000). The pH of the mixture was recorded soon after treatment with the enzyme cocktail (pH ~ 8) and after 10 min. The IVPD (%) was calculated using Eq. (6.5). According to the power curve equation of the three-enzyme (trypsin-chymotrypsin-peptidase) pH drop method of Hsu et al. (1977):

$$\text{IVDP (\%)} = 210.46 - 18.10 \times \text{pH}_{10\ \text{min}} \quad (6.4)$$

A more accurate interpretation of the equation used in this study is:

$$\text{IVDP (\%)} = 210.46 - [18.10\ (\text{pH}_{0\ \text{min}} - \Delta\text{pH}_{10\ \text{min}})] \quad (6.5)$$

where $pH_{0\ min}$ is the initial pH and $\triangle pH_{10\ min}$ is the change in pH over 10 min from $pH_{0\ min}$.

6.2.4.2.7. Amino acid in-vitro protein digestibility scores

The amino acid score (AAS) is derived by dividing the relative abundance of the essential amino acid expressed as (mg of amino acid / g test protein) by the relative abundance of the corresponding amino acid from the 1991 FAO and WHO reference values for a 2 to 5-year-old child (FAO, 1991). The lowest ratio resulting from the limiting amino acids was selected as the amino acid score (AAS) that was calculated using Eq. (6.6). The reference amino acid composition for the limiting amino acids Methionine (MET) + Cysteine (CYS) is 25 mg/g protein.

$$AAS = \frac{mg\ amino\ acid\ per\ gram\ of\ protein\ (sample)}{mg\ amino\ acid\ per\ gram\ of\ protein\ (reference)} \quad (6.6)$$

The *in-vitro* protein digestibility corrected amino acid score (IVPDCAAS) was determined as given in Eq. (6.7)

$$\text{IVPDCAAS (\%)} = \text{AAS} \times \text{IVPD (\%)} \quad (6.7)$$

6.2.4.2.8. Trypsin inhibitory activity

Trypsin inhibitory activity was ascertained according to Alonso et al. (2000) and Kakade et al. (1974) with few modifications. TRIS-buffer (50 mM) solution was prepared with $CaCl_2.H_2O$ and tris-(hydroxymethyl) aminomethane and adjusted to pH 8.2 (Kakade et al., 1974). The substrate solution was freshly prepared by dissolving 110 mg of L-BApNA in 2 mL of dimethyl sulfoxide and brought up to 250 mL with TRIS buffer pre-heated to 37 °C (Kakade et al., 1974). Trypsin inhibitory activity levels were estimated against 0.1 mg/mL trypsin (13000 to 20000 BAEE U/mg protein) enzyme solution in TRIS buffer.

Trypsin inhibitors were extracted from 350 mg of faba bean PCs/PIs in 2.5 mL TRIS buffer by homogenization (accuSpin 400, Fisher Scientific, Osterode, NI, Germany) for 1 min. The samples were centrifuged at 1095 ×g for 5 min. The residual pellet was resolubilized, homogenized and centrifuged following the above steps. The supernatants were pooled, incubated at 4 °C overnight, and centrifuged again at 1095 ×g for 5 min. The tubes containing 200 µL of the extracts were incubated at 37 °C in a shaking water bath (Jeio Tech BS-06/11/21/31, Seoul, South Korea). After 10 min, 250 µL of 0.1 mg/mL trypsin enzyme solution and 550 µL of TRIS buffer pre-heated to 37 °C were added and allowed to react for 3 min. The reagent blank contained 0.5 mL of 30% acetic acid, 250 µL of trypsin and 750 µL of distilled water. Thereafter, 2.5 mL of 1 mM L-BApNA substrate solution pre-warmed to 37 °C was added and vortexed for 10 s. After 7 min of incubation in the shaking water bath at 37 °C, 0.5 mL of 30% acetic acid was added to terminate the reaction. The absorbance due to the release of *p*-nitroaniline was measured at 410 nm (Alonso et al., 2000; Kakade et al., 1974). Trypsin inhibitory activity levels of the samples were expressed as trypsin units inhibited (TUI)/mg sample dry weight or TUI/mg nitrogen dry weight. One trypsin unit was defined as an increase of 0.02 absorbance units at 410 nm per 10 mL of the reaction mixture (Liu

et al., 2021) according to Eq. (6.8), where A_{410R} is reference absorbance; A_{410RB}, reference blank; A_{410S}, sample reading; and A_{410SB}, sample blank.

$$TUI/\ mg\ sample\ dry\ weight = \frac{\{(A_{410R} - A_{410RB}) - (A_{410S} - A_{410SB})\} \times 50}{sample\ dry\ weight\ (mg)} \quad (6.8)$$

6.2.4.2.9. Raffinose family oligosaccharides, total tannins and crude fat contents

HPLC determination of the individual raffinose family oligosaccharides, raffinose, stachyose, and verbascose, spectrophotometric analysis of total tannins at 700 nm and gravimetric analysis of crude fat contents were performed according to the methods previously described (Chapter 4, Section 4.2.4.2.2).

6.2.4.2.10. Electrophoretic protein profiles

The PCs and PIs as well as the non-precipitated fraction from W_{35}-M were analysed for their low molecular weight protein profiles using sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE) according to Hong et al. (2017) with few modifications. Briefly, faba bean protein samples (1 mg/mL) were diluted with Laemmli sample buffer containing 5% β-mercaptoethanol at 1:1 ratio, vortexed for 15 s and heated to 85 °C for 10 min in an Eppendorf Thermomixer® Dry Block Heating Shaker (Mississauga, ON, Canada). Samples (15 µL) were loaded in triplicate along with the molecular weight markers (10 µL) on precast Mini-PROTEAN gels (4−20% gradient gels) and run at 90 V for 2 h in a Mini-PROTEAN electrophoresis tetra cell (Bio-Rad, Hercules, CA, USA) in 10X tank buffer (0.25 M Tris base (2-amino-2-hydroxymethyl-propane-1,3-diol), 1.92 M glycine and 1% SDS in double distilled water) diluted 1X. To visualise the protein bands, the gels were stained in Coomassie Brilliant Blue-R-250 in water, methanol and

acetic acid (4:5:1, v:v:v) for 2 h and destained overnight in the destaining buffer solution (5:4:1 distilled water:methanol:acetic acid, v:v:v).

The gels were scanned in an Azure Biosystems C200 bioanalytical imaging system (Dublin, CA, USA). Prestained Protein Ladder (PageRuler Plus, Thermo Scientific, Mississauga, ON, Canada) ranging from 10 to 250 kDa was used as the molecular weight marker. One band per sample was selected and identified based on literature (Ayala-Rodriguez et al., 2022; Nivala et al., 2017; Warsame et al., 2020) as well as the relative migration distances of the targeted bands to the molecular weight markers. Legumin/Vicilin (L/V) ratios were calculated (Martinez et al., 2016) based on the sum of the relative percentages of 11S and 7S protein fractions assessed on three bands per sample using the densitometry option of Image J software v.1.53k software (National Institute of Health, Bethesda, MD, USA).

6.2.5. Statistical analysis

All the results expressed as mean ± SD on a dry weight basis (db) were based on duplicate analyses of three independent replicate samples (n = 6) unless otherwise specified. Significant differences between samples were tested by two-way analysis of variance (ANOVA) followed by Duncan's multiple range test ($P < 0.05$). Two data sets were compared according to the two-sample t-test ($P < 0.05$). All these statistical analyses were carried out using SAS Studio University Version for Windows (SAS Institute Inc., Cary, NC, USA).

6.3. RESULTS AND DISCUSSION

6.3.1. Mineral composition

The micro-elemental composition of faba bean cvs. Snowbird and Athena (Table 6.1) showcases a broad array of minerals with higher levels of K, P, S, Mg and Cl^-. As reported previously, K, Ca and Mg were among the dominating elements present on both the abaxial (external surface) and adaxial (split surface) surfaces of LT and HT faba bean cotyledons (Chapter 3, Table 3.1). The freeze-dried protein bodies of faba beans were reported to encompass K, P, Mg, S, Cl and trace levels of Ca as globoid crystals embedded in amorphous protein (Lotta & Buttrose, 1977). These minerals present in protein bodies likely support the release of salt-soluble proteins during the extraction of proteins.

Although the previously reported ash contents of dehulled LT (3.7 %) and HT (3.5%) faba bean flours were not significantly ($P > 0.05$) different, according to Table 6.1, LT has substantially ($P < 0.05$) higher levels of minerals compared to HT except for Cl^-, Cu and Ni. Higher levels of tannins (2.0 ± 0.1 mg tannic acid equivalent (TAE)/g sample, db) were reported in HT as compared to LT (0.5 ± 0.4 mg TAE/g sample, db) dehulled bean flours (Chapter 4, Table 4.2). Tannins can be classified into hydrolysable and condensed tannins and the latter (proanthocyanidins), which are flavan-3-ol polymers have a high affinity to cross-link with minerals (i.e. Fe, Zn) and restrict their bioavailability (Multari et al., 2015).

Table 6.1. Mineral composition (µg/g dry weight) of faba beans.

Mineral	Snowbird (LT)	Athena (HT)
K	14145.96 ± 335.08[a]	10995.20 ± 69.25[b]
P	6760.08 ± 119.16[a]	4293.45 ± 48.34[b]
S	2813.11 ± 57.65[a]	1937.68 ± 17.30[b]
Mg	2000.38 ± 29.67[a]	1112.86 ± 11.10[b]
Cl^-*	1343.99 ± 45.79[b]	1649.89 ± 8.33[a]
Ca	924.74 ± 39.5[a]	355.67 ± 14.29[b]
Zn	233.48 ± 6.57[a]	37.17 ± 0.26[b]
Na	117.67 ± 1.58[a]	102.35 ± 1.84[b]
Fe	106.78 ± 4.86[a]	62.33 ± 1.28[b]
Al	19.04 ± 3.64[a]	5.35 ± 2.10[b]
Mn	21.79 ± 0.24[a]	9.73 ± 0.24[b]
Cu	11.72 ± 0.25[b]	16.67 ± 0.28[a]
B	9.37 ± 0.30[a]	6.76 ± 0.21[b]
Ni	2.20 ± 0.10[b]	4.69 ± 0.08[a]

[a-b]Mean(n = 3) ± SD values in the same row followed by different superscripts are significantly different at $P < 0.05$ according to the two-sample t-test.

Levels of Cr, Co, As, Cd and Pb were below the detection limits (0.002, 0.002, 0.005, 0.001 and 0.006 mg/L, respectively).
*Chloride content was measured by a colourimetric method (Gallery Beermaster Plus). The rest of the minerals were assessed by ICP-OES (Inductively Coupled Plasma-Optimal Emission Spectroscopy)

From a dietary standpoint, deficiencies associated with Fe, Zn, Mg and Ca have detrimental effects on health (Multari et al., 2016). When compared to the daily allowance values (mg/day) of Fe (10 to 18), Zn (5 to11), Mg (130 to 420) and Ca (1000 to 1300) for 4 to 8 years old children and adults recommended by Health Canada (2005), both LT and HT can be considered significant sources of Fe (LT: 10.7 ± 0.5 and HT: 6.2 ± 0.1 mg/100 g), Zn (LT: 23.3 ± 0.7 and HT: 3.7 ± 0.0 mg/100 g), Mg (LT: 200.0 ± 3.0 and HT: 111.3 ± 1.1 mg/100 g), and Ca (LT: 92.5 ± 4.0 and HT: 35.6 ± 1.4 mg/100 g).

According to Khan et al. (2015), in 40 Saudi Arabian faba bean cultivars, the Fe content was 14.6 to 15.8 mg/100 g and the Zn content was 5.8 to 7.2 mg/100 g. In another study, the Fe contents of 15 Swedish faba bean cultivars ranged from 1.8 to 21.3 mg/100 g, whereas their Zn contents ranged from 0.9 to 5.2 mg/100 g (Mayer Labba et al., 2021). These results were comparable to those obtained in the present study for the Canadian cultivars. The Zn, Mg, Fe and K contents of HT were comparable to the findings of Millar et al. (2019). The mineral content of both the cvs. LT and HT were substantially higher than the minerals detected in the form of isotopes (Multari et al., 2016), particularly, K, Mg, Ca, Na, Fe and Mn contents were 3-fold higher in LT and 2-fold higher in HT. Faba bean cvs. LT and HT are significant sources of minerals but their composition can be heavily influenced by the cultivar, its origin, the geographical conditions and to a lesser extent to the analytical technique of detection (Multari et al., 2016).

6.3.3. Extractability of protein, starch and dietary fibre

All the different wet fractionation processes investigated in this study yielded three main fractions enriched in starch, dietary fibre and protein. In addition, a starch- and protein-rich intermediate brown layer (Fig. 6.1) and a dark brown layer mainly containing the insoluble protein were also obtained. This insoluble protein layer was washed with the solvent and collected as part of the protein fraction for mass balance calculations, except for the WS_{23}-D method with extraction at S/F = 3 followed by dialysis (Table 6.2). This insoluble protein fraction was only 1.4% of the input although the protein content was 87.8% for LT and 79.4% for HT. The PIs obtained using the WS_{23}-D method were 92.0% (LT) and 91.3% (HT) protein, excluding the insoluble proteins. This method also yielded starch isolates of 92.3% and 91.5% purity for LT and HT, respectively; however, their recovery values were low for both cultivars (38.0%). Some

of the starch ended up in the intermediate layer as well as the dietary fibre fraction and less than 0.5% of starch was recovered in the protein fraction. Both WS_{23}-D and WS_{23}-U methods shared similar extraction steps till step 15 of Fig. 6.1. Similarly, W_{35}-D, W_{35}-M, W_{35}-A and W_{35}-H methods shared the same extraction steps till step 15 of Fig. 6.1. Therefore, in both cases similar results related to starch, dietary fibre and intermediate fractions were obtained (Tables 6.2-6.3).

WS_{23}-D method (Table 6.2) resulted in greater protein purity and yield as compared to WS_{23}-U and S_{23}-D methods possibly due to the salting-in effect resulting from the naturally present minerals during the first water extraction step as well as the salts introduced in the second extraction step. In the ultrafiltration step, protein extracts were passed through a semi-permeable membrane to concentrate the proteins, whereby molecules (10 to 1,000 kDa) larger than the MWCO (6-8 kDa) of the membrane were retained and smaller molecules (mono-, di-, and oligosaccharides, phytic acid, off-flavour compounds and salts) permeated through the membrane (Arntfield & Maskus, 2011; Nichols & Cheryan, 1981).

However, proteins of compact tertiary structures can still escape the ultrafiltration membrane if the MWCO of the membrane is not ten times smaller than the MW of the proteins (Arntfield & Maskus, 2011; Nichols & Cheryan, 1981). Another possible reason is that solute-solute interactions can lead the added salts (NaCl) and naturally present minerals to bind to the proteins and remain in the permeate (Nichols & Cheryan, 1981). Although a retentate washing step was used in the ultrafiltration step to minimise these interactions, it did not improve protein purity as compared to dialysis. During dialysis, water was changed every day for two days, which likely enhanced the removal of the salts and small molecules and improved the purity as compared to ultrafiltration.

Table 6.2. Chemical composition, yield and recovery values (%) of salt-extracted fractions of faba beans on a dry matter basis.

Fraction	Component	Snowbird (LT)	Athena (HT)
WS_{23}-D_{23} (Water and 1% salt extraction (S/F = 3) + Dialysis at 23 °C)			
Soluble Protein	Yield	23.98 ± 0.78[a]	20.37 ± 1.51[b]
	Protein (N×6.25)	92.00 ± 0.54[a] (64.78 ± 2.47)	91.29 ± 2.53[a] (54.62 ± 2.68)
	Starch	0.32 ± 0.03[b] (0.15 ± 0.02)	1.33 ± 0.23[a] (0.35 ± 0.11)
Insoluble Protein	Yield	1.36 ± 0.05[a]	1.36 ± 0.56[a]
	Protein (N×6.25)	87.85 ± 2.40[a] (3.49 ± 0.04)	79.44 ± 0.45[b] (3.17 ± 1.28)
	Starch	6.72 ± 0.04[a] (0.18 ± 0.01)	4.08 ± 0.01[b] (0.11 ± 0.05)
Starch	Yield	20.13 ± 0.23[a]	20.60 ± 0.30[a]
	Protein (N×6.25)	0.40 ± 0.12[b] (0.23 ± 0.07)	1.06 ± 0.15[a] (0.64 ± 0.09)
	Starch	92.26 ± 0.18[a] (37.64 ± 0.36)	91.48 ± 1.06[b] (38.18 ± 0.56)
Dietary Fibre	Yield	15.80 ± 1.80[b]	17.34 ± 0.66[a]
	Protein (N×6.25)	22.51 ± 1.27[a] (10.48 ± 1.78)	20.20 ± 0.96[b] (10.29 ± 0.11)
	Starch	38.01 ± 0.43[a] (12.17 ± 1.42)	32.75 ± 0.28[b] (11.51 ± 0.42)
	Total dietary fibre	41.92 ± 0.43[b] (61.27 ± 7.60)	55.79 ± 0.82[a] (7.83 ± 0.60)
Intermediate	Yield	5.87 ± 0.61[b]	6.92 ± 0.52[a]
	Protein (N×6.25)	21.72 ± 0.30[a] (3.74 ± 0.35)	16.36 ± 0.32[b] (3.33 ± 0.24)
	Starch	54.01 ± 1.72[b] (6.41 ± 0.58)	55.79 ± 0.82[a] (7.83 ± 0.60)
WS_{23}-U_{23} (Water and 1% salt extraction at 23 °C (S/F = 3) + Ultra-filtration at 23 °C)			
Protein	Yield	15.76 ± 0.91[a]	12.85 ± 1.77[b]
	Protein (N×6.25)	50.00 ± 0.61[b] (23.13 ± 1.17)	58.87 ± 1.79[a] (22.21 ± 2.55)
	Starch	4.92 ± 0.60[a] (1.57 ± 0.22)	4.55 ± 0.22[b] (1.19 ± 0.20)
S_{23}-D (Salt extraction at 23 °C (S/F = 2) + Dialysis at 4 °C)			
Protein	Yield	20.27 ± 0.77[b]	22.10 ± 1.61[a]
	Protein (N×6.25)	79.14 ± 0.13[b] (47.10 ± 1.72)	81.58 ± 0.50[a] (54.64 ±1.21)
	Starch	5.29 ± 0.10[b] (2.17 ± 0.08)	7.34 ± 0.39[a] (3.38 ±0.13)
Starch	Yield	27.78 ± 0.43[a]	27.25 ± 0.39[a]
	Protein (N×6.25)	1.42 ± 0.13[a] (1.16 ± 0.12)	1.39 ± 0.04[b] (1.11 ± 0.04)
	Starch	90.29 ± 1.53[a] (50.83 ± 1.27)	82.42 ± 1.13[b] (45.50 ± 0.08)
Dietary Fibre	Yield	19.10 ± 0.18[a]	15.33 ± 1.00[b]
	Protein (N×6.25)	22.57 ± 0.50[b] (12.66 ± 0.35)	23.75 ± 0.14[a] (10.71 ± 0.57)
	Starch	37.83 ± 0.46[a] (14.65 ± 0.21)	33.53 ± 1.16[b] (10.43 ± 0.86)
	Total dietary fibre	35.08 ± 1.72[a] (61.95 ± 3.57)	31.29 ± 1.47[b] (44.28 ± 2.75)
Intermediate	Yield	17.26 ± 0.60[a]	17.46 ± 0.39[a]
	Protein (N×6.25)	39.51 ± 0.46[a] (20.02 ± 0.82)	38.79 ± 0.04[b] (19.91 ± 0.35)
	Starch	38.01 ± 0.43[a] (13.29 ± 0.47)	33.47 ± 1.23[b] (11.85 ±0.58)

[a-b]Mean (n = 3) ± SD values within a row with different letters are significantly different ($P < 0.05$), according to the two-sample t-test. S/F: solvent/feed ratio (v/w). Recovery values (%) given within brackets were based on the initial compositional analyses of protein (LT: 34.06 ± 0.29% and HT: 34.08 ± 0.15%), starch (LT: 49.53 ± 0.37% and HT: 46.34 ± 0.30%) and dietary fibre (LT: 10.82 ± 0.58% and HT: 10.55 ±0.47%) contents of the dehulled bean flour on a dry matter basis.

Table 6.3. Chemical composition, yield and recovery values (%) of water-extracted fractions of faba beans on a dry matter basis.

Fraction	Component	Snowbird (LT)	Athena (HT)
W_{35}-D (Water extraction at 35 °C (S/F = 2) + Dialysis at 4 °C)			
Protein	Yield	28.48 ± 0.49[a]	27.51 ± 0.28[b]
	Protein (N×6.25)	93.23 ± 0.11[b] (77.94 ± 1.36)	96.43 ± 0.29[a] (78.02 ± 0.99)
	Starch	2.85 ± 0.09[a] (1.65 ± 0.06)	0.44 ± 0.40[b] (0.11 ± 0.10)
Starch	Yield	25.59 ± 0.79[a]	23.82 ± 1.04[b]
	Protein (N×6.25)	1.48 ± 1.20[b] (1.11 ± 0.19)	1.59 ± 0.08[a] (1.12 ± 0.10)
	Starch	94.33 ± 1.41[b] (48.90 ± 1.03)	97.84 ± 0.27[a] (50.29 ± 2.24)
Dietary Fibre	Yield	24.20 ± 0.20[a]	23.44 ± 0.66[a]
	Protein (N×6.25)	16.76 ± 0.21[a] (11.91 ± 0.06)	15.51 ± 0.05[b] (10.69 ± 0.32)
	Starch	34.69 ± 0.36[b] (17.01 ± 0.31)	36.41 ± 1.00[a] (18.42 ± 0.93)
	Total dietary fibre	39.96 ± 0.51[a] (89.39 ± 1.84)	40.41 ± 0.34[a] (89.76 ± 1.96)
Intermediate	Yield	9.29 ± 0.39[a]	9.35 ± 0.72[a]
	Protein (N×6.25)	18.56 ± 1.27[b] (5.05 ± 0.27)	28.01 ± 0.26[a] (7.71 ± 0.66)
	Starch	66.66 ± 0.87[a] (12.55 ± 0.65)	55.99 ± 1.90[b] (11.29 ± 0.82)
	Total dietary fibre	11.05 ± 1.05[a] (9.50 ± 1.29)	10.04 ± 0.19[a] (8.90 ± 0.55)
W_{35}-M (Water extraction at 35 °C (S/F = 2) + Micellization)			
Protein	Yield	11.67 ± 0.59[a]	12.11 ± 0.29[a]
	Protein (N×6.25)	99.18 ± 0.10[a] (33.99 ± 1.74)	98.73 ± 0.22[a] (35.17 ± 0.75)
	Starch	0.50 ± 0.15[a] (0.12 ± 0.04)	0.44 ± 0.40[a] (0.11 ± 0.10)
Non-precipitated	Yield	14.38 ± 0.02[b]	20.13 ± 0.34[a]
	Protein (N×6.25)	63.53 ± 0.19[a] (26.82 ± 0.07)	58.06 ± 0.06[b] (34.38 ± 0.58)
	Starch	35.71 ± 0.22[a] (10.41 ± 0.07)	21.41 ± 1.04[b] (18.40 ± 0.28)
W_{35}-A (Water extraction at 35 °C (S/F = 2) + Acid precipitation)			
Protein	Yield	20.51 ± 1.17[a]	19.77 ± 0.49[a]
	Protein (N×6.25)	95.02 ± 0.62[a] (57.23 ± 3.62)	96.45 ± 0.26[a] (56.07 ± 1.26)
	Starch	1.56 ± 0.03[a] (0.65 ± 0.03)	1.36 ± 0.04[b] (0.51 ± 0.02)
Non-precipitated	Yield	6.64 ± 0.45[b]	9.66 ± 0.30[a]
	Protein (N×6.25)	38.33 ± 0.31[a] (7.48 ± 0.48)	38.43 ± 0.21[a] (10.92 ± 0.40)
	Starch	3.85 ± 0.07[b] (0.52 ± 0.04)	5.76 ± 0.35[a] (1.20 ± 0.09)
W_{35}-H (Water extraction at 35 °C (S/F = 2) + Heat treatment)			
Protein	Yield	19.85 ± 1.02[a]	18.98 ± 1.06[a]
	Protein (N×6.25)	77.41 ± 0.19[b] (45.13 ± 2.42)	79.61 ± 1.37[a] (44.44 ± 2.47)
	Starch	5.88 ± 0.94[b] (2.37 ± 0.41)	7.07 ± 0.65[a] (3.06 ± 0.30)
Non-precipitated	Yield	7.37 ± 0.44[a]	7.57 ± 0.51[a]
	Protein (N×6.25)	65.42 ± 0.36[a] (14.15 ± 0.90)	58.06 ± 0.06[b] (12.93 ± 0.87)
	Starch	18.83 ± 0.47[a] (2.81 ± 0.13)	19.05 ± 0.38[a] (3.11 ± 0.21)

Table 6.3. Continued

Fraction	Component	Snowbird (LT)	Athena (HT)

		W_{23}-D_{23} (Water extraction at (S/F = 2) + Dialysis 23 °C)	
Protein	Yield	24.36 ± 0.91[a]	21.50 ± 1.31[b]
	Protein (N×6.25)	82.26 ± 0.24[b] (58.82 ± 2.03)	87.49 ± 1.06[a] (55.20 ± 2.82)
	Starch	3.80 ± 0.03[b] (1.88 ± 0.09)	7.57 ± 0.49[a] (3.30 ± 0.33)
Starch	Yield	27.62 ± 0.62[a]	27.75 ± 2.26[a]
	Protein (N×6.25)	1.32 ± 0.06[a] (1.07 ± 0.03)	1.22 ± 0.13[a] (0.99 ± 0.10)
	Starch	95.54 ± 0.70[a] (53.47 ± 1.42)	88.78 ± 2.03[b] (49.91 ± 3.78)
Dietary Fibre	Yield	21.81 ± 0.77[b]	23.10 ± 1.23[a]
	Protein (N×6.25)	16.43 ± 0.37[b] (10.52 ± 0.59)	26.00 ± 0.17[a] (15.92 ± 1.04)
	Starch	19.30 ± 0.31[a] (8.53 ± 0.40)	17.94 ± 1.37[b] (7.60 ± 0.09)
	Total dietary fibre	38.42 ± 0.91[b] (77.39 ± 0.91)	40.73 ± 1.11[a] (78.42 ± 2.69)
Intermediate	Yield	5.02 ± 0.06[b]	9.58 0.50[a]
	Protein (N×6.25)	46.35 ± 0.31[a] (6.84 ± 0.12)	41.19 ± 0.30[b] (11.58 ± 0.65)
	Starch	47.18 ± 0.09[b] (4.80 ± 0.06)	49.63 ± 1.09[a] (9.63 ± 0.56)

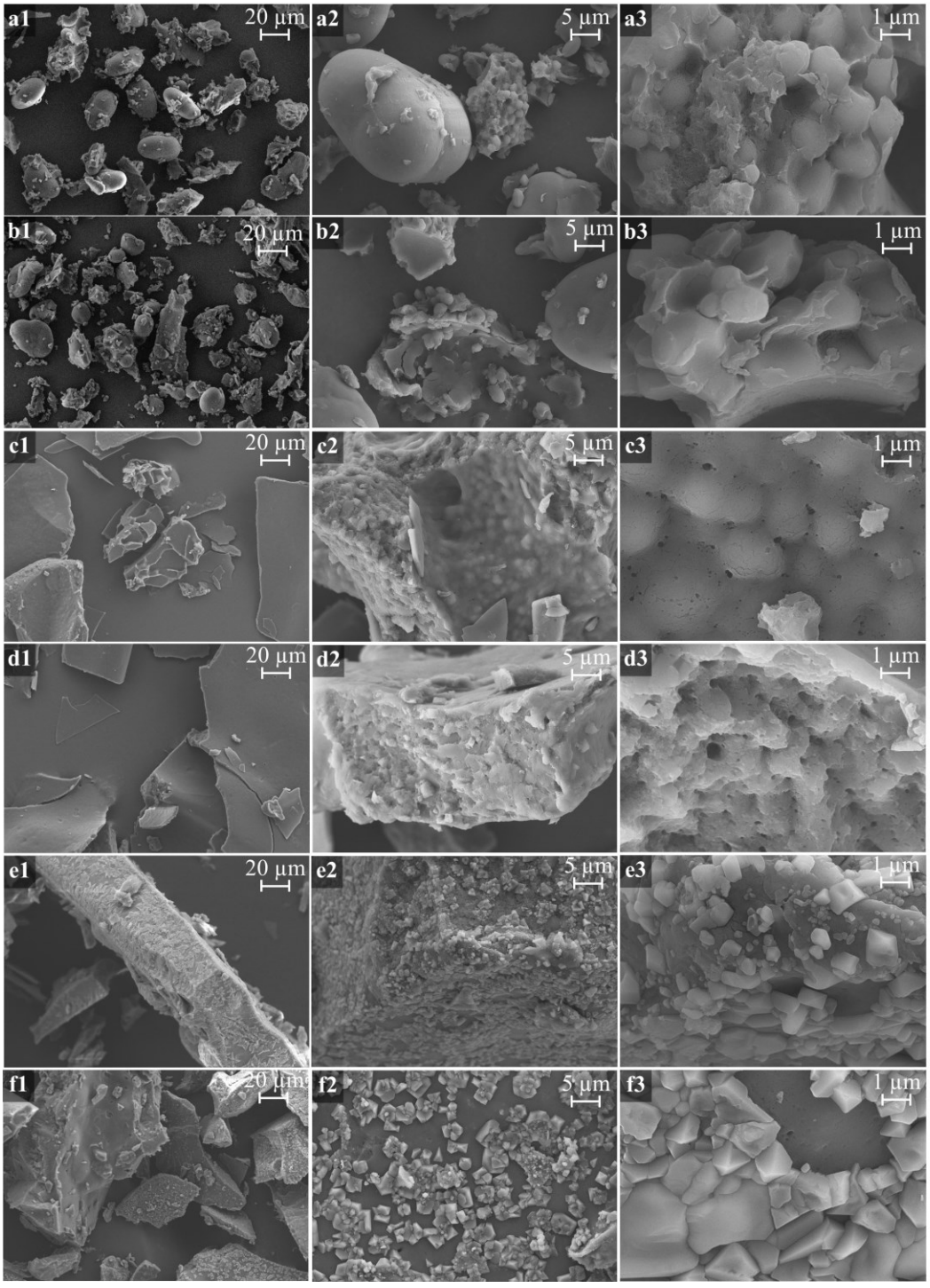
[a,b]Mean (n = 3) ± SD values within a row with different letters are significantly different ($P < 0.05$), according to the two-sample t-test. S/F: solvent/feed ratio (v/w). Recovery values (%) given within brackets were based on the initial compositional analyses of protein (LT: 34.06 ± 0.29% and HT: 34.08 ± 0.15%), starch (LT: 49.53 ± 0.37% and HT: 46.34 ± 0.30%) and dietary fibre (LT: 10.82 ± 0.58% and HT: 10.55 ±0.47%) contents of the dehulled bean flour on a dry matter basis.

Utilising 1% $NaCl_{(aq)}$ solution at S/F = 2 (S_{23}) resulted in lower protein purities and recoveries as compared to WS_{23}-D_{23} regardless of the cultivar (Table 6.2). The intermediate fraction of the S_{23} method, a by-product of no commercial value, resulted in a 3-fold greater yield as compared to WS_{23}. This intermediate layer is the result of the inability to separate the protein bodies and starch granules of similar size and density (Schutyser et al., 2015). The purities and recoveries of the starch and dietary fibre fractions were significantly ($P < 0.05$) higher with WS_{23} as compared to S_{23}.

A low S/F ratio (2 or 3) was used in this study as opposed to the S/F ratios of 10 or 20 most commonly employed in the conventional AA method at the industrial scale. During alkali extraction both protein and soluble carbohydrates (Vasanthan & Temelli, 2008) are solubilized, and higher S/F ratios are needed to avoid challenges associated with the handling of highly viscous extracts. In the proposed low S/F ratio method, the salts present in the resulting liquid extracts would be substantially more concentrated than using a high S/F ratio, as the naturally present

minerals in the feed are dissolved in a smaller amount of solvent. Although solutes of MW less than the MWCO (6-8 kDa) of the semipermeable dialysis membrane should permeate as a result of random molecular motions across the concentration gradient, the formation of salt crystals as evidenced by the SEM images (Fig. 6.2) must have interfered with their permeation through the dialysis membrane. It is possible that when S/F = 3 with 1% added $NaCl_{(aq)}$ solution was employed, protein precipitation and salt crystallization (Fig. 6.2) were experienced as an outcome of the salting-out effect. According to the SEM images (Fig. 6.2e1-f3), the salt crystals were sized from 100 nm to 3 μm, while the MWCO was only 6 to 8 kDa (1.2 to 1.4 nm radius).

In comparison to the S_{23} method, the W_{35} method engaging dialysis at 4 °C (D) not only resulted (Table 6.3) in protein fractions of high purity (LT: 93.2% and HT: 96.4%) but also high recovery (78.0%), in agreement with the protein solubility results of Fig. 6.3. These values were significantly higher as compared to W_{23}-D method (Table 6.3) in agreement with Fig. 6.3. This could mean that the naturally present minerals facilitate the salting-in effect and at 35 °C water plays a crucial role in the disentanglement of flour particles and enhances the solubility of the salt-soluble globulins as well as the water-soluble albumins. Also, dialysis at 4 °C is favourable for isolating the proteins over 23 °C. According to the SEM images, globular protein structures were observed in the PIs obtained by W_{35}-D (Fig. 6.2c1-d3) similar to the protein storage organelles observed in the dehulled bean flours of both the cultivars (Fig. 6.2a1-b3), which appear morphologically different from the rest of the PCs/PIs. Additionally, this method (W_{35}-D) yielded salt-free dietary fibre fractions with significantly improved yield, purity and recovery values and starch fractions with higher purities (Table 6.3).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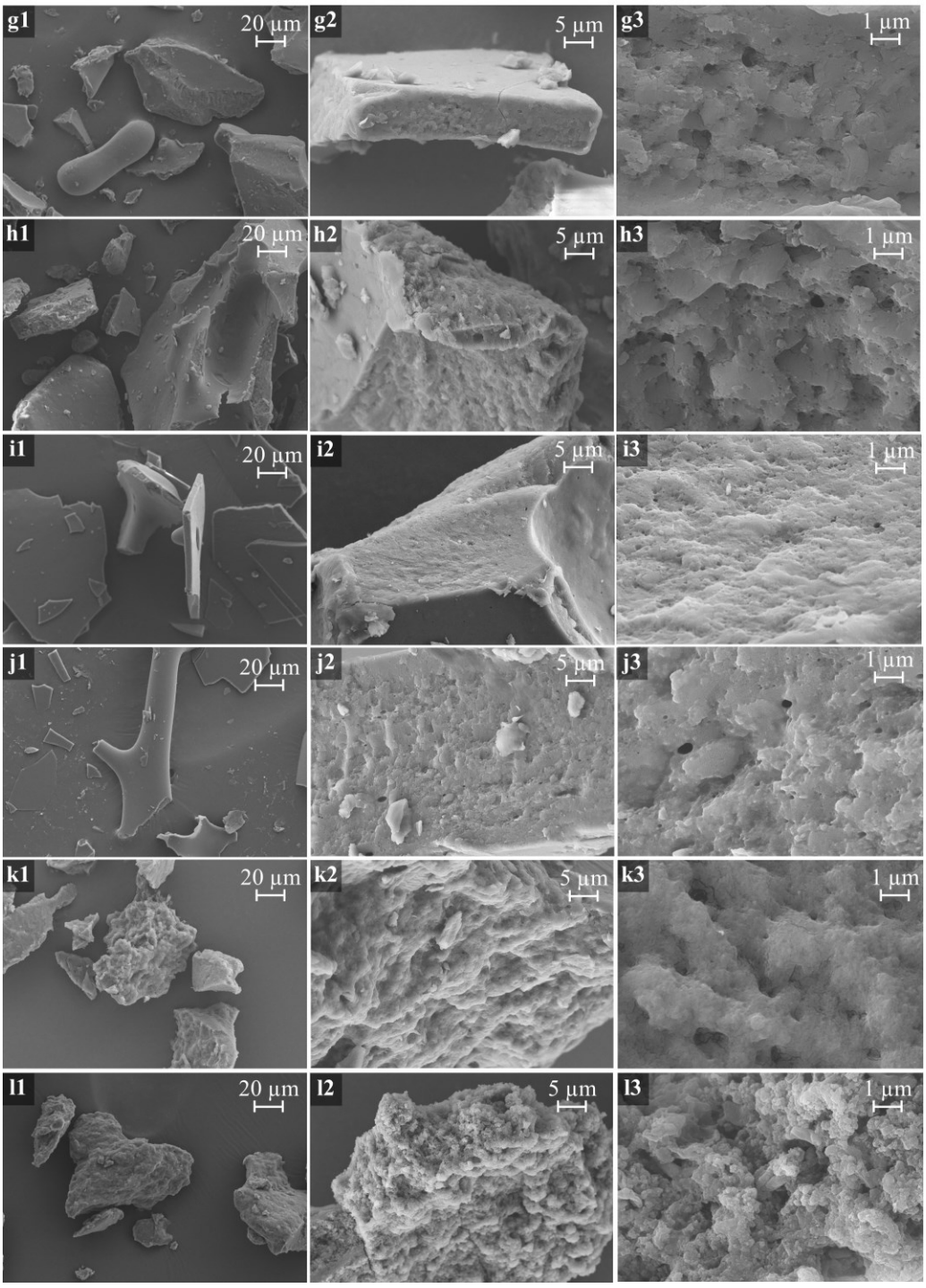
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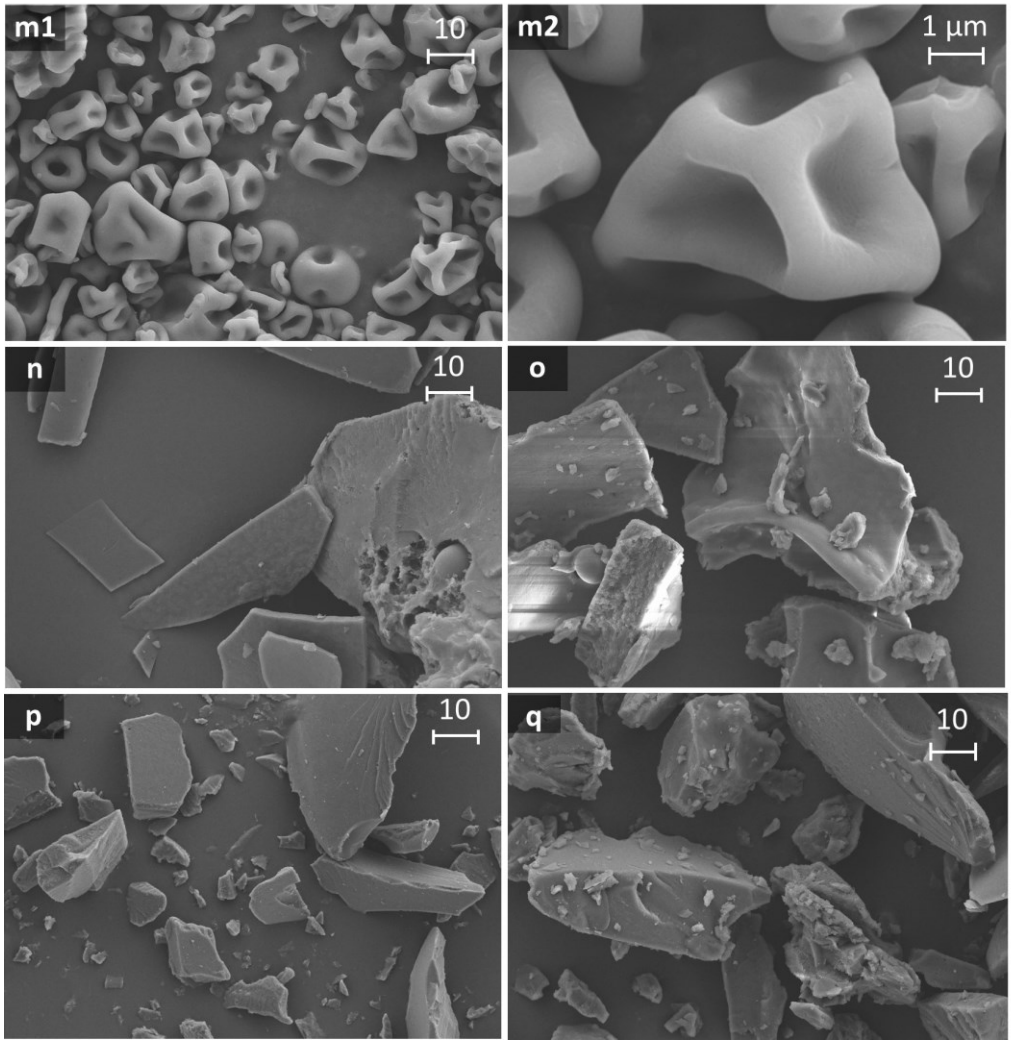


Fig. 6.2. SEM images of faba bean fractions resulting from wet fractionation of dehulled faba bean flour using different methods.

SEM images of (a,b) dehulled faba bean flour; and protein concentrates or isolates resulting from: (c,d) water extraction at 35 °C + dialysis, (e, f) $NaCl_{(aq)}$ extraction at 23 °C + dialysis, (g, h) water extraction at 35 °C + micellization, (i,j) water extraction at 35 °C + isoelectric precipitation, (k,l) alkali extraction + isoelectric precipitation, (m) water extraction at 23 °C + ultrafiltration + spray drying HT and (n,o) water extraction at 23 °C + dialysis, and (p,q) non-precipitated fractions of micellization.
Cultivars: Snowbird (LT) (a,c,e,g,i,k,n,p) and Athena (HT) (b,d,f,h,j,l,m,o,q). (1: ×500, 2: ×2000, 3: ×10000, m1: ×2000, m2: ×10000 and n-q: ×1000 magnification). P: protein; S: starch granule.

The highest protein purities (98.7 to 99.0%) were attained with the W_{35}-M method; however, the corresponding yield (12%) and recovery values (LT: 34.0% and HT: 35.2%) of the protein fractions were lower than the rest of the water extraction methods regardless of the cultivar (Table 6.3). The protein and non-precipitated fractions (Fig. 6.2p-q) of the W_{35}-M method together accounted for 60 to 65% of the proteins in total. Based on the raffinose family oligosaccharides analyses, significant amounts of sucrose (LT: 88.40 ± 1.24 and HT: 115.17 ± 0.44 mg/g), raffinose (LT: 3.08 ± 0.20 and HT: 3.39 ± 0.08 mg/g), stachyose (LT: 43.55 ± 0.86 and HT: 48.52 ± 0.11 mg/g) and verbascose (LT: 82.19 ± 1.43 and HT: 84.37 ± 0.06 mg/g) were detected in the W_{35}-M non-precipitated fractions. In the W_{35}-M method, proteins undergo a series of dissociation steps and form protein micelles as a result of the interactions between the amphiphilic protein molecules leading to the stable arrangement of β-sheets (Murray et al., 1979), which then appear as sub-cellular micelles (Fig. 6.2c1-d3) similar to the findings reported in Chapters 4 (Fig. 4.5) and 5 (Fig. 5.6). According to the previous studies reported in Chapters 4 and 5, the proteins recovered in the protein fractions resulting from hybrid fractionation of faba beans employing pearling (52-61%) or ACAPS (48-63%) dry fractionation techniques followed by wet fractionation employing micellization were higher than the PIs obtained by W_{35}-M, although the purity levels remained the same. Therefore, micellization is more effective in recovering the proteins when used in conjunction with dry fractionation methods than being used alone. To maximise protein recovery, a more suitable alternative would be to produce a protein + fibre enriched fraction by pearling or ACAPS for minimally processed food applications and then to subject the remaining dietary fibre-depleted fraction to aqueous fractionation to isolate the proteins.

The W_{35}-A method resulted in protein isolates of high purity for both LT (95.0%) and HT (96.4%) along with 57.2% and 56.1% protein recoveries, respectively (Table 6.3). Nearly 7.5% (LT) and 11.0% (HT) of the proteins were recovered in the non-precipitated fractions. The PCs obtained by W_{35}-H had the least protein purity and recovery values when compared to the rest of the PCs/PIs obtained by W_{35} along with 14.2% (LT) and 12.9% (HT) protein recovered in the non-precipitated fraction. The AA method based on alkaline extraction followed by isoelectric precipitation of the proteins was employed as the control since it is the conventional wet fractionation process commonly used in the industry. The protein purity of the PCs (LT: 87.1% and HT: 86.3%) obtained by AA (Table 6.4) of both cultivars was lower than the PIs obtained by W_{35}-D (Table 6.3). This is possible because 16.2% (LT) and 12.9% (HT) of the proteins were not precipitated at the isoelectric point (Table 6.4). AA method mainly yields globulins followed by glutelins whereas both W and S methods yield a mixture of globulins and albumins (Johnston et al., 2015).

In general (Tables 6.3 to 6.4), the yield, purity and recovery values were significantly ($P < 0.05$) lower for HT compared to LT, probably due to the interference of tannins in protein isolation. It is noteworthy that the PCs of AA would have undergone denaturation leading to the unfolding and stretching of molecular structures exposing their interior hydrophobic groups (Karaca et al., 2011) followed by the formation of aggregates of nanomolecular particles (Fig. 6.2k1-l3).

Table 6.4. Chemical composition, yield and recovery values (%) of faba bean fractions resulting from the pH shift method (AA: alkali extraction + acid precipitation) on a dry matter basis.

Fraction	Component	Snowbird (LT)	Athena (HT)
Protein	Yield	26.39 ± 0.37[b]	28.43 ± 1.07[a]
	Protein (N×6.25)	87.11 ± 1.36[a] (67.50 ± 1.95)	86.29 ± 0.24[a] (71.30 ± 1.14)
	Starch	3.62 ± 0.57[b] (1.94 ± 0.33)	7.28 ± 0.61[a] (4.42 ± 0.42)
Non-precipitated	Yield	18.34 ± 1.68[a]	12.36 ± 1.18[b]
	Protein (N×6.25)	30.05 ± 0.19[b] (16.18 ± 1.43)	35.51 ± 0.10[a] (12.91 ± 1.27)
	Starch	3.77 ± 0.12[b] (1.40 ± 0.16)	5.75 ± 0.37[a] (1.53 ± 0.07)
Starch	Yield	24.54 ± 1.25[a]	24.44 ± 1.20[a]
	Protein (N×6.25)	6.05 ± 0.16[a] (4.37 ± 0.33)	6.10 ± 0.29[a] (4.38 ± 0.03)
	Starch	88.71 ± 1.06[a] (41.81 ± 1.66)	84.11 ± 0.95[b] (44.36 ± 2.17)
Dietary Fibre	Yield	25.88 ± 1.59[a]	24.60 ± 0.53[a]
	Protein (N×6.25)	5.38 ± 0.12[b] (4.09 ± 0.19)	8.23 ± 0.21[a] (5.95 ± 0.07)
	Starch	57.99 ± 1.96[a] (30.37 ± 0.86)	54.36 ±0.78[b] (28.86 ± 0.88)
	Total dietary fibre	24.07 ± 0.11[b] (57.55 ±3.33)	31.97 ± 1.76[a] (74.52 ±3.74)
Intermediate	Yield	1.59 ± 0.29[a]	1.30 ± 0.36[a]
	Protein (N×6.25)	12.56 ± 0.14[b] (0.39 ± 0.35)	19.23 ± 0.21[a] (0.74 ± 0.21)
	Starch	14.71 ± 1.36[b] (0.47 ± 0.10)	18.36 ± 0.65[a] (0.51 ± 0.13)

[a,b]Mean (n = 3) ± SD values within a row with different letters are significantly different ($P < 0.05$), according to the two-sample t-test. S/F: solvent/feed ratio (v/w). Recovery values (%) given within brackets were based on the initial compositional analyses of protein (LT: 34.06 ± 0.29% and HT: 34.08 ± 0.15%), starch (LT: 49.53 ± 0.37% and HT: 46.34 ± 0.30%) and dietary fibre (LT: 10.82 ± 0.58% and HT: 10.55 ±0.47%) contents of the dehulled bean flour on a dry matter basis.

There are concerns related to the scalability of wet fractionation processes involving dialysis, micellization or ultrafiltration operations. From an economic standpoint, no differences were reported between the AA method and membrane isolation of soy proteins or ultrafiltration of faba bean proteins (Lawhon et al., 1979; Olsen & Anderson, 1978). A scale-up of the water extraction method with HT faba bean dehulled flour at a S/F ratio of 3 followed by ultrafiltration and spray drying (HT-W_{23}-US) yielded 16.46 ± 0.12% protein fraction, 32.85 ± 0.33% starch fraction, 16.38% dietary fibre fraction along with a 7.93 ± 1.72% intermediate fraction. The purity and corresponding recovery values of protein, starch and dietary fibre of the protein fraction were 82.80 ± 0.03% and 40.08 ± 0.28%, 1.32 ± 0.01% and 0.47 ± 0.00%, and 8.01 ± 0.10% and 12.50 ± 0.24%, respectively. The purity of PCs obtained from HT by W_{23}-US was

substantially less than that of PIs obtained by W_{35}-D possibly because the extraction was carried out at 23 °C instead of 35 °C; dialysis was replaced by ultrafiltration, and the protein fraction was spray dried (Fig. 6.2. m1-m2) instead of freeze drying. However, the protein values were higher than that of PCs obtained by WS_{23}-U, mainly due to the salting-in effect. Lower yields can be explained by the loss of samples in the spray drying system.

The recovery results reported in Tables 6.2 to 6.4 are based on the compositional analyses results of LT and HT dehulled bean flour protein (LT: 34.06 ± 0.29% and HT: 34.08 ± 0.15%), starch (LT: 49.53 ± 0.37% and HT: 46.34 ± 0.30%) and dietary fibre (LT: 10.82 ± 0.58% and HT: 10.55 ± 0.47%) contents on a dry matter basis (Chapter 4, Table 4.2). The protein contents of the inputs and all output fractions were estimated using a nitrogen-to-protein conversion factor of 6.25. Based on the crude fat analysis results, the PIs of W_{35}-D (LT: 1.7 ± 0.3% and HT:2.2 ± 0.5%) had considerably ($P < 0.05$) less crude fat in comparison to those of W_{35}-M (LT: 4.9 ± 0.4% and HT: 4.3 ± 0.1 %). The corresponding LT and HT fibre, starch and intermediate fractions of the W_{35}-M method also contained 1.47 ± 0.22% and 1.67 ± 0.48%, 0.75 ± 0.20% and 0.63 ± 0.26%, and 2.15 ± 0.74% and 2.13 ± 0.05% crude fat, respectively. Although widely accepted, using the traditional conversion factor of N × 6.25 has resulted in protein contents above 100% for the faba bean protein isolates based on dry matter, similar to the literature (Mariotti et al., 2008). Therefore, there is a need to establish a protein conversion factor specific to faba bean proteins based on their amino acid composition. However, based on the comparisons of yield, purity and recovery values of protein as discussed above, it can be concluded that the W_{35}-D method is a mild and chemical-free alternative to the conventional AA method. In addition, the significant reduction in the S/F ratio will lead to substantial savings.

6.3.2. Protein solubility

According to Osborne's protein solubility-based classification (Osborne, 1924), three-fourths of the faba bean storage proteins were salt-soluble globulins and less than one-fourth were water-soluble albumins, followed by trace levels of ethanol-soluble prolamins and dilute acid- or alkali-soluble glutelins (Appendix B: Table B.1). Globulin (73.6 to 75.8%) and glutelin (1.2 to 11.5%) values reported by El Fiel et al. (2002) were equivalent to the findings of this study. However, their albumin levels (1.4 to 2.2%) were almost 10-fold less (22.3 to 22.8%) and the prolamin (2.7 to 3.5%) values were 10-fold higher than the values (0.2%) obtained here. These discrepancies can be due to the differences in the cultivars, the impact of minerals and anti-nutritional factors on protein solubility, and to a lesser extent the protein profiling approach. There were no significant ($P > 0.05$) differences between the two cultivars investigated in terms of their protein solubility-based profiles (Appendix B: Table B.2), despite the significant variations in their mineral and tannin contents.

Since 74 to 75% of the faba bean storage proteins account for the salt-soluble globulins, the solubility of faba bean proteins was investigated as a function of varying $NaCl_{(aq)}$ concentrations against water. Salts can greatly influence the solubility and stability of macromolecules, especially proteins (Arakawa & Timasheff, 1984). At low concentrations, the salt molecules can increase the solubility of proteins, which is referred to as 'salting-in' by stabilizing the protein molecule by weakening attractive interactions (Ahmed et al., 2012; Dahal & Schmit, 2018). Whereas at high salt concentrations, protein-protein interactions become energetically more favourable than protein-solvent interaction, thereby decreasing electrostatic repulsions and enhancing hydrophobic interaction. The increase in hydrophobic interactions would result in a higher tendency for the proteins to form insoluble aggregates, thus decreasing protein solubility known as the 'salting-out' effect (Ahmed et al., 2012).

Surprisingly, LT protein solubility was considerably ($P < 0.05$) higher in demineralised water (83.6 ± 0.5%) as compared to using 0.5% or 0.75% $NaCl_{(aq)}$ solution and was not significantly different from using a 2% $NaCl_{(aq)}$ solution (Fig. 6.3a) due to the salting-in effect resulting from the naturally present minerals. HT protein solubility in demineralised water (77.1 ± 0.8%) was similar to that of 0.75% or 1% $NaCl_{(aq)}$ solution but higher than that of 0.25%.

The protein fractions used in the solubility analyses were obtained by using a S/F ratio of 3 (v/w) and varying added NaCl concentrations (0 to 6%). Although the nature of the minerals or counter ion effects were not confirmed in this study, the minerals (~3%) inherent in faba bean dehulled bean flour (Table 6.1) must be creating an optimal condition to release the salt-soluble proteins from the flour matrix when water or $NaCl_{(aq)}$ solutions of low concentration were used as a solvent. The demineralised water used in these experiments had less than 2 µS/cm conductivity and 0.1 ppm hardness, which were comparable to that of distilled water. Thus, the minute levels of minerals present in the water were not expected to influence the solubility of proteins.

Protein solubility drastically dropped with the added NaCl concentration increasing from 2 to 6%, due to the salting-out effect for both LT and HT (Fig. 6.3a). A similar trend has been previously reported with chickpea flour, where the solubility of protein was greatest at 0.4 M NaCl (~2.3%) and decreased with increasing salt concentration due to the salting-out effect. In the same study, the protein solubility was less with 0.2 M NaCl solution as compared to using water (Osman et al., 2005). Similar to LT in 2% $NaCl_{(aq)}$ solution, cowpea and pigeon pea in 0.4 M $NaCl_{(aq)}$ solutions reached maximum protein solubility (Ahmed et al., 2012).

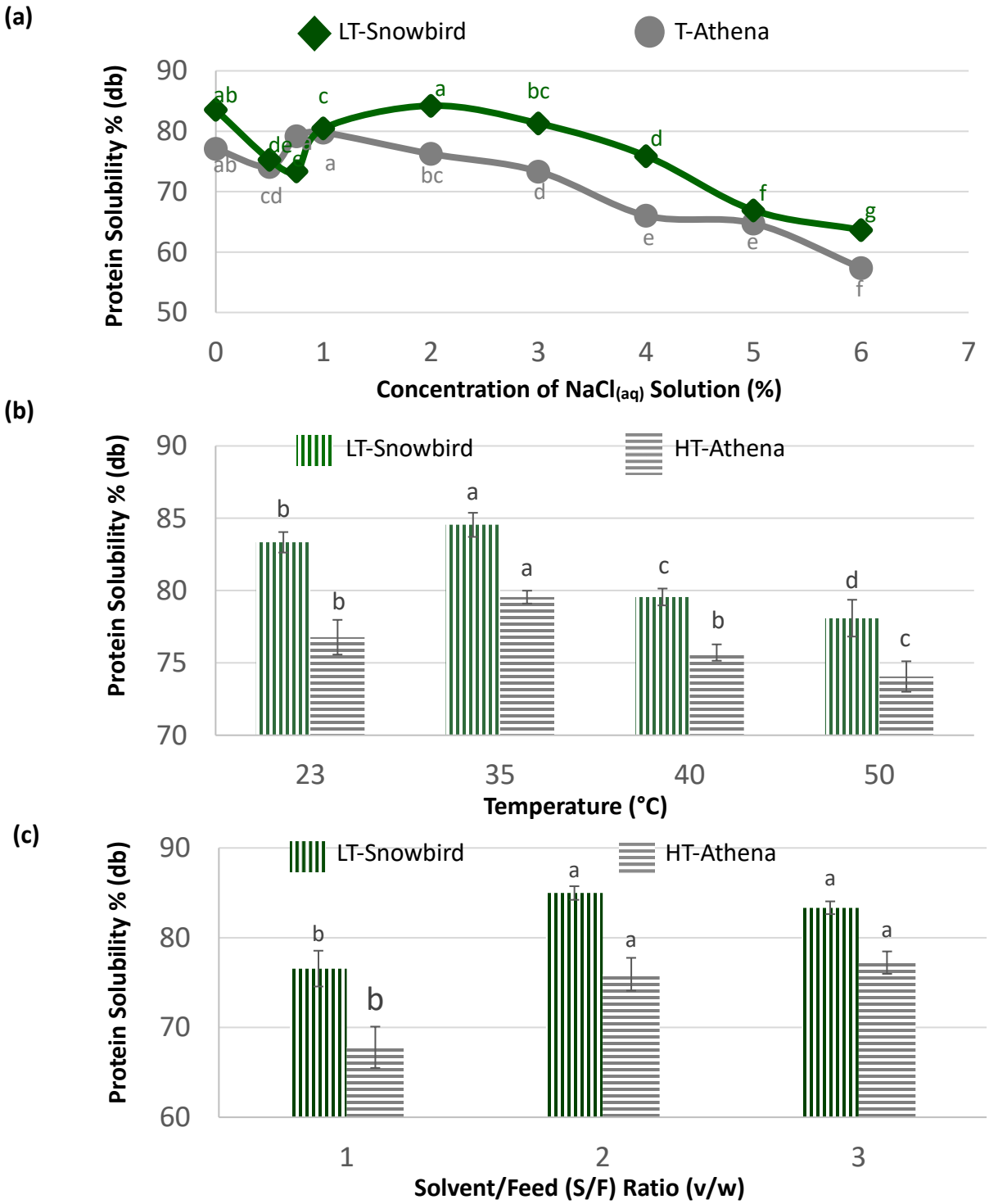


Fig. 6.3. Effect of (a) NaCl concentration (%), (b) temperature and (c) solvent/feed (v/w) ratio on faba bean protein solubility (%) on a dry matter basis.

[a-g]Mean (n = 6) ± SD values for a particular cultivar followed by different letters are significantly different ($P < 0.05$), according to Duncan's multiple range test.

The naturally present minerals as globoid structures likely support the release of salt-soluble proteins during protein extraction. Arakawa and Timasheff (1984) reported that the divalent cations interacting and binding with proteins can increase surface tension thereby enhancing salt inclusion and leading to a decreased preferential hydration. It was shown that the preferential hydration decreased in the order of SO_4^{2-}>COO^->CH_3^->Cl^- regardless of the cationic species in use, whereas it increased in the order of Mn^{2+}<Ni^{2+}<Ca^{2+}<Ba^{2+}<Mg^{2+}<Na^+<K^+ (Arakawa & Timasheff, 1984; Dahal & Scmit, 2018) similar to the ranking of ions in the charge of globular proteins. Therefore, the presence of divalent cations should decrease faba bean protein solubility but can be complicated by the heterogeneity of the protein-water interface (Dahal & Scmit, 2018). The solubility of faba bean proteins in water as a function of temperature is illustrated in Fig. 6.3b. It is noteworthy that both LT (84.6 ± 0.8%) and HT (79.6 ± 0.4%) protein solubilities were substantially (P < 0.05) higher at 35 °C as compared to 23 °C. LT protein solubility gradually reduced at 40 °C and 50 °C as compared to 35 °C whereas for HT no significant differences were observed between 23 °C and 40 °C but the protein solubility reduced to 74.1 ± 1.0% at 50 °C. It is possible that at 35 °C, the enthalpy of the flour matrix increases and helps release the proteins from the flour matrix as well as solubilise them in water. Increasing the temperature beyond 40 °C interfered with protein solubility plausibly due to the breakage of the various interactions of the proteins and their partial denaturation.

The solubility of faba bean proteins in water as a function of S/F is shown in Fig. 6.3c. No significant (P > 0.05) differences were observed in protein solubility between S/F ratios of 2 (LT: 85.0 ± 0.8% and HT: 76.0 ± 1.8%) and 3 (LT: 83.3 ± 0.7% and HT: 77.2 ± 1.3%). However, at S/F = 1, substantially lower protein solubilities for both LT (76.6 ± 2.0%) and HT (67.8 ± 2.3%) were observed probably due to the salting out effect as the naturally present minerals in the feed

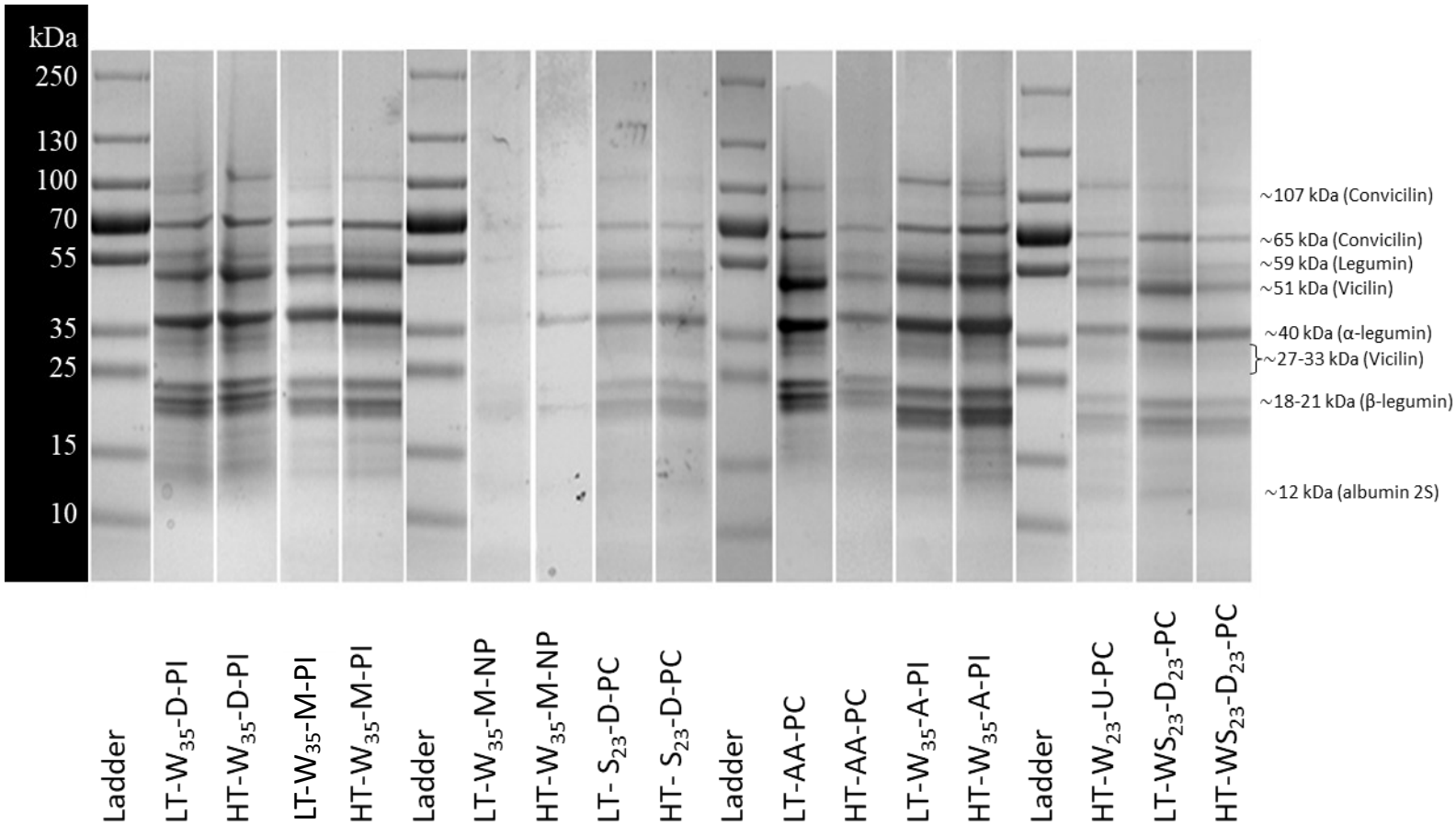
are dissolved in a smaller amount of solvent. Therefore, the use of S/F = 2 would be sufficient to achieve maximum extractability of faba bean proteins, which would minimize solvent use.

6.3.4. Electrophoretic protein profiles

The salt-soluble globulins can be subdivided into two high-molecular-weight proteins, the hexameric 11S legumin with a MW of ~340 kDa and the trimeric 7S vicilin protein with a MW of ~158 kDa (Multari et al., 2015; Wright & Boulter, 1973). Convicilin is regarded as a subunit of the 7S vicilin (Chen et al., 2019). The bands visible at ~65 and ~54 kDa, ~59 kDa, and ~51 kDa and ~18-21 kDa in the SDS-PAGE profiles presented in Fig. 6.4 likely correspond to the subunits of the globulins convicilin, legumin, and vicilin, respectively (Nivala et al., 2017; Warsame et al., 2020). Legumin subunits are composed of acidic (α-legumin) and basic (β-legumin) chains linked by a single disulphide bond appearing as bands around ~40 kDa and ~18 to 21 kDa, respectively (Nivala et al., 2017; Warsame et al., 2018). The three bands of β-legumins appearing from ~18 to 21 kDa (Fig. 6.4b) are comparable to the findings of Nivala et al. (2017).

The electrophoretic polypeptide bands of PIs obtained by W_{35}-D, W_{35}-M, and W_{35}-A appearing below 15 kDa from 12 to14 kDa for LT and HT as well as of W_{35}-M of HT around 10 kDa correspond to the subunits of 2S albumin. Although most of the polypeptide bands of faba bean protein fractions of the different methods employed appearing in Fig. 6.4a are globulins (convicilin, legumin and vicilin), the PIs of W_{35} methods also contain faint albumin bands.

(a)


kDa
250
130
100
70
55
35
25
15
10
~107 kDa (Convicilin)
~65 kDa (Convicilin)
~59 kDa (Legumin)
~51 kDa (Vicilin)
~40 kDa (α-legumin)
~27-33 kDa (Vicilin)
~18-21 kDa (β-legumin)
~12 kDa (albumin 2S)
Ladder
LT-W35-D-PI
HT-W35-D-PI
LT-W35-M-PI
HT-W35-M-PI
Ladder
LT-W35-M-NP
HT-W35-M-NP
LT- S23-D-PC
HT- S23-D-PC
Ladder
LT-AA-PC
HT-AA-PC
LT-W35-A-PI
HT-W35-A-PI
Ladder
HT-W23-U-PC
LT-WS23-D23-PC
HT-WS23-D23-PC

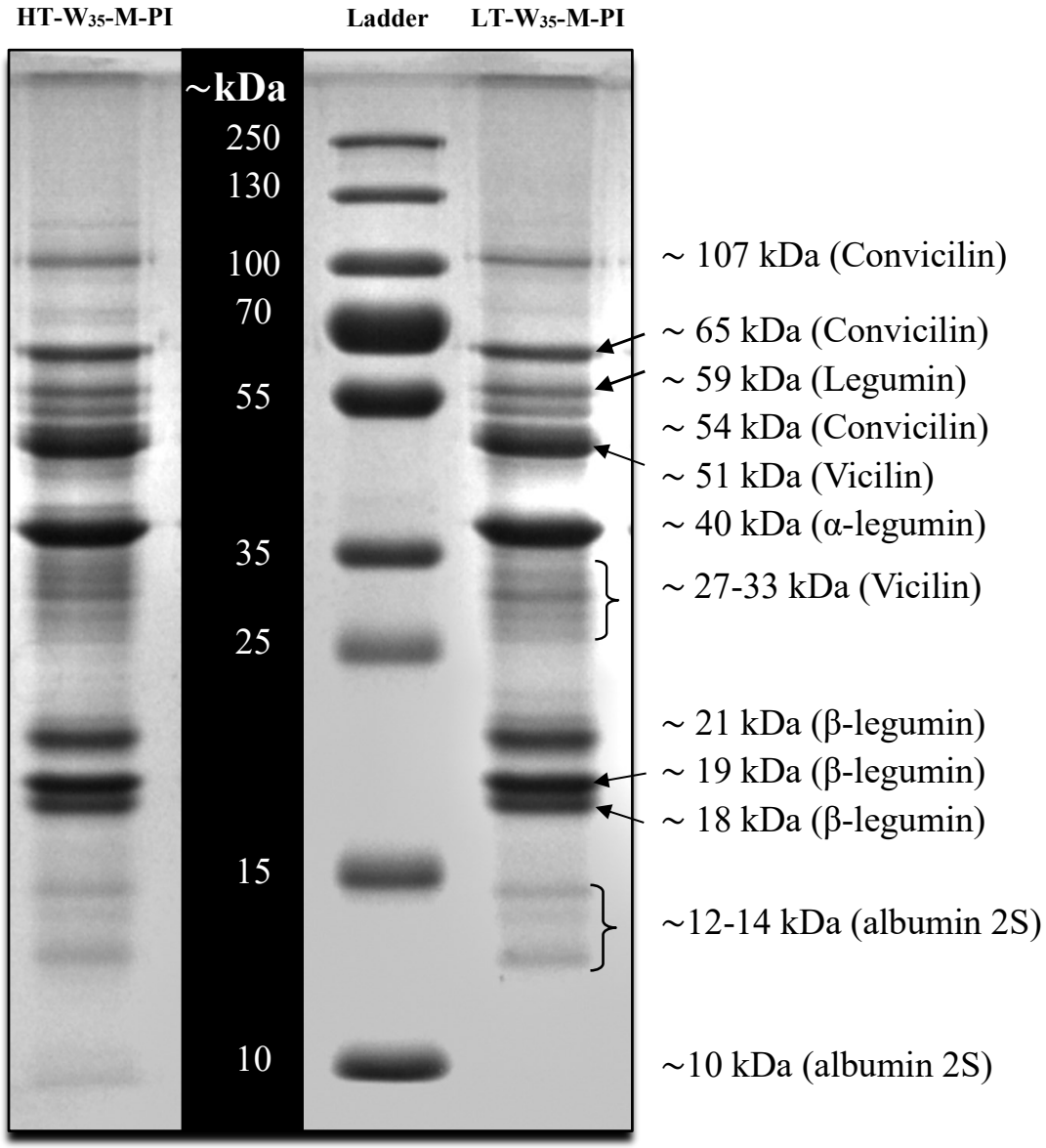


Fig. 6.4. Electrophoretic profiles of faba bean protein fractions determined by SDS-PAGE.

(a) Lanes of protein isolates (PIs) and non-precipitated (NP) fractions of Snowbird (LT) and Athena (HT) faba bean cultivars treated with water at either (W_{23}) or 35 °C (W_{35}) followed by either dialysis at 4 °C (D), micellization (M) or acid precipitation (A) or treated with water and 1% $NaCl_{(aq)}$ solution (WS_{23}) or 1% $NaCl_{(aq)}$ solution (S_{23}) at 23 °C followed by dialysis (D). HT-W_{23}-U-PC: S/F = 3 (v/w) ultrafiltered and spray-dried. Initial sample concentration for analysis was 1 mg/mL, and (b) sample concentration was 1.5 mg/mL.

Therefore, the SDS-PAGE-related observations confirm that the PIs of W_{35} methods are composed of mainly globulins but also albumins to a lesser extent. The salts present in the PCs of S could have interfered with the SDS-PAGE-based protein profiling, as the bands in lanes 9 and 10 appear to be faint compared to the rest of the lanes. The non-precipitated fraction of micellization was also investigated for its protein profile. Although proteins were present, the corresponding polypeptide bands in lanes 7 and 8 appear less concentrated compared to the rest possibly due to the cross-linking of the proteins with other polymers. Although substantial differences could be observed between the band intensities of different PIs, the protein profiles were similar. There were no obvious differences in the SDS-PAGE protein profiles between the two cultivars investigated.

The L/V ratios were calculated based on the sum of the relative percentages of L [α-legumin (~40 kDa) + β-legumin chains (~18 to 21 kDa)] and V (~50 kDa) proteins and presented in Appendix B: Table B.2. The L/V ratios of faba bean PCs and PIs were within the range of 2.73 to 3.76, which were comparable to the findings of Martinez et al. (2016). The PIs of W_{35}-D and W_{35}-M had the highest L/V ratios followed by the PCs of AA. The PCs of S_{23}-D of both cultivars had the lowest L/V ratios probably due to the interference caused by the salts as the legumins are less soluble in $salt_{(aq)}$ solutions compared to vicilins (Osborne, 1924). In addition, the legumins of PCs of AA must have selectively precipitated due to the combined effect of pH and ionic strength. Other parameters such as the cultivar (i.e., genotype), growth conditions of the geographic location and the protein extraction steps can also influence the L/V ratio of proteins (Martinez et al., 2016). The correlation of L/V ratios altered by different processing conditions to their functionality warrants further investigation.

6.3.5. Protein quality

Amino acid composition of the faba bean cultivars resulting from both performic acid and hydrochloric acid hydrolysis along with protein digestibility, IVPD, AASs, IVPDCAAS and TUI/mg are given in Table 6.5. The amino acid composition and TUI are expressed as (mg amino acid/g N) to minimise the oversight of using a N-to-protein conversion factor of 6.25. In the present study, GLU and ARG were the most abundant in both LT and HT faba bean PIs regardless of the isolation technique. Based on Table 6.5, the W_{35}-D method resulted in an overall higher amino acid content as compared to the rest of the methods, except for the amino acid LYS, which was high in the PIs of W_{35}-M. Tryptophan (TRY) and proline (PRO) contents were not analysed as part of the amino acid profiling; therefore, a N-to-protein conversion factor could not be reached. The sulphur-containing amino acids MET and CYS were limiting with AAS of 1.05 and 1.05, 0.94 and 0.93, and 0.91 and 0.92 for the PIs of W_{35}-D, W_{35}-M and AA of LT and HT, respectively, relative to the established reference values of the FAO based on the nutritional requirements of a 3 to 5-year-old child (FAO, 2009).

These results confirm that faba bean proteins are a good source of S-containing amino acids MET and CYS, but the levels are dependent upon the protein isolation technique. While globulins are usually rich in GLU, ASP, LEU and ARG, water-soluble albumins are rich in sulphur-containing amino acids. Alcohol-soluble prolamins present to a lesser extent are rich in LEU, GLU and PRO but devoid of LYS and TRY (El Fiel et al., 2002), followed by glutelins, which to some extent are similar in amino acid composition to that of prolamins, but with higher levels of GLY, MET and HIS (El Fiel et al., 2002; Multari et al., 2015).

Table 6.5. Amino acid composition (mg amino acid/g N) and quality parameters of faba bean protein fractions on a dry matter basis.

Component		W_{35}-D-PI		W_{35}-M-PI		AA-PC	
		LT	HT	LT	HT	LT	HT
Essential amino acids (mg amino acid/g N)	HIS	526.70 ± 67.69[a]	479.57 ± 95.66[a]	368.35 ± 24.32[a]	317.54 ± 31.56[a]	393.30 ± 44.14[a]	344.89 ± 36.49[a]
	ILE	497.77 ± 17.85[a]	504.44 ± 16.08[a]	434.98 ± 19.57[a]	475.35 ± 19.57[a]	537.16 ± 21.97[a]	592.26 ± 14.34[a]
	LEU	755.52 ± 69.73[a]	561.40 ± 29.61[b]	699.97 ± 27.11[a]	526.10 ± 57.82[b]	575.91 ± 70.86[a]	517.81 ± 21.74[a]
	LYS	314.46 ± 33.29[a]	269.72 ± 93.93[b]	397.78 ± 27.49[a]	282.87 ± 42.24[b]	212.03 ± 35.72[a]	263.83 ± 76.73[a]
	MET	142.16 ± 3.32[a]	148.07 ± 1.61[a]	132.22 ± 6.54[a]	124.64 ± 15.98[a]	124.46 ± 6.03[a]	125.92 ± 3.61[a]
	PHE	566.46 ± 26.03[a]	581.22 ± 21.58[a]	512.07 ± 25.73[a]	572.69 ± 49.62[a]	644.54 ± 27.17[a]	715.83 ± 18.16[a]
	THR	552.40 ± 24.00[a]	543.71 ± 17.41[a]	451.12 ± 19.09[a]	462.84 ± 22.52[a]	540.15 ± 21.84[a]	561.13 ± 10.71[a]
	VAL	325.74 ± 42.36[a]	246.71 ± 68.99[b]	243.57 ± 39.10[a]	191.48 ± 26.05[a]	208.31 ± 31.19[a]	172.19 ± 6.70[a]
Non-essential amino acids (mg amino acid/g N)	ALA	687.62 ± 60.83[a]	555.06 ± 160.32[b]	426.42 ± 31.85[a]	356.06 ± 42.10[b]	428.70 ± 117.04[a]	319.51 ± 18.38[b]
	ARG	1638.88 ± 169.74[a]	1434.07 ± 259.51[a]	1476.76 ± 56.54[a]	876.47 ± 90.55[b]	1232.85 ± 114.51[a]	1025.65 ± 73.00[b]
	ASP	826.16 ± 64.06[a]	730.14 ± 107.87[b]	724.57 ± 18.77[a]	500.30 ± 61.21[b]	658.76 ± 54.16[a]	606.82 ± 19.10[b]
	CYS	33.26 ± 0.55[a]	31.99 ± 0.73[a]	26.38 ± 1.24[a]	25.37 ± 0.72[a]	26.95 ± 0.45[a]	28.79 ± 1.20[a]
	GLU	2011.27 ± 168.67[a]	1774.74 ± 301.58[b]	1590.35 ± 43.45[a]	1193.51 ± 135.84[b]	1520.46 ± 121.57[a]	1349.18 ± 59.98[b]
	GLY	865.67 ± 82.71[a]	779.62 ± 142.04[b]	575.41 ± 24.90[a]	450.88 ± 46.19[b]	660.92 ± 44.85[a]	521.27 ± 71.78[b]
	SER	672.71 ± 53.45[a]	615.08 ± 73.49[a]	509.06 ± 20.43[a]	423.99 ± 51.40[a]	567.99 ± 32.63[a]	506.16 ± 26.73[b]
	TYR	400.18 ± 12.35[a]	406.62 ± 18.55[a]	191.27 ± 12.04[b]	288.53 ± 40.53[a]	426.26 ± 18.39[b]	455.73 ± 11.43[a]
	N%	14.37 ± 0.04[a]	14.72 ± 0.00[a]	16.15 ± 0.01[a]	15.84 ± 0.00[a]	14.07 ± 0.01[a]	13.67 ± 0.03[a]
	IVPD	83.50 ± 1.62[a]	82.83 ± 0.18[a]	83.68 ± 0.28[a]	84.28 ± 1.32[a]	83.86 ± 1.00[a]	84.70 ± 0.55[a]
	AAS	1.05 ± 0.01[a]	1.05 ± 0.01[a]	0.94 ± 0.05[a]	0.93 ± 0.05[a]	0.91 ± 0.04[a]	0.92 ± 0.04[a]
	IVPDCAAS	88.30 ± 1.05b[a]	87.17 ± 0.81[a]	78.34 ± 3.80[a]	78.78 ± 3.93[a]	76.62 ± 3.74[a]	77.68 ± 3.20[a]
	TUI/mg N	0.96 ± 0.07[a]	0.76 ± 0.05[b]	0.33 ± 0.08[a]	0.17 ± 0.02	not detected	not detected
	Sucrose (mg/g)	4.76 ± 0.51[b]	6.90 ± 0.77[a]	10.32 ± 0.10[a]	9.83 ± 0.06[a]	2.11 ± 0.06[b]	2.73 ± 0.18[a]
	Raffinose (mg/g)	1.26 ± 0.34[a]	1.50 ± 0.03[a]	2.60 ± 0.76[a]	2.59 ± 0.34[a]	1.39 ± 0.21[b]	1.79 ± 0.27[a]
	Stachyose (mg/g)	4.82 ± 1.10[a]	2.29 ± 0.11[b]	2.00 ± 0.42[a]	1.84 ± 0.44[a]	0.48 ± 0.14[a]	0.47 ± 0.13[a]
	Verbascose (mg/g)	8.92 ± 0.17[a]	5.07 ± 0.30[b]	4.78 ± 0.41[a]	3.41 ± 0.18[b]	1.29 ± 0.09[b]	1.38 ± 0.12[a]
	Total Tannins (mg TAE/g)	0.86 ± 0.08[b]	1.12 ± 0.02[a]	0.42 ± 0.12[b]	0.82 ± 0.27[a]	0.22 ± 0.00[b]	1.36 ± 0.00[a]

[a-b]Mean (n = 3) ± SD values within a row for a particular extraction method followed by different letters are significantly different ($P < 0.05$), according to a two-sample t-test. n = 6 for TUI/mg N

TAE: tannic acid equivalent; LT: Snowbird; HT: Athena; W_{35}-D: water extraction at 35 °C followed by dialysis at 4° C; W_{35}-M: water extraction at 35 °C followed by micellization; AA: alkali extraction followed by acid precipitation

HIS, histidine; ILE: isoleucine; LEU: leucine; LYS: lysine; MET: methionine; PHE: phenylalanine; THR: threonine; VAL: valine; ALA: alanine; ARG: arginine; ASP: aspartic acid; CYS: cysteine; GLU: glutamic acid; GLY: glycine; SER: serine; TYR: tyrosine; IVPD: *in-vitro* protein digestibility; AAS: amino acid score; IVPDCAAS: *in-vitro* protein digestibility corrected amino acid score; TUI: trypsin units inhibited.

The W_{35}-D method is capable of extracting both the globulins and albumins, unlike the AA method in which the sulphur-rich albumins are lost during the extraction process (Johnston et al., 2015). Based on the amino acid profile of the PIs of W_{35}-M, it can be confirmed that the levels of LYS have increased during the micellization process. The non-limiting presence of LYS is complementary with cereals rich in sulphur-containing amino acids for well-balanced nutrition (Acevedo-Pacheco & Serna-Saldívar, 2016). The basic amino acids LYS, HIS and ARG side chains containing amino, imidazolyl, and guanidine groups can be protonated under acidic conditions to form micelles (Xie et al., 2021) as observed in Figs. 6.2i1-j3, which are comparable to the morphological characteristics of the PIs of W_{35}-M (Figs. 6.2g1-h3). Amongst the essential amino acids, LEU was abundant followed by PHE and THR in the PIs of W_{35}-D. In comparison to the literature (Ayala-Rodriguez et al., 2022; Multari et al., 2015), amino acid levels of LT and HT faba bean proteins were similar or relatively higher.

Amino acid composition is an essential factor in evaluating the nutritional quality of a dietary protein source. Adequate quantities of LYS, MET and TRY are considered necessary in food that is of high nutritional value for human consumption. The IVPD values were not significantly ($P > 0.05$) different between the cultivars and the protein extraction method (Table 6.5). However, the IVPDCAAS values were higher for the PIs of W_{35}-D followed by W_{35}-M and AA. The IVPD values were the lowest and the trypsin inhibitory activity levels were the highest for the PCs of S_{23}-D of LT (78.15 ± 0.51% and 1.32 ± 0.05 TUI/mg N) and HT (79.30 ± 0.73% and 0.70 ± 0.10 TUI/mg N) faba bean cultivars. As higher trypsin inhibitors have the potential to reduce protein digestibility, lower trypsin inhibitory activity levels are desirable. However, the trypsin inhibitory activity levels of the PCs of S_{23}-D (LT: 0.16 TUI/mg dw and HT: 0.09 TUI/mg dw) were still significantly lower than that of literature results of faba bean

flour (4.47 ± 0.21, 5.45 ±1.11 and 5.96 ± 0.27 TUI/ mg dw) reported by Alsonso et al. (2000), Millar et al. (2019) and Shi et al. (2017), respectively, confirming the effectiveness of wet fractionation on trypsin inhibitory activity reduction. In contrast to the faba bean flour trypsin inhibitory activity, soybeans (58 and 63 TUI/mg dw) and chickpeas (28 and 29 TUI/mg dw) were significantly higher in trypsin inhibitory activity (Pisulewska & Pisulewski, 2000). Faba bean trypsin inhibitory activity levels were comparable to that of peas (4.2 and 4.5 TUI/mg dw) and lentils (3.4 TUI/mg dw) reported by Pisulewska and Pisulewski (2000).

Although trypsin inhibitors are proteins and concentrating or isolating proteins can also increase trypsin inhibitory activity, their levels in the PC/PI fractions obtained in the present study were lower than the levels (0.43 ± 0.11 TUI/mg dw) reported in faba bean flours subjected to extrusion, which has been claimed to be effective not only in reducing the activity of trypsin inhibitors but also chymotrypsin and α-amylase inhibitors (Alonso et al., 2000). It is important to note that the trypsin inhibitory activity levels shown in Table 6.5 are presented in TUI/mg N dw, which are 6 to 8-fold higher than TUI/mg dw values. In the present study, trypsin inhibitory activity was not detected in the PCs of AA probably due to some level of denaturation and inactivation of trypsin inhibitors under extreme pH extraction conditions. Similarly, hydrothermal treatment of presoaked faba beans was found to be 100% effective in eliminating trypsin inhibitory activity (Shi et al., 2017). However, the proposed mild wet fractionation methods were not 100% effective in eliminating trypsin inhibitory activity. The proposed wet fractionation conditions could have had minimal impact on the native protein secondary structure conformation and functionality, as opposed to the AA method. The faba bean trypsin inhibitors have been previously reported to have a MW of 18 kDa (Gupta et al., 2000), which must have been retained during the dialysis step of the W_{35}-D method. The β-legumin band

appearing at ~18 kDa (Gupta et al., 2000) in Fig. 6.4 must be overlapping with the bands corresponding to trypsin inhibitors with a similar MW.

According to Multari et al. (2015), the isolation of proteins minimises notable levels of anti-nutrients such as phytic acid, tannins and vicine and convicine. Accordingly, the total tannin contents of HT whole bean flour (10.782 ± 0.725 mg TAE/g) were reduced by 5-fold by dehulling (Chapter 4, Table 4.2) whereas a further 2 to 3-fold reduction was achieved by the isolation of the proteins. Therefore, the isolation of HT faba bean proteins opens new dimensions for the utilisation of the underutilised high tannin cultivars in food applications.

Although LT is a low-tannin cultivar, the total tannin contents have been reduced by 2-fold by dehulling except for PIs of LT and HT W_{35}-D and PC of HT-AA (Table 6.5). The protein secondary conformational shifts of the HT faba bean PI due to the removal of tannins have been previously confirmed (Chapter 4, Figure 4.1). Although tannins are frequently considered anti-nutritional factors, they can also reduce the risk of many diseases due to their health-beneficial properties (Multari et al., 2015). The sucrose, raffinose, stachyose and verbascose contents of LT and HT dehulled faba bean flours were 23.69 ± 3.84 and 29.63 ± 5.37, 1.36 ± 0.11 and 1.66 ± 0.05, 10.16 ± 0.73 and 12.37 ± 0.33, and 17.52 ± 2.03 and 16.51 ± 1.10 mg/g sample dw, respectively (Chapter 4, Table 4.2). These values were reduced in the corresponding protein fractions, in particular, the levels of stachyose and verbascose (Table 6.5). Therefore, the isolation of faba bean proteins was efficient not only in reducing the levels of total tannins but also the levels of raffinose family oligosaccharides.

6.4. CONCLUSIONS

Faba bean proteins mainly consisted of salt-soluble globulins according to Osborne's protein solubility-based classification regardless of the cultivar. However, protein extraction using salt solutions resulted in significantly lower protein contents and recoveries as compared to the other fractionation approaches. Instead of using high S/F ratios of 10 and 20 often adopted by the industry for wet fractionation using alkali extraction, S/F ratios of 2 and 3 used in this study showed high protein solubility in water at 35 °C as opposed to using 0.5%, 0.75% or 1% salt.

The presence of minerals in relatively high concentrations could have facilitated the salting-in effect of the proteins and releasing of the faba bean proteins from the flour matrix. At the same time, using a low S/F ratio in the WS and S methods led to the salting-out effect. The optimised W_{35}-D method proposed in the present study generated protein isolates with 28% yield with a purity of 93% (LT) to 96% (HT) and 78% protein recovery, which is substantially higher than those obtained by the conventional pH-shift method and $NaCl_{(aq)}$ extraction methods. The proposed method also yielded starch isolate (94-98%) and dietary fibre concentrate (40%) with 89 to 90% of the dietary fibre recovered as value-added co-products. The advantages of the mild fractionation conditions employed are obvious in reducing the water, energy and chemical consumption, as well as a reduction of trypsin inhibitory activity, raffinose family oligosaccharides and total tannins. The AAS of the PIs of W_{35}-D were higher as the limiting sulphur-containing amino acids-rich albumins were retained along with the globulins. Although large-scale extraction of this method was carried out with few modifications, further work is warranted to scale up this process for potential industrial food applications.

At present, the technologies used by the plant protein industries are highly cost-intensive due to the extensive use of water and chemicals, subsequent effluent water treatment and recycling and dialysis as well as spray drying of the extracted final protein slurry to capture the finished product in a dry form with minimal sodium levels. This seriously concerns the industry for its long-term sustainability. Therefore, multiple efforts to adjust/revise the processing approaches are being investigated by the industry in order to improve cost efficiency and environmental sustainability. It is believed that the outcome of the present study will be useful and can guide the plant protein refining industry in its technological innovation and product improvement efforts.

CHAPTER 7: Impact of mild extraction of faba bean (*Vicia faba* L.) proteins against conventional methods on their secondary structure and physicochemical characteristics[6]

[6]A version of this chapter will be published: Jeganathan, B., Vasanthan, T., & Temelli, F. Impact of mild extraction of faba bean (*Vicia faba* L.) proteins against conventional methods on their secondary structure and physicochemical characteristics.

7.1. INTRODUCTION

There is renewed interest in the cultivation and utilization of faba beans (*Vicia faba* L.) as an alternative protein source to fully exploited soybeans (Multari et al., 2015). The isolation of protein fractions from faba beans could enhance their utilisation as functional food ingredients, through the removal of components that diminish their food value (Landry et al., 2016; Multari et al., 2015). The ability of the proteins to bind to both water and fat, foam, emulsify or gel are some of the techno-functional qualities that can be viewed as the expressions of the three molecular aspects of proteins: (1) size and shape-related hydrodynamic/rheological properties, (2) hydration properties, and (3) protein surface activity-related properties (Damodaran, 2017; Day, 2013; Shevkani et al., 2019).

These properties of plant proteins can be impacted by the alterations in protein structure (primary, secondary and tertiary) brought on by the environment the protein is exposed to during processing (Damodaran, 2017; Day, 2013; Shevkani et al., 2019). Despite their high nutritional value, growing worldwide interest, and potential to be explored as a functional food ingredient, faba bean protein utilization as a techno-functional ingredient in plant-based foods is relatively limited in the food industry (Boye et al., 2010). Faba bean proteins have been subjected to laboratory research as a culinary ingredient and proven to have both promising (Karaca et al., 2011; Langton et al., 2020; Shi & Nickerson, 2022) and challenging techno-functional properties (Nivala et al., 2017, 2021), depending on the processing conditions.

The flour particle size is a decisive measure of the final product quality (Ahmed et al., 2016), which is positively correlated ($r = 0.9$, $P < 0.0001$) to the protein and dietary fibre contents but negatively correlated to the starch contents (Chapter 5, Fig. 5.5). However, the correlation between particle size and inherent physicochemical characteristics of grain flour

remains unclear. On the other hand, the thermal properties of faba bean proteins vary with the moisture content (Ricci et al., 2018), thus investigating the thermal behaviour of proteins at both low and high moisture contents for extrusion or thermally processed food applications is pivotal.

The electrophoretic polypeptide bands of faba bean proteins were mainly composed of globulins (74-76%) and albumins (22-23%) as reported in Chapter 6 (Section 6.3.2) as well as in the literature (Multari et al., 2015). The abundance of legumin (11S) as compared to vicilin (7S) is reported to have an impact on their physicochemical properties (Shevkani et al., 2019). These two protein types generally differ in size, structure, and amino acid composition, consequently enabling them to show distinct functional attributes. Legumins are less soluble in aqueous salt solutions ($salt_{(aq)}$) compared to vicilins (Osborne, 1924) due to their increased structural constraints resulting from their disulphide bonds, which restrict interfacial activities (Shi & Nickerson, 2022).

Further utilization of Canadian faba beans in food applications requires an in-depth understanding of the impact of different wet fractionation methods on the physiochemical attributes as they can ultimately influence the functional properties. Like any other legume proteins, faba bean proteins are conventionally extracted by the pH-shift method. Protein denaturation under alkali extraction followed by acid precipitation can lower the quality and utility of the protein components (Jafari et al., 2016). This study was designed to provide insight into the physicochemical attributes of the protein concentrates/protein isolates (PCs/PIs) resulting from mild fractionation methods explored previously (Chapter 6): water extraction at 35 ºC followed by dialysis at 4 °C (W_{35}-D); water extraction at 35 ºC followed by micellization (W_{35}-M); $NaCl_{(aq)}$ solution (1% (w/v)) extraction at 23 ºC followed by

dialysis at 4 °C (S_{23}-D) in comparison to the conventional alkali extraction followed by acid precipitation (AA).

Based on the above, the objective of the present study was to characterise faba bean PCs/PIs resulting from mild extraction in comparison to the conventional pH-shift method. As the cultivar and environmental factors also play a crucial role in the composition and structure of the proteins and their interactions with other macromolecules (Day, 2013; Multari et al., 2015), both low-tannin Snowbird (LT) and high-tannin, Athena (HT) cultivars were selected for this study. This study was designed to provide insight into the protein secondary structural conformational changes using Fourier transform infrared spectroscopy (FTIR) and circular dichroism (CD); thermal property changes using differential scanning calorimetry (DSC); particle size distribution by laser light diffraction principle; and X-ray diffraction (XRD) patterns of LT and HT W_{35}-D-PIs, W_{35}-M-PIs, S_{23}-D-PCs; and AA-PCs.

7.2. MATERIALS AND METHODS

7.2.1. Materials

The faba bean fractions W_{35}-D-PIs, W_{35}-M-PIs, S_{23}-D-PCs; and AA-PCs were obtained from LT and HT as described in Chapter 6 (Section 6.2.3). Bovine serum albumin (BSA) standard solution ampules of 2 mg/mL concentration were purchased from Thermo Scientific (Rockford, IL, USA). The rest of the chemicals were acquired from Sigma-Aldrich Canada Co. (Saint Louis, MO, USA).

7.2.2. Characterisation of physicochemical properties

7.2.2.1. Particle size distribution

The particle size distribution (PSD) of faba bean PC/PI dispersions was measured using a Malvern static laser light diffraction unit (Mastersizer 3000, Malvern Instruments Ltd, Worcestershire, England, UK), covering a size range of 0.01–3000 μm. Protein samples of 1% (w/v) concentration were dispersed in ultrapure water and shaken in a hand shaker (Burrell Wrist Action Shaker, 75–765 BT, Pittsburgh, PA, USA) at a setting of 10 for 2 h and left overnight at 4 °C. The particle refractive index used was 1.33 and the absorptive index was 0.1. Protein dispersions equilibrated to 23 °C were introduced into the dispersing unit using ultrapure water as the dispersant until a laser obscuration of 12% was achieved. Particle size was defined in terms of volume percentiles: 10^{th} (Dv[10]), median (Dv[50]) and 90^{th} percentile (Dv[90]), volume-weighted mean particle diameter (D[4,3]), and surface-area weighted mean particle diameter (D[3,2]).

7.2.2.2. Protein secondary structural changes: Fourier transform infrared spectroscopy

Attenuated total reflectance-Fourier transform infrared (ATR-FTIR) spectra of the wet fractionated protein isolates were evaluated following the procedure described in Chapter 4 (Section 4.2.7). The amide II region peaks were also baseline corrected and integrated for the peak intensities under the Gadgets tab in OriginPro 9.9.5.167 Learning Edition (Origin Lab Corporation, Northampton, MA, USA).

7.2.2.3. Protein secondary structural changes: Circular Dichroism

The protein secondary structural motifs of faba bean LT and HT W_{35}-D-PIs and AA-PCs were investigated by Circular Dichroism (CD) spectropolarimeter (DSM 17 CD OLIS Inc., Athens, GA, USA) located at the Gunning/Lemieux Chemistry Centre, University of Alberta. Faba bean protein clear solutions diluted to 1 mg/mL in 20 mmol/L sodium phosphate buffer (pH 7.2) were used for the analysis. The protein contents of the LT and HT W_{35}-D-PIs and AA-PCs were measured according to the micro-burette procedure described by Greenfield (2007). The CD spectra were recorded at far-ultraviolet (far-UV) regions as a function of wavelength (185-260 nm) at 25 °C using a quartz cuvette with a 1 mm path length. All the CD spectra were obtained as the average of three consecutive scans with automatic subtraction of respective buffer spectra (Greenfield, 2007).

Based on the thorough description of the principles and equations used for the interpretation of CD data by Greenfield (2007) and Kelly et al. (2005), the data obtained directly from the CD instrument is the difference in absorption between the left-handed and the right-handed polarized light (ΔA_λ) as a function of wavelength (λ), which can be expressed as:

$$\Delta A_\lambda = [\Delta \varepsilon_\lambda] \times l \times c \quad (7.1)$$

where, $\Delta\varepsilon_\lambda$ is the difference in molar absorptivity for oppositely polarized light expressed in $(mol/L)^{-1}\ cm^{-1}$ as the absorption is dimensionless, l is the path length through the cell (cm) and c is the molar concentration of the sample (mol/L). CD data reported as ellipticity ($[\theta]_\lambda$) at λ in degrees is related to the absorbance by a factor of 32.98, which suggests that the measured ellipticity should obey Beer−Lambert's law.

$$[\theta]_\lambda = 32.98\ \Delta A_\lambda \quad (7.2)$$

The mean residue ellipticity at wavelength λ ($[\theta]_{mw,\lambda}$) in units of deg cm^2 dmol^{-1} is obtained from Eq. (7.3).

$$[\theta]_{mrw,\ \lambda} = \frac{\text{mrw} \times [\theta]_{\lambda}}{10 \times l \times c} \tag{7.3}$$

where mrw is the mean residual weight (average molecular weight) used in place of the molecular weight. The following equation can be used to calculate mrw,

$$mrw = \frac{\text{M}}{(n-1)} \tag{7.4}$$

where M is essentially the molecular mass of the polypeptide chain in g/mol divided by the number of backbone amides (n-1) with n being the number of amino acids in the chain. The mrw is 110 ± 5 g mol^{-1} for most proteins (Kelly et al., 2005), which was used in this study similar to soy proteins (Jiang, Wang et al., 2015). The molar ellipticity at wavelength λ ($[\theta]_{molar,\lambda}$) in units of deg cm^2 dmol^{-1} corrected for concentration is determined using Eq. (7.5):

$$[\theta]_{molar,\ \lambda} = \frac{100 \times \theta_{\lambda}}{m \times d} \tag{7.5}$$

where, m is the molar concentration of the solute. Alternatively, conversion from molar extinction to molar ellipticity uses a factor of 3298 (Eq. (7.6)).

$$[\theta]_{molar,\ \lambda} = 3298 \Delta\varepsilon_{\lambda} \tag{7.6}$$

The relative contents of protein secondary structures were estimated by deconvoluting the CD spectra into secondary structure fractions using the SELCON3, CONTIN/LL, and CDSSTR algorithms in CDPro Software by referring to CD spectra of protein sets with known secondary structures (Sreerama & Woody, 2009). The regular α-helix, distorted α-helix (ends), regular β-sheet, distorted β-sheet, β-turns, and unordered (other) were the six secondary structures estimated. For simplicity, the total percentages of both α-helix and β-sheet were obtained by adding the corresponding regular and distorted fractions, i.e. β-sheet total = β-sheet regular + β-sheet ends (Greenfield, 2007; Sreerama & Woody, 2009).

7.2.2.4. X-ray diffraction pattern

X-ray diffraction (XRD) patterns of the faba bean PCs/PIs were measured using a Rigaku, Ultima IV X-ray diffractometer (Akishima-shi, Tokyo, Japan) using a Co-Kα X-ray tube with a D/Tex Ultra with Fe filter counter operating at 38 kV and 38 mA. Faba bean PCs/PIs were mounted onto zero background plates without the use of ethanol or water. Diffractograms were taken between 5° and 60° 2θ range at a scanning rate of 1.20°/min with the step size maintained at 0.0500° and divergence slit width at 0.67° while the focusing geometry was on Bragg Brentano mode (Saini et al., 2018; Vilas Dhumal et al., 2019).

Data were converted using JADE MDI 9.6 software (Newton Square, PA, USA) and phase identification was carried out using DIFFRAC.EVA software (V6) with the 2021 ICDD PDF 4+ and PDF 4+/Organics databases (International Centre for Diffraction Data, Newtown Square, PA, USA). With reference to these interpretations, the data were plotted in OriginPro 2022b 9.9.5.167 learning edition software (Origin Lab Corporation, Northampton, MA, USA). A Gaussian function was used for multi-curve peak fitting. Interplanar spacing also known as d-spacing is the distance between two consecutive planes and was determined using Bragg's equation (Eq. (7.7)) where n is the integer defining the order of diffraction (1), λ is the wavelength of X-ray radiation (0.1542) used in this study, d is the interlayer spacing between diffraction lattice, and θ is the measured diffraction angle (Bragg & Bragg, 1913).

$$n\,\lambda = 2d\,Sin\,\theta \tag{7.7}$$

7.2.2.5. Differential Scanning Calorimetry (DSC)

LT and HT PCs/PIs were conditioned to reach 12% moisture content in airtight containers at room temperature (23 ºC) in a 50% relative humidity chamber maintained with a

saturated solution of calcium nitrate tetrahydrate (Ricci et al., 2018). Sample weights were checked daily until 120 g/kg moisture content was achieved. Approximately 3-4 mg of the samples were hermetically sealed in aluminium DSC pans (Tzero Hermetic Pan, TA Instruments Inc., Hayesville, NC, USA) and calorimetric measurements were performed against an empty sealed pan as the reference in a differential scanning calorimeter (Q100, TA Instruments Inc., New Castle, DE, USA) based on the methods previously described by Perez et al. (2016) and Ricci et al. (2018) with few modifications.

The scanning temperature ranged from 30 to 240 °C during the first heating cycle at a 10 °C/min heating ramp and was isothermal for 5 min. Samples were cooled to -10 °C at 10 °C/min and the second heating was performed to 240 °C at 5 °C/min. Both LT and HT W_{35}-D-PIs were also conditioned in stainless steel pans (PerkinElmer Inc., Shelton, CT, USA) with excess water (75%) and equilibrated for 16 h (Nivala et al., 2017) to compare the thermal properties of high-moisture samples against low-moisture samples. The thermal transitions of proteins in terms of denaturation temperature (T_d, ˚C) and enthalpy of transition (ΔH, J/g) of dry protein in the first heating cycle and glass transition temperature (T_g, ˚C) at the inflection point in the second heating cycle for the low-moisture samples were derived from the thermograms using Universal Analysis 2000 Software 4.5A Version (TA Instruments Inc., New Castle, DE, USA).

7.2.3. Statistical Analysis

All the results expressed as mean ± SD on a dry weight (dw) basis were based on the analyses of three independent replicate samples (n = 3) unless otherwise specified. Significant differences ($P < 0.05$) between samples were tested by two-way analysis of variance (ANOVA)

followed by mean separation using Duncan's multiple range test. Additionally, Pearson's correlation analysis was performed ($P < 0.05$) to understand the correlation between the protein purity and corresponding glass transition temperature. Additionally, Pearson's correlation analysis was performed ($P < 0.05$) to understand the correlation between the protein purity and corresponding glass transition temperature and endothermic enthalpy change at denaturation temperature. All these statistical analyses were carried out using SAS Studio University Version for Windows (SAS Institute Inc., Cary, NC, USA).

7.3. RESULTS AND DISCUSSION

7.3.1. Particle size distribution of faba bean proteins

The volume-weighted particle size distribution trends presented in Fig. 7.1 prove that the wet fractionation method is a more significant determinant of the PSD pattern as compared to the cultivar. Both the W_{35}-D-PIs and S_{23}-D-PCs showed trimodal distribution, while W_{35}-M-PIs resulted in a bimodal distribution pattern. However, the AA-PCs exhibited monomodal distribution plausibly due to the alkali extraction and acid precipitation conditions leading to the unfolding of the protein structures and aggregation into larger particles of uniform size. The precipitation and lyophilization steps may facilitate the formation of large particles. Langton et al. (2020) also observed the large aggregates forming in the AA method as compared to the salt extraction method. This difference in particle size in suspensions could be attributed to the differences in the extraction process. Langton et al. (2020) also observed the large aggregates forming in the AA method as compared to the salt extraction method. This difference in particle size in suspensions could be attributed to the differences in the extraction process.

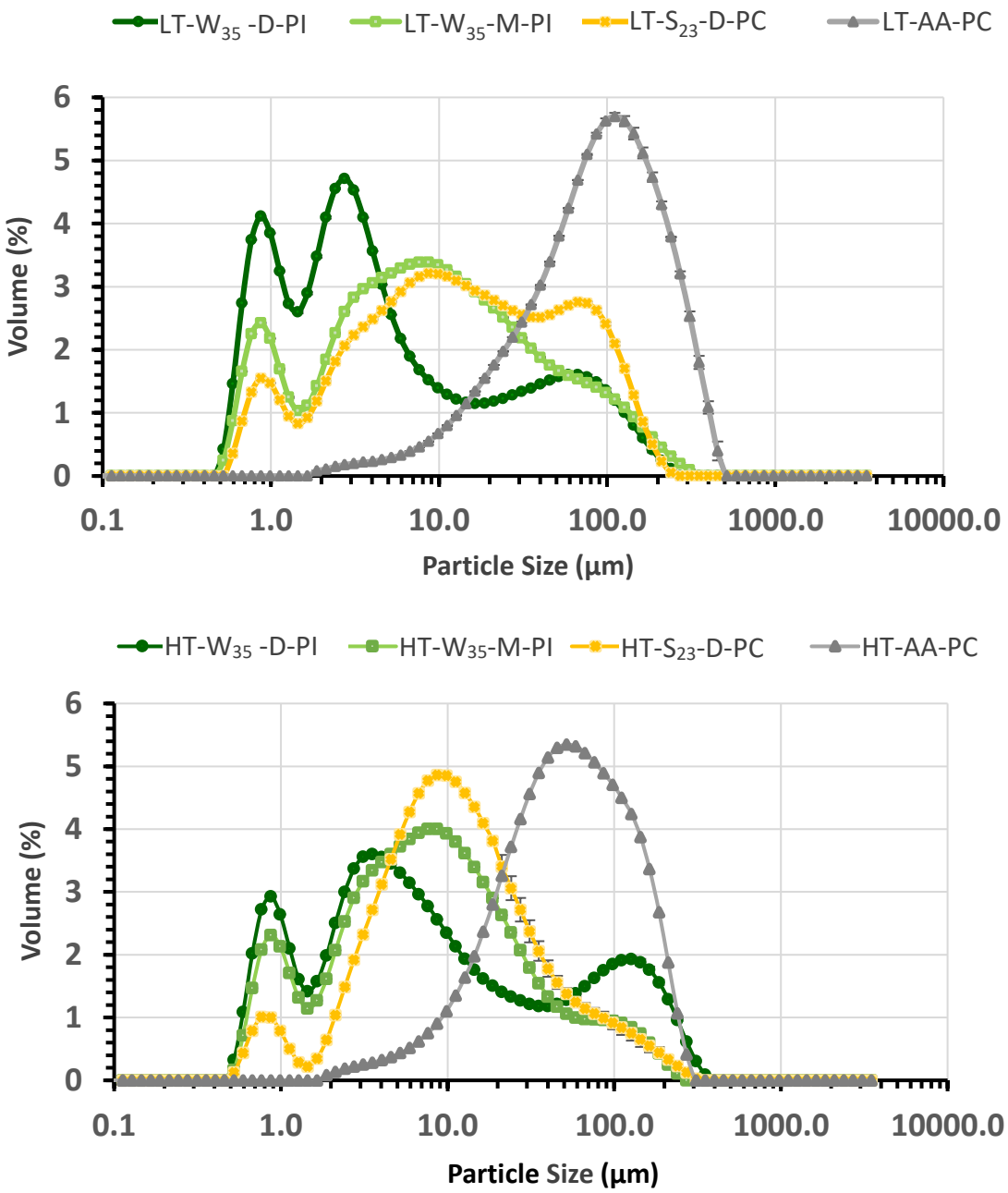


Fig. 7.1. Volume-weighted particle size distribution of 1% (w/v) faba bean protein concentrates /protein isolates (PCs/PIs)-water suspensions of cultivars Snowbird (LT) and Athena (HT).

Mean (n = 3) ± SD. W_{35}: water extraction at 35 °C; S_{23}: 1% $NaCl_{(aq)}$ solution extraction at 23 °C; M: micellization; D: dialysis at 4 °C; and AA: alkali extraction followed by acid precipitation.

Lentil PIs extracted by isoelectric precipitation also resulted in larger particles as compared to the ultrafiltered PIs (Alonso-Miravalles et al., 2019). According to Table 7.1, the volume-weighted diameters (D[4,3]) of LT (123 μm) and HT (76 μm) AA-PCs were significantly ($P < 0.05$) larger than the other PCs/PIs evaluated as well as the lentil-PIs reported by (Alonso-Miravalles et al., 2019). Similarly, the surface-area weighted diameter (D[3,2]) was also significantly ($P < 0.05$) larger for both LT (44 μm) and HT (32 μm) AA-PCs. Additionally, more than 90% of the dispersed particles were smaller than 268 μm for LT and 167 μm for HT AA-PCs. These large particles could be resulting from the aggregation of the denatured proteins that probably interacted with each other. Starch granules in the range of 12-33 μm could be correlating to the Dv[10] values of AA-PCs and Dv[50] values of the S_{23}-D-PCs, which contain nearly 5.29-7.34% starch content as reported previously (Chapter 6, Table 6.2).

The denatured protein aggregates may adhere to the surface of starch granules present in the PCs, similar to the findings of Jiang et al. (2016) resulting from the microwave-heating of faba bean flours. A substantial portion of the S_{23}-D-PC particles were larger (Dv[90]): 94 μm for LT and 58 μm for HT) and only 10% of the particles were smaller than 2 and 3 μm, respectively, which were comparable to those of the LT and HT PIs. The overall particle size (Dv[90]), was larger for LT, except for W_{35}-D-PI for which HT is larger. Emulsification capacity and stability as well as the textural and rheological characteristics of proteins are dependent upon their particle size distribution (McClements, 2015a; Tabilo-Minizaga et., 2019). Further work is warranted to establish the correlation between the particle size distribution of proteins and their corresponding functional characteristics.

Although the tannins are significantly reduced during the extraction process (Chapter 6, Table 6.5), the different levels of residual tannins may be affecting the interactions between proteins and having an impact on the particle size. Almost 50% of the W_{35}-D-PIs were either smaller or larger than 4 µm (LT) and 7 µm (HT), which were substantially smaller than the Dv[50] values of the rest. These data suggest that W_{35}-D-PIs have a more dispersed PSD followed by W_{35}-M-PIs and S_{23}-D-PCs; thus, the PIs can be classified as small and easily dispersible particles, yet less uniform as compared to the AA-PCs. It is noteworthy that the mild extraction techniques did not have an impact on the particle size in protein-water suspension, as shown by particle size distribution analysis.

Table 7.1. Particle diameters of 1% water suspension of Snowbird (LT) and Athena (HT) faba bean PCs/PIs obtained from four different wet fractionation methods.

Sample	Dv[10] (µm)	Dv[50] (µm)	Dv[90] (µm)	D[3,2] (µm)	D[4,3] (µm)
LT-W_{35}-D-PI	0.92 ± 0.00^{c}	3.64 ± 0.02^{d}	69.03 ± 0.23^{d}	2.45 ± 0.01^{d}	20.10 ± 0.10^{d}
LT-W_{35}-M-PI	1.16 ± 0.01^{bc}	9.33 ± 0.08^{c}	75.67 ± 0.49^{c}	3.88 ± 0.02^{c}	26.43 ± 0.15^{c}
LT-S_{23}-D-PC	1.98 ± 0.01^{b}	14.27 ± 0.12^{b}	94.07 ± 1.01^{b}	5.38 ± 0.02^{b}	32.83 ± 0.40^{b}
LT-AA-PC	21.37 ± 0.06^{a}	96.23 ± 0.31^{a}	268.00 ± 1.00^{a}	44.03 ± 0.06^{a}	123.00 ± 0.00^{a}
HT-W_{35}-D-PI	1.03 ± 0.01^{c}	6.75 ± 0.04^{d}	130.0 ± 1.73^{b}	3.31 ± 0.01^{c}	36.60 ± 0.36^{b}
HT-W_{35}-M-PI	1.22 ± 0.01^{c}	8.00 ± 0.03^{c}	50.53 ± 1.25^{c}	3.81 ± 0.01^{c}	20.10 ± 0.26^{c}
HT-S_{23}-D-PC	3.00 ± 0.03^{b}	11.37 ± 0.15^{b}	57.70 ± 3.00^{c}	5.99 ± 0.06^{b}	24.33 ± 1.17^{d}
HT-AA-PC	15.93 ± 0.12^{a}	57.53 ± 0.81^{a}	166.67 ± 2.31^{a}	32.33 ± 0.29^{a}	76.07 ± 1.10^{a}

[a-d]Mean ± SD (n=3) values for a particular cultivar followed by different letters within the column are significantly different ($P < 0.05$), according to Duncan's multiple range test

Dv(10): 10th volume percentile; Dv(50): 50th volume percentile; Dv(90): 90th volume percentile; D[3,2]: surface-area weighted mean particle diameter; and D[4,3]: volume-weighted mean particle diameter. Dv(10) is the size in microns below which 10% of the sample is represented. Dv(50) is the micron size at which 50% of the sample is either smaller or larger. Dv(90) is the size of the particle below which 90% of the sample fits

Faba bean protein concentrates/isolates (PCs/PIs) of cultivars Snowbird (LT) and Athena (HT) treated with water at 35 °C followed by either dialysis at 4 °C (W_{35}-D) or micellization (W_{35}-M); or treated with 1% $NaCl_{(aq)}$ solution followed by dialysis at 4 °C (S_{23}-D); or alkali extracted, and acid precipitated (AA).

7.3.2. Protein secondary structural changes: fourier transform infrared spectroscopy

Conformational changes of faba bean protein secondary structures or any modifications of the functional groups attached to the backbone of the proteins can be assessed based on the FTIR spectral characteristics of the fingerprint region of the PIs extracted using different methods. The main FTIR spectral characteristics of both LT (Fig. 7.2a) and HT (Fig. 7.2b) faba bean PI samples were comparable to those of the dehulled bean flours and hybrid fractionated PIs discussed in Chapters 4 and 5 (Sections 4.3.3.2 and 5.3.4.2) as well as the findings of Johnson et al. (2020) and Wang et al. (2013).

The strong band at ~3275 cm^{-1} can be attributed to the O-H contraction vibration of water (Lan et al., 2019) and the stretching vibrations of N-H in the characteristic amide A region, which does not depend on the backbone conformation, rather on the strength of the hydrogen bond (Junior et al., 2015; Kong & Yu, 2007); whereas, the bands at ~2926 and ~2956 cm^{-1} correspond to the asymmetric stretching vibrations of C-H in methylene groups (Johnson et al., 2020; Lan et al., 2019).

Amongst the three main bands of the protein fingerprint region, namely amide I, II and III, the C=O stretching vibration of the peptide linkages appearing at ~1634 cm^{-1} in the amide I region is most widely used to understand the secondary structure backbone conformation. On the other hand, the conformationally sensitive amide II signal at ~1522 cm^{-1}, which is typically attributed to the C-N stretching vibrations and coupling of symmetric N-H in-plane bending vibrations are less straightforward in establishing a correlation with the protein secondary structure (Chen et al., 2013; Johnson et al., 2020).

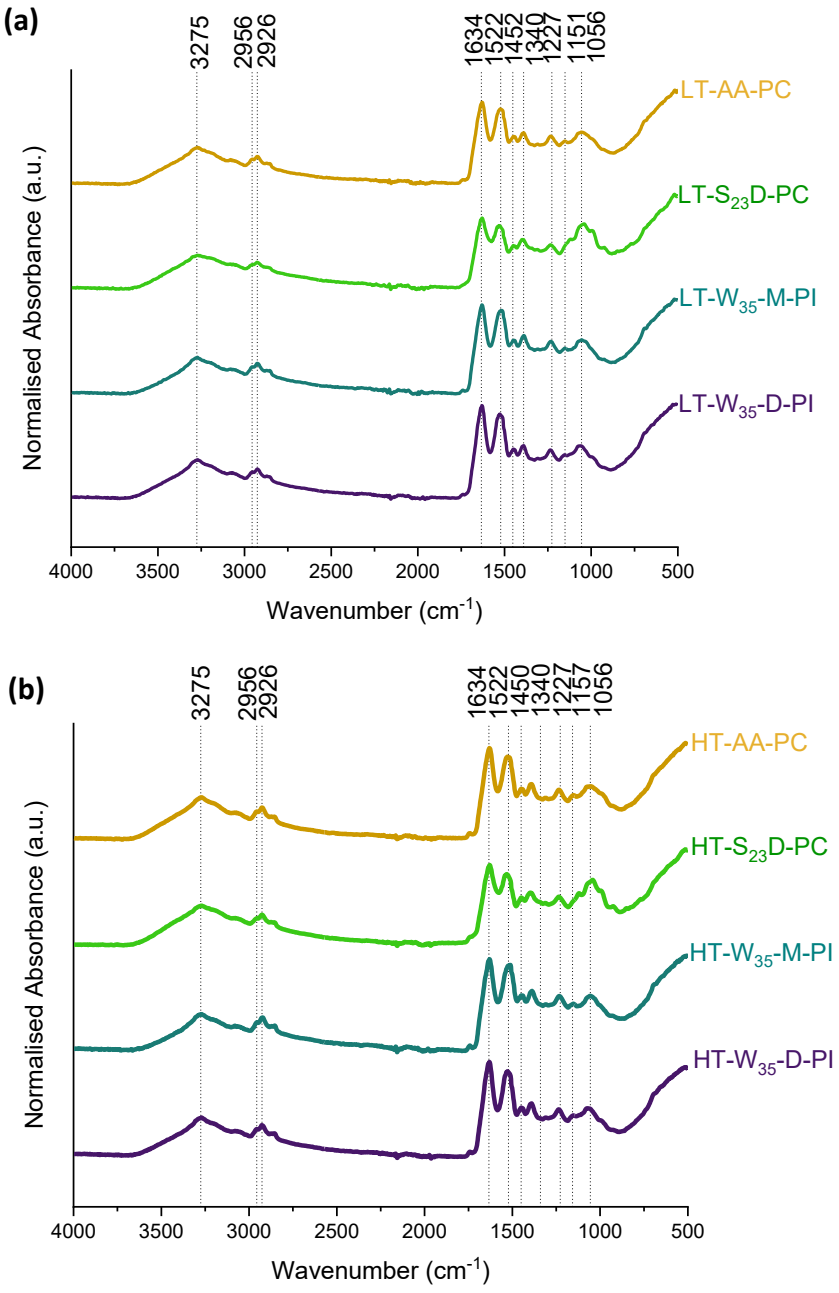


Fig. 7.2. Main FTIR spectral characteristics of wet fractionated faba bean proteins.

Faba bean protein concentrates/isolates (PCs/PIs) of cultivars (a) Snowbird (LT) and (b) Athena (HT) treated with water at 35 °C followed by either dialysis at 4 °C (W_{35}-D) or micellization (W_{35}-M); or treated with 1% $NaCl_{(aq)}$ solution followed by dialysis at 4 °C (S_{23}-D); or alkali extracted, and acid precipitated (AA).

The changes in the amide II band location or the intensity can reflect conformational changes in the protein tertiary structure (Chen et al., 2013). The intensities of the amide II region of W_{35}-D-PIs were 3 folds higher than those for S_{23}-D-PCs and the intensities of both W_{35}-M-PIs and AA-PCs were 2.5 folds higher than those of the corresponding parent flour presented in Chapters 4 and 5 (Figs. 4.6 and 5.7). The amide II peak intensities of S_{23}-D-PCs were similar to those of the parent flour, regardless of the cultivar, confirming that the extraction method is a more significant factor influencing protein tertiary structure as compared to the variations associated with the cultivar. Increased intensities of the amide II bands can be attributed to the gradual unfolding of protein tertiary structures and greater involvement of C-N stretching and N-H bending vibrations, correlating to strong H-bonds. In contrast, lower intensities can relate to groups involved in H-bonds being buried within the hydrophobic protein core and becoming more inaccessible to the solvent (Chen et al., 2013). The SEM images (Fig. 6.2) of S_{23}-D-PCs of both cultivars from presented in Chapter 6 showcase the salt crystal deposits on the protein surface. It is possible that these crystal deposits are restricting the formation of stronger H-bonds as well as the solvent's access to the proteins.

The weak amide III band at ~1227 cm^{-1} can be primarily assigned to the in-plane N-H bending and coupling of the C-N stretching overtone vibrations on the plane as well as weak contributions from C-C stretching and C=O in-plane bending vibrations (Chen et al., 2013). The bands of amide III are strongly dependent on the details of the hydrogen bonding, the nature of the side chains and the field of force. The peaks identified at ~1450 and ~1340 cm^{-1} correspond to the stereochemistry of the bending vibrations of CH_2 and CH_3, respectively. Both the spectral bands at ~1151 and ~1056 cm^{-1} correspond to strong C-OH stretching vibrations of carbohydrates (Johnson et al., 2020; Lan et al., 2019; Wang et al., 2013).

The second derivative FTIR spectra of the amide I region (1600-1700 cm^{-1}) of W_{35}-D-PIs, W_{35}-M-PIs and S_{23}-D-PCs of LT (Fig. 7.3a) and HT (Fig. 7.3b) primarily consisted of 8 and 7 bands, respectively, due to the diminishing of the band at ~1665 cm^{-1} in LT and bands at ~1650 and ~1665 cm^{-1} in HT. However, AA-PCs had 9 bands similar to the parent flours appearing at ~1609 cm^{-1} (vibrations of amino acid side chain residues), ~1620 cm^{-1} (intermolecular β-sheets), ~1628 cm^{-1} (β-sheets), ~1638 cm^{-1} (β-sheets), ~1647 cm^{-1} (α-helix/random coils), ~1657 cm^{-1} (α-helix), ~1665 cm^{-1} (β-turns), ~1675 cm^{-1} (β-sheets) and ~1687 cm^{-1} (β-sheets/turns), comparable to the findings of Kong & Yu (2007) and Yang et al. (2018). Unlike LT, HT dehulled bean flour had the corresponding peaks appearing at 1628, 1638, 1647, 1657, 1665, 1675 and 1687 cm^{-1} shifted to ~1630, 1640, 1650, 1661, 1669, 1681 and 1693 cm^{-1} respectively, which was the case for HT-AA-PC as well. These shifts in the amide I region can originate only from a change in the protein structure, highlighting the potential interactions between tannins and protein secondary structures. However, both LT and HT PIs/PCs resulting from W_{35}-D, W_{35}-M and S_{23}-D shared similar IR spectral characteristics to that of LT dehulled bean flour resulting from the major reduction of tannins during the protein isolation steps. Although the hydroxyl groups of tannins have a high affinity towards proteins, tannin-protein complexes are generally unstable as the hydrogen bonds can continually break and re-form (Rodríguez-Espinosa et al., 2019).

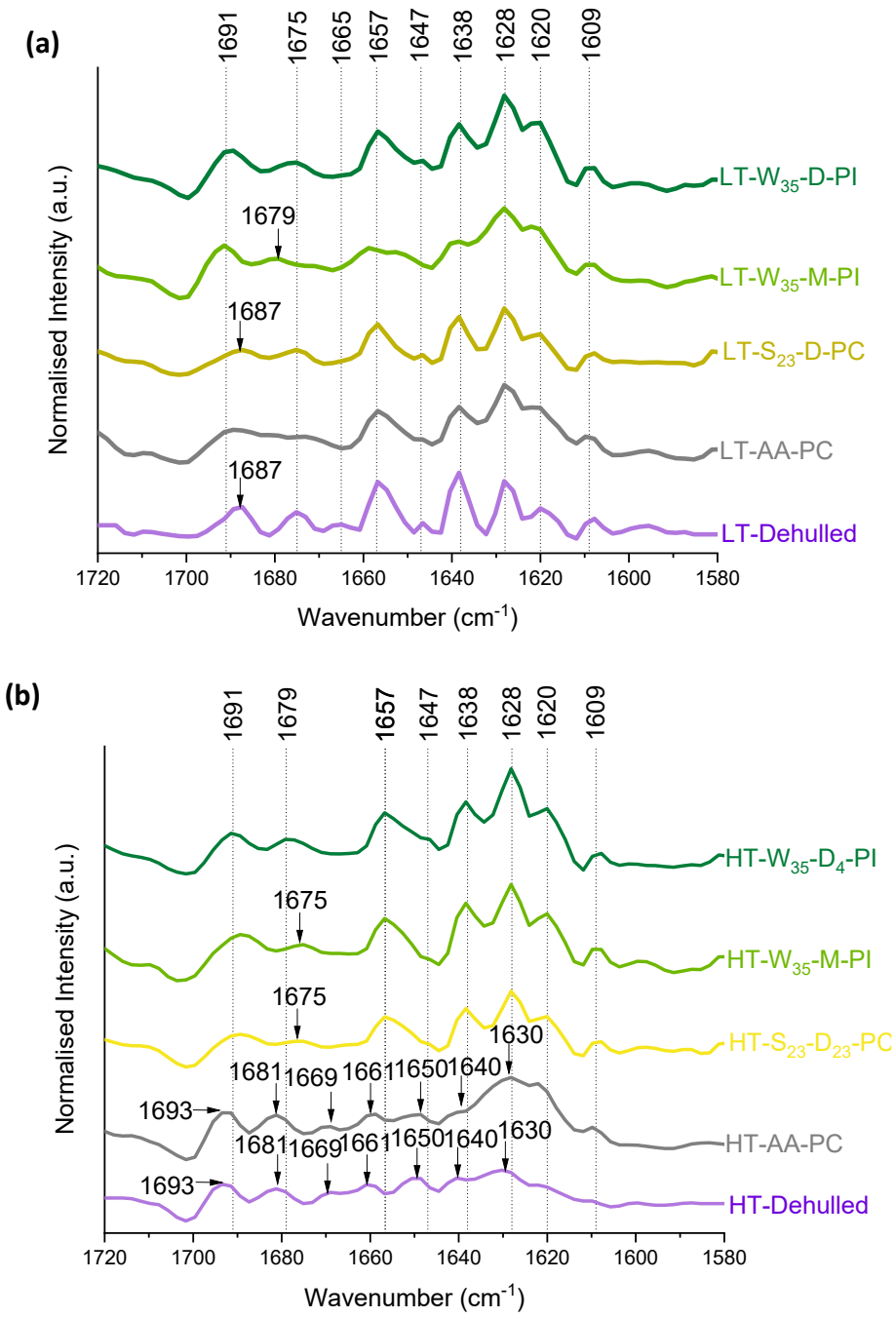


Fig. 7.3. FTIR second derivative spectral peak identification of amide I vibrational region (1600-1700 cm^{-1}) attributed to secondary structures of faba bean proteins.

Faba bean protein concentrates/isolates (PCs/PIs) of cultivars (a) Snowbird (LT) and (b) Athena (HT) treated with water at 35 °C followed by either dialysis at 4 °C (W_{35}-D) or micellization (W_{35}-M); or treated with 1% $NaCl_{(aq)}$ solution followed by dialysis at 4 °C (S_{23}-D); or alkali extracted, and acid precipitated (AA). Spectra for dehulled bean flours (Fig. 4.6) are included here for ease of comparison.

The band assignment discussed above can be altered by some minor or rare structures. It has been reported that the unordered structures generally referred to as random coils assigned to the IR band between 1640 and 1648 cm^{-1} can overlap with the α-helices (Kong & Yu, 2007; Yang et al., 2018). Although the vibrations of amino acid side chains are assigned to the IR band at 1609 cm^{-1} (Yang et al., 2018), approximately 30% of the amino acid residues were reported to be residing in β-turn conformations (Kong & Yu, 2007).

The regions of the polypeptide chain where the chain direction changes are identified as reverse turns, which comprise the widely distributed β-turns, as well as the less prevalent γ-turns and α-turns and may include the well-defined Ω-loops frequently occurring at the surfaces of globular proteins. Thus, turns are primarily composed of hydrophilic residues. However, since β-turns appear at ~1665 cm^{-1} near the characteristic IR band representing 3_{10}-helices, they can become more challenging to identify due to their non-periodic nature and heterogeneity (Marcelino & Gierasch, 2008). Also, the bands appearing from 1695−1690 cm^{-1} have been assigned to β-sheets by many authors, while some assign it to β-turns (Johnson et al., 2020; Kong & Yu, 2007). These complications imply that there is no simple correlation between the IR spectra and secondary structural components and caution needs to be exercised in the interpretation of the IR spectra of proteins.

The overall peak intensities of the amide I region (Figs. 7.4 and 7.5) were decreasing in the following order: W_{35}-M-PI > W_{35}-D-PI > AA-PC > S_{23}-D-PC regardless of the cultivar. The micellization of the proteins leads to the stable arrangement of β-sheet structures, which are amphiphilic in nature with hydrophilic groups exposed to water and hydrophobic moieties hidden in the core of the globular proteins to avoid water (Murray et al., 1979).

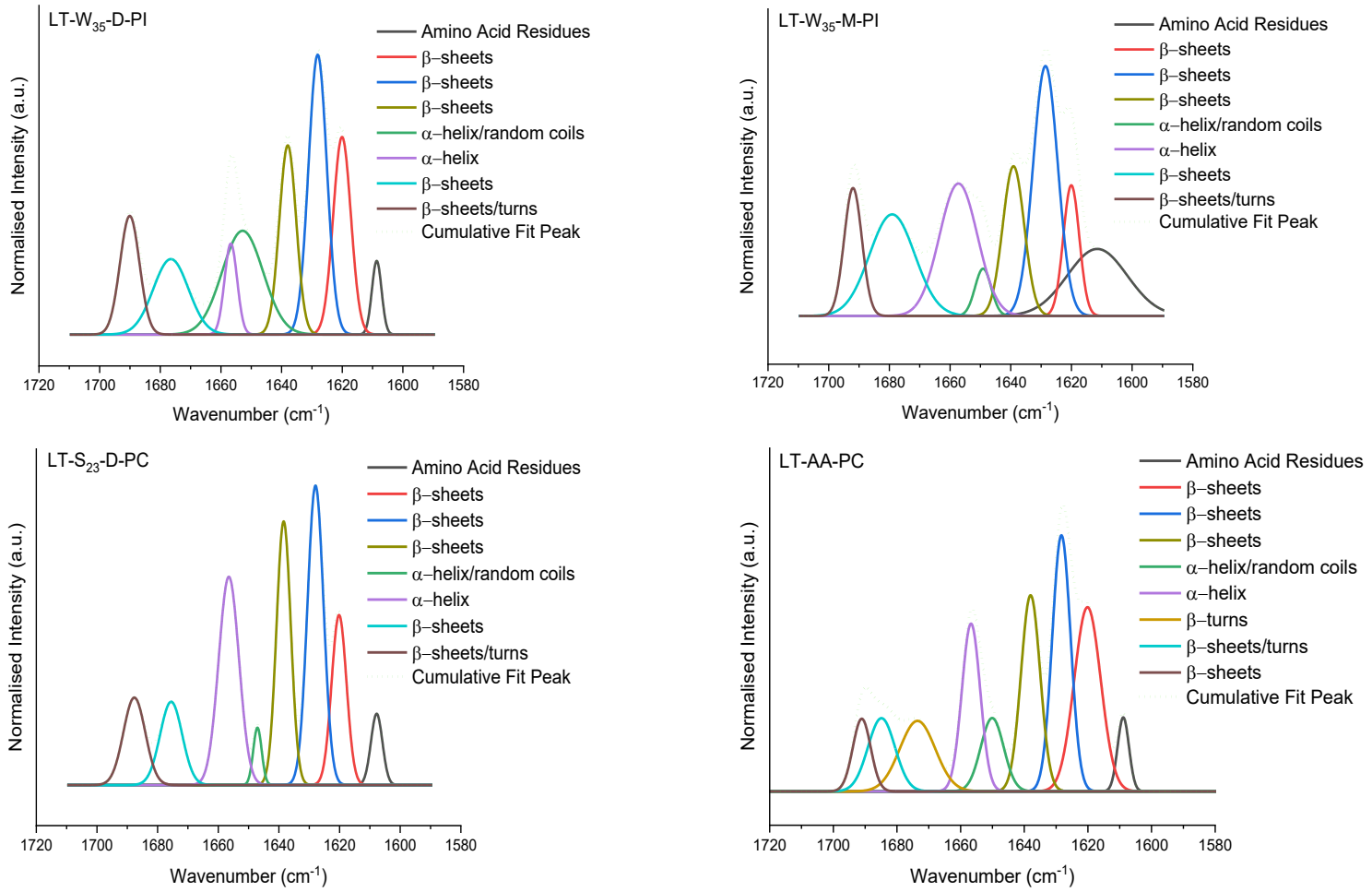


Fig. 7.4. Curve-fitted second derivative peaks of amide I vibrational region (1600-1700 cm^{-1}) attributed to secondary structures of Snowbird (LT) protein concentrates (PCs) and isolates (PIs).

W_{35}: water extraction at 35 °C; S_{23}: 1% $NaCl_{(aq)}$ solution extraction at 23 °C; M: micellization, D: dialysis at 4 °C; and AA: alkali extraction followed by acid precipitation.

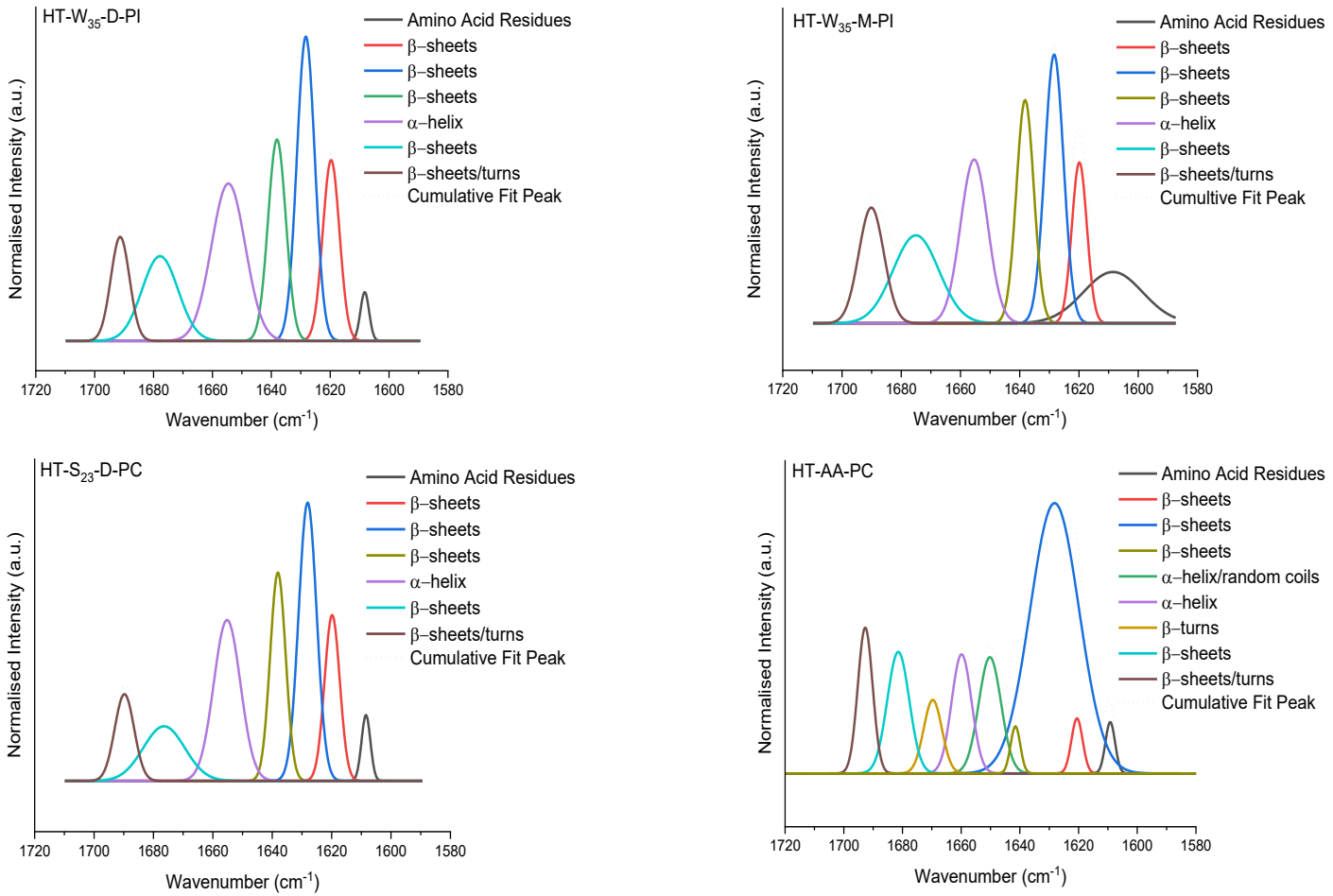


Fig. 7.5. Curve-fitted second derivative peaks of amide I vibrational region (1600-1700 cm^{-1}) attributed to secondary structures of Athena (HT) protein concentrates (PCs) and isolates (PIs).

W_{35}: water extraction at 35 °C; S_{23}: 1% $NaCl_{(aq)}$ solution extraction at 23 °C; M: micellization, D: dialysis at 4 °C; and AA: alkali extraction followed by acid precipitation.

Although most of the hydrophobic residues might be buried in the core of the globular proteins, phenylalanine and tyrosine could remain on the surface due to their bulky nature (Jarpa-Parra et al., 2014). Micellization could take place to some extent in the W_{35}-D-PIs as the samples were dialysed at 4 °C for 48 h. This could be the reason for the overall increase in the peak intensities of β-sheets in W_{35}-D-PIs similar to W_{35}-M-PIs. The highest intensities recorded for peaks at ∼1628 and 1675 cm^{-1} could relate to antiparallel β-sheets as the predominant secondary structures and the β-sheet peak intensities at 1620 cm^{-1} could mean strong protein aggregates at the protein interface similar to the findings of Jarpa-Parra et al. (2014; 2016). Such a spectrum indicated that most of the secondary structures were intact after wet fractionation.

According to the relative proportions of β-sheets expressed in Table 7.2, the W_{35}-M-PIs were similar to their parent flours, while the W_{35}-D-PIs had 66% β-sheets. The relative proportions of all the signals for W_{35}-M and W_{35}-D PIs except for β-turn vibrations at 1665 cm^{-1} for both LT and HT and α-helix vibrations at 1650 cm^{-1} for HT had increased. The increase in the relative peak intensities of the adjacent bands could be overshadowing these bands. Such a spectrum indicates that most of the secondary structures were intact after wet fractionation. A disruption in one to three hydrogen bonds or a few hydrophobic contacts can readily lead to a cooperative conformational shift in proteins since they are flexible and are in a metastable state in their natural state (Damodaran, 2017). According to the amino acid profiles reported in Chapter 6 (Table 6.5), faba beans are rich in polar and hydrophilic amino acids such as glutamic acid, arginine, aspartic acid and lysine, which contribute to favourable hydrophilic interactions with water molecules through hydrogen bonds and electrostatic interactions. The presence of glycine residue close to the asparagine residue enables turn structures by providing supplementary flexibility to the main chain (Jarpa-Parra et al., 2014).

Table 7.2. Content of secondary structures of faba bean proteins by FTIR analysis.

Fractions	Protein secondary structures (%)				
	α-helix	**β-sheet**	**β-turns**	**unordered**	**amino acid residues**
LT Dehulled	~23.24	~57.16	~14.57	~1.38	~3.65
LT-W_{35}-D-PI	~16.39	~66.35	~9.47	~4.68	~3.11
LT-W_{35}-M-PI	~17.88	~57.13	~8.05	~2.71	~14.23
LT-S_{23}-D-PC	~20.77	~63.45	~9.38	~2.38	~4.03
LT-AA-PC	~12.44	~64.09	~13.26	~6.79	~3.41
HT Dehulled	~10.05	~58.16	~15.23	~15.61	~0.96
HT-W_{35}-D-PI	~23.34	~66.24	~8.55	–	~1.87
HT-W_{35}-M-PI	~17.27	~59.65	~11.27	–	~11.80
HT-S_{23}-D-PC	~21.92	~66.52	~8.57	–	~2.99
HT-AA-PC	~9.13	~65.47	~13.49	~10.00	~1.91

Values were calculated based on the following band assignment: ~1609 cm^{-1} for amino acid residues, ~1618 cm^{-1}, ~1628 cm^{-1}, ~1638 cm^{-1}, ~1681 $cm^{-}$and ~1693 cm^{-1} for β-sheets, ~1649 cm^{-1} for random coils, ~1655 cm^{-1} as α-helix and ~1664 cm^{-1} and ~1687 cm^{-1} as β-turns (Kong & Yu, 2007).

Protein concentrates or isolates (PCs/PIs) of Snowbird (LT) and Athena (HT) faba bean cultivars were treated with water at 35 °C followed by either dialysis at 4 °C (W_{35}-D) or micellization (W_{35}-M); or treated with 1% $NaCl_{(aq)}$ solution at 23 °C followed by dialysis at 4 °C (S_{23}-D); or alkali-extracted and acid precipitated (AA).

The substantial drop in peak intensities (Figs. 7.4 and 7.5) of the AA-PCs at 1657 and 1689 cm^{-1} for LT and at 1629 and 1641 cm^{-1} for HT signifies the partial unfolding of the protein secondary structures. This could be associated with the dissociation of hydrogen from carbonyl and sulphate groups and the disruption of hydrogen bonds at alkali extraction conditions that could have increased protein surface charge, leading to partial protein denaturation. The diminishing of highly ordered secondary structures denotes the weakening of hydrogen bond strength and hydrophobic interactions of the peptide groups. The decreased hydrogen bond strength in a partially unfolded peptide group may facilitate the formation of permanently disordered PCs/PIs (Ricci et al., 2018). However, the curve-fitted second derivative spectra of

the amide I region of the AA-PCs (Fig. 7.5) confirmed that the majority of the protein secondary structures remained regardless of the harsh extraction conditions.

The overall peak intensities of the S_{23}-D-PCs were comparable to those of the parent flours except for the diminishing of the peak corresponding to β-turns at 1665 cm^{-1} for both LT and HT and at 1650 cm^{-1} for HT. This is plausibly due to the interference caused by the salt deposits on the protein surface as discussed earlier. According to the protein secondary structure content presented in Table 7.2, the β-sheet content of LT W_{35}-D-PIs (66%) was the highest followed by AA-PCs (64%), S_{23}-D-PCs (63%), and W_{35}-M-PIs (57%), which was not different from that of the parent flour (57%). A similar trend was observed with HT however, the β-sheet levels of W_{35}-D-PI and AA-PC were not different from each other. Therefore, ≥60% of the protein structures can be attributed to β-sheets, which is comparable to the level of β-sheets reported in soybean PIs by Hwang and Damodaran (1996). According to Jarpa-Parra et al. (2015), nearly two-thirds of the amino acid residues of the lentil legumin-like proteins were corresponding to β-sheets (1632 cm^{-1}) and β-turns (1660 cm^{-1}) as the major protein secondary structures at pH 3, 5 and 7. The α-helix percentage calculated solely based on the ~1655 cm^{-1} band intensity was 23% for the LT parent flours, which was comparable to that of both LT (21%) and HT (22%) S_{23}-D-PCs. However, α-helix levels of LT W_{35}-D-PIs, W_{35}-M-PIs and AA-PCs were 16, 18 and 12%, respectively. In contrast, the overall α-helix levels were only 10% in the HT dehulled bean flour as well as AA-PCs, which could be due to the influence of tannins. However, these levels were 23% and 17% for HT W_{35}-D-PI and W_{35}-M-PI, respectively, plausibly due to the reduction of tannins during the protein isolation step. The presence of α-helix structures could contribute to the formation of more stable multilayer interfaces by getting bundled side by side with hydrophilic side chains extending towards the

aqueous phase and hydrophobic side chains penetrating the hydrophobic part of the interface through the creation of more non-covalent intramolecular interactions similar to the lentil legumin-like proteins (Jarpa-Parra et al., 2015). The peak intensities of β-turns were integrated from the peaks at ~1665 and 1688 cm^{-1}. Both the AA-PCs and the corresponding parent flours had similar levels of β-turns, however, these values were half-fold in W_{35}-D-PIs, W_{35}-M-PIs and S_{23}-D-PCs in LT except for W_{35}-M-PIs in HT. The amino acid residue vibrations (1611 cm^{-1}) were significantly higher for the W_{35}-M-PIs as compared to the rest for both cultivars and the rest were comparable to each other.

Based on the abovementioned findings, it can be concluded that wet fractionation processes explored in this study led to minimal protein secondary structure conformational changes in W_{35}-M-PIs, W_{35}-D-PIs and S_{23}-D-PCs as compared to AA-PCs, which may be due to the reduction of tannins during processing. Although all the bands originally observed in the parent flour were also observed in the AA-PCs, the overall intensities were reduced. The large β-sheets contributed to ≥60% of the protein secondary structures of faba bean proteins.

7.3.3. Protein secondary structural changes: circular dichroism patterns

Circular dichroism is an efficient tool for the rapid determination of the secondary structure conformation, stability, interactions, folding and binding properties of purified proteins (Greenfield, 2007). However, there is limited work on the far-UV spectra of faba bean PIs. CD is based on the unequal absorption of the right- and left-handed circularly polarised light when asymmetric molecules interact with light to different extents and have elliptical polarisation (Kelly et al., 2005). A CD signal will be observed due to the chiral structure of the chromophores or the covalently linked chiral centre of a molecule placed in an asymmetric

environment (Kelly et al., 2005). The differences in the structural elements of the polypeptide backbone aligned in arrays can have their optical transitions shifted or divided into multiple transitions resulting in characteristic CD spectra in the far-UV (Greenfield, 2007).

W_{35}-D-PIs and AA-PCs of both LT and HT cvs. were analysed for CD spectra as shown in Fig. 7.6. The far-UV spectra of the PCs and PIs at 240 nm and below are principally due to the peptide bonds. The negative weak but broad shoulder from ~205-220 nm predominantly corresponds to parallel and anti-parallel β-sheet structures. The distinctive negative peak at ~216-218 nm and positive peak at ~195 nm correspond to anti-parallel β-sheets (Greenfield, 2007). Dominant β-sheet conformation is generally expected for legumins and these findings were comparable to the findings of Nivala et al. (2021). The α-helical secondary structures correspond to the positive band centred around 193 nm and negative bands around 208 and 222 nm (Greenfield, 2007; Kelly et al., 2005). The disordered proteins have low ellipticity above 210 nm and negative peaks near 195 nm. The chromophores of interest include the peptide bond (absorption below 240 nm), aromatic amino acid side chains (absorption in the range 260 to 320 nm) and disulphide bonds (weak broad absorption bands centred around 260 nm). Because the spectra of proteins are dependent on their conformation, CD can be used to assess the extent of structural changes taking place (Greenfield, 2007). In addition, non-protein co-factors can absorb over a wide spectral range (Kelly et al., 2005).

The far-UV CD spectra of the HT-AA-PC show a shift in the negative peak towards 200 nm and the diminishing of the positive peak around 195 nm (Fig. 7.6). The shift relates to the loss of ordered structures and the increase of random coil conformation and molecular flexibility. The spectrum for the heat-denatured faba bean protein isolates has been reported to show similarity to the molten globule state of bovine β-lactoglobulin (Nivala et al., 2021), which

are comparable to the HT-AA-PC spectrum. The six secondary structures estimated in CD Pro are regular α-helices, distorted α-helices, regular β-sheets, distorted β-sheets, β-turns, and unordered structures as shown in Table 7.3.

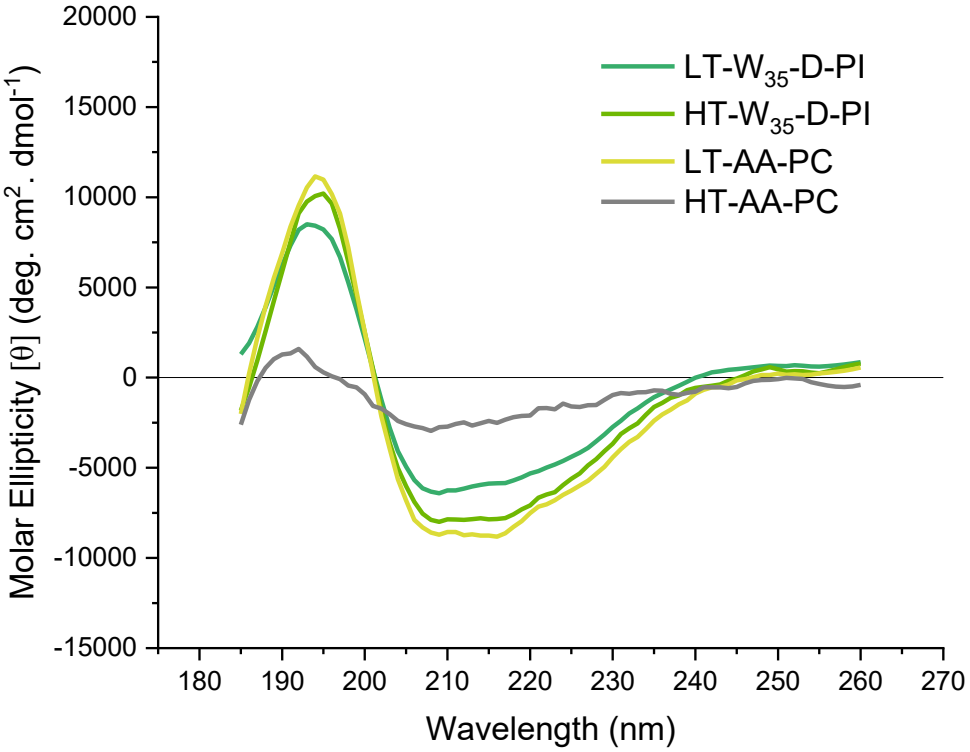


Fig. 7.6. Far-UV circular dichroism spectra of Snowbird (LT) and Athena (HT) protein fractions.

W_{35}: water extracted at 35 °C followed by dialysis at 4 °C (W_{35}-D); and AA: alkali extraction followed by acid precipitation.

Table 7.3. Protein secondary structure of faba bean proteins analysed by circular dichroism.

Fraction	Method	Protein secondary structure (%)							
		α-helix regular	α-helix ends	α-helix total	β-sheet regular	β-sheet ends	β-sheet total	β-turns	Other
LT-W_{35}-D-PI	SELCON3[3]	8.4	9.0	17.4	17.8	9.9	27.7	18.2	36.0
LT-W_{35}-D-PI	SELCON3[9]	4.7	7.9	12.6	25.6	11.0	36.6	17.8	24.0
LT-W_{35}-D-PI	CDSSTR[3]	6.7	8.3	15.0	21.9	10.8	32.7	21.3	29.8
LT-W_{35}-D-PI	CDSSTR[9]	2.8	6.6	9.4	24.8	11.4	36.2	22.5	31.2
LT-W_{35}-D-PI	CONTIN[3]	8.9	8.7	17.6	20.1	10.7	30.8	22.2	29.4
LT-W_{35}-D-PI	CONTIN[9]	8.7	9.1	17.8	20.3	10.6	30.9	22.5	28.8
LT-AA-PC	SELCON3[3]	10.6	10.7	21.3	20.6	11.3	27.7	22.2	24.8
LT-AA-PC	SELCON3[9]	8.9	9.3	18.2	20.8	11.2	32.0	22.9	27.1
LT-AA-PC	CDSSTR[3]	11.7	11.9	23.6	13.7	9.9	32.7	24.3	28.6
LT-AA-PC	CDSSTR[9]	8.9	9.6	18.5	20.1	11.3	31.4	22.1	28.0
LT-AA-PC	CONTIN[3]	10.0	9.8	19.8	18.8	11.2	30.8	23.3	26.8
LT-AA-PC	CONTIN[9]	8.2	8.6	16.8	18.8	10.6	29.4	23.6	30.0
HT-W_{35}-D-PI	SELCON3[3]	9.0	9.5	18.5	20.5	11.5	32.0	22.5	27.7
HT-W_{35}-D-PI	SELCON3[9]	10.7	10.2	20.9	19.8	10.8	27.7	22.5	27.1
HT-W_{35}-D-PI	CDSSTR[3]	10.4	10.1	20.5	15.4	10.6	26.0	23.7	29.9
HT-W_{35}-D-PI	CDSSTR[9]	9.8	10.1	19.9	15.8	10.0	32.7	23.4	30.6
HT-W_{35}-D-PI	CONTIN[3]	9.3	9.6	18.9	18.8	11.4	30.2	22.8	28.0
HT-W_{35}-D-PI	CONTIN[9]	9.0	10.2	19.2	19.3	10.8	30.8	23.4	27.3
HT-AA-PC	SELCON3[3]	4.4	5.9	10.3	27.0	16.7	43.7	19.1	13.5
HT-AA-PC	SELCON3[9]	4.4	5.9	10.3	27.0	16.7	43.7	19.1	13.5
HT-AA-PC	CDSSTR[3]	1.0	4.3	5.3	24.6	12.8	37.4	23.5	33.7
HT-AA-PC	CDSSTR[9]	1.8	4.6	6.4	23.6	12.6	36.2	24.7	33.4
HT-AA-PC	CONTIN[3]	0.6	4.0	4.6	27.0	14.2	41.2	21.8	32.4
HT-AA-PC	CONTIN[9]	0.6	4.2	4.8	27.3	14.1	41.4	22.1	31.7

The results for faba bean proteins summarized in this table were derived from the three algorithms (SELCON3, CONTIN/LL, and CDSSTR) and two (3rd and 9th sets) of the seven reference protein sets in CDPro software package (Greenfield, 2007; Sreerama & Woody, 2009).

Faba bean protein concentrates/isolates (PCs/PIs) of cultivars Snowbird (LT) and Athena (HT) treated with water at 35 °C followed by either dialysis at 4 °C (W_{35}-D) or alkali extracted, and acid precipitated (AA).

According to Table 7.3, except for the SELCON3 algorithm results of LT-W_{35}-D-PI, β-sheets were the predominant protein secondary structures of all the PCs and PIs regardless of the cultivar, which were comparable to the FTIR results. However, the β-sheets were followed by other undefined unordered structures, β-turns and α-helix, which contradicted the findings of FTIR results. Some denatured states of the proteins can possess more residual folded structure than others (Damodaran 2017). The α-helix levels of AA-PCs were only 4-5% according to Table 7.3, which is substantially lower than the rest. The protein composition of the faba bean PCs and PIs used in the current study comprised legumin (L) and vicilin (V) polypeptides with a higher (L/V) ratio for W_{35}-D-PIs and W_{35}-M-PIs as compared to S_{23}-D-PCs of LT and HT as discussed in Chapter 6 (Appendix B: B.2). The multi-protein profile of the plant material can hinder proper dichroic data acquisition (Liu et al., 2019; Nivala et al., 2021), which could have influenced the results. Also, the decreased hydrogen bond strength in a partially unfolded peptide group may facilitate the formation of disordered PCs/PIs (Ricci et al., 2018).

7.3.4. X-ray diffraction patterns of the protein fractions

XRD is a useful technique to investigate the crystalline nature of materials, such as amorphous, semi-crystalline, and crystalline structures. To the best of the author's knowledge, XRD patterns of faba bean PCs/PIs are reported for the first time in this study. Since W_{35}-D-PIs were screened as the best alternatives to AA-PCs, only the W_{35}-D-PIs and AA-PCs of both LT and HT cvs. were chosen for the XRD evaluation. The diffractograms presented in Fig. 7.7 indicate a pre-dominantly amorphous nature of the faba bean PIs and the possibility of several peaks between the 2θ range 9° to 25°. However, one small intensity peak around 11° and a high-

intensity peak around 23-24° 2θ were observed for all the PCs and PIs regardless of the cultivar. These two characteristic broad peaks at 11° and 23-24° 2θ should correspond to the α-helix and β-sheet structures of the faba bean protein secondary conformation, respectively, similar to the findings of (Chen et al., 2013) for 7S and 11S soy globulins. However, Wang et al. (2006) reported three diffraction peaks for soy PIs appearing at 8.5, 19.5 and 24.5°. In contrast, only two characteristic peaks were observed for lentil PIs (Joshi et al., 2011), soy PIs at 9° and 19° (Wang et al., 2014) as well as whey PIs at 8.83° and 19.66° (González-Martínez et al., 2017) similar to the results of this study. These findings are also complementary to the FTIR and CD findings discussed above confirming that the β-sheet structures are the most significant protein secondary structures of faba bean proteins.

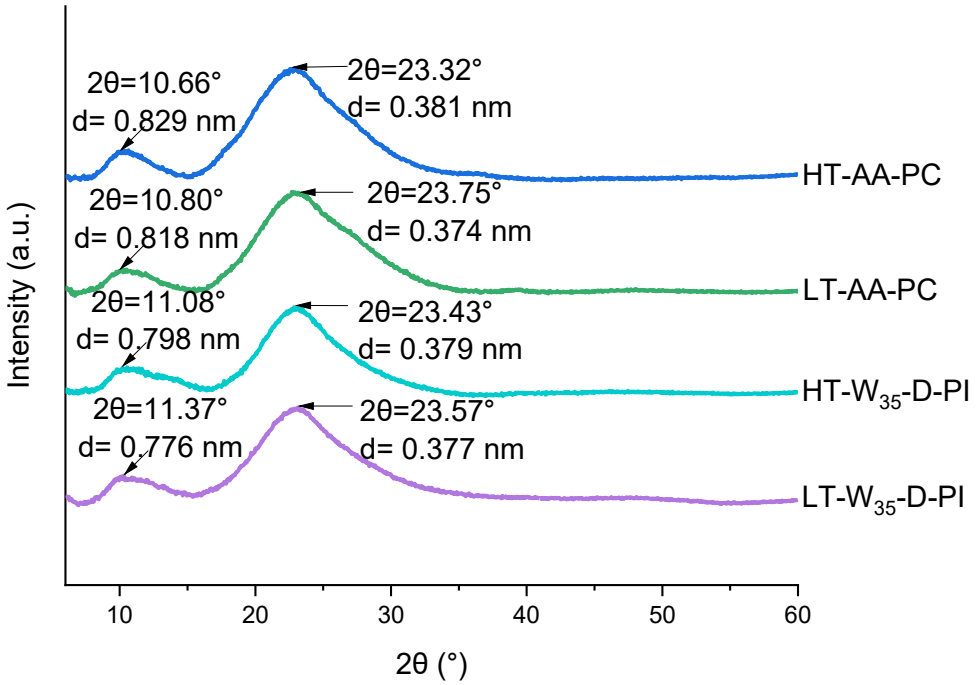


Fig. 7.7. X-ray diffraction patterns of faba bean protein concentrates/isolates (PCs/PIs) of Snowbird (LT) and Athena (HT) cultivars.

Water extraction at 35 °C followed by dialysis at 4 °C (W_{35}-D), and alkali extracted and acid precipitated (AA).

The intensities of HT-AA-PC diffraction peaks were the highest followed by LT-AA-PC while being close to each other. This may be because the AA-PCs might have partially denatured and crystallized during the alkali extraction and isoelectric precipitation steps, respectively. LT and HT W_{35}-D-PIs demonstrated low d-spacing values as compared to the rest (Fig. 7.7) corresponding to 11° 2θ. However, the peak intensities of both LT and HT W_{35}-D-PIs were 1.1 and 1.5 folds lower than the intensities of LT-AA-PC and HT-AA-PC, respectively. The characteristic peaks for LT faba bean starch were reported to be at 15°, 17° and 23° 2θ (Dong et al., 2022). The <8% starch present in faba bean AA-PCs must be influencing the intensities corresponding to 23° 2θ of the proteins. The XRD results, along with FTIR and CD findings, allow the depiction of the pattern of faba bean PC/PI secondary structural conformation changes. Instead of forming bigger aggregates via hydrophobic interactions between unfolded secondary structures of PCs/PIs, the formation of hydrogen bonding and/or electrostatic interaction can lead to the loss of inherent ordered structures, leading to greater amorphous state and smaller particle size (Lan et al., 2019).

7.3.5. Thermal behaviour of the protein fractions

DSC analysis was performed to evaluate the impact of different extraction methods on the extent of protein denaturation and the results are presented in Fig. 7.8 and Table 7.4. As moisture content is critical in terms of the thermal attributes of the proteins, W_{35}-D-PIs from both cultivars were conditioned to reach low (~12%) and high (~75%) moisture levels to compare their thermal behaviour. The low-moisture conditioned W_{35}-D-PIs had very high denaturation temperatures (LT: 170.51 ºC and HT: 169.72 ºC) as compared to the high-moisture samples (LT: 94.51 ºC and HT: 94.63 ºC), as shown in Fig. 7.8a and b and Table 7.4.

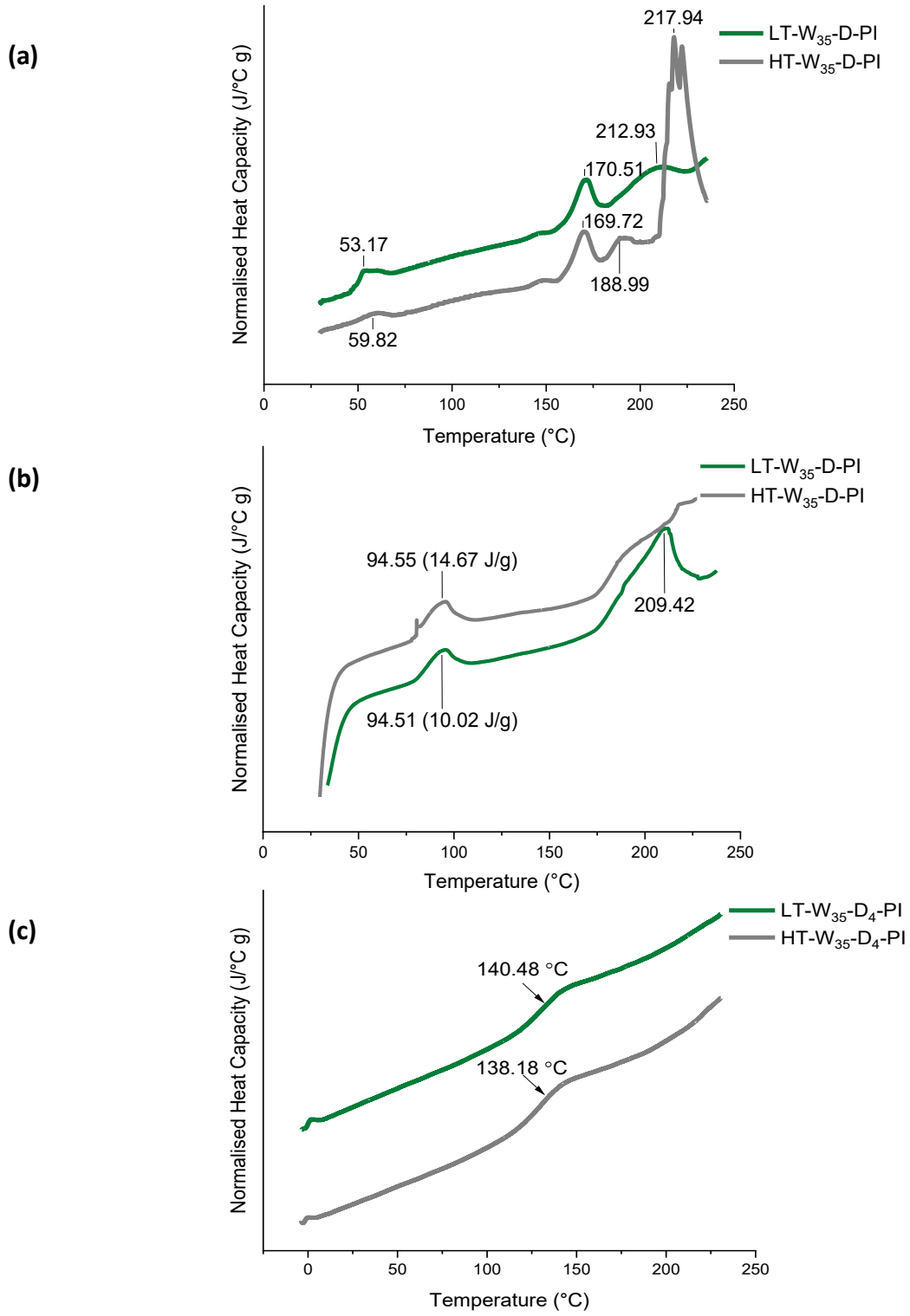


Fig. 7.8. DSC thermograms of Snowbird (LT) and Athena (HT) faba bean protein isolates (PIs) conditioned to low and high moisture contents.

(a) first heating cycle of low-moisture (~12%) and (b) high-moisture (~75%) PIs, and (c) second heating cycle of low-moisture (~12%) PIs extracted with water at 35 ºC and dialysed at 4 ºC (W_{35}-D). Endothermic peaks are upward.

Table 7.4. Starch gelatinisation (T_s), protein denaturation (T_{d1}, T_{d2}), protein degradation (T_{dg}) and glass transition (T_g) temperatures, and gelatinisation (ΔH_g) and denaturation (ΔH_{d1}, ΔH_{d2}) enthalpies of faba bean protein concentrates/isolates (PCs/PIs).

Sample	Starch gelatinisation	Enthalpy of starch gelatinisation	Protein denaturation temperature	Enthalpy of denaturation of proteins	Protein denaturation temperature	Enthalpy of denaturation of proteins	Protein degradation temperature	Glass transition temperature
	T_s (°C)	ΔH_g (J/g)	T_{d1} (°C)	ΔH_{d1} (J/g)	T_{d2} (°C)	ΔH_{d2} (J/g)	T_{dg} (°C)	T_g (°C)
LT-W_{35}-D-PI	53.17	5.77	170.51	10.24	-	-	212.93	138.19
LT-W_{35}-M-PI	-	-	171.39	9.755	-	-	217.65	146.22
LT-S_{23}-D-PC	68.46	5.82	140.77	0.69	160.78	5.20	216.42	108.84
LT-AA-PC	-	-	183.54	5.80	212.30	2.13	229.21	138.03
HT-W_{35}-D-PI	59.82	1.61	169.72	10.31	188.99	3.97	217.94	140.48
HT-W_{35}-M-PI	-	-	144.39	24.70	-	-	217.12	146.63
HT-S_{23}-D-PC	66.20	2.34	131.72	0.67	155.84	2.51	219.81	103.71
HT-AA-PC	-	-	172.08	3.79	196.42	0.31	219.68	137.98

Faba bean PCs/PIs of cultivars Snowbird (LT) and Athena (HT) treated with water at 35 °C followed by either dialysis at 4 °C (W_{35}-D) or micellization (W_{35}-M); or treated with 1% $NaCl_{(aq)}$ solution at 23 °C followed by dialysis at 4 °C (S_{23}-D); or alkali extracted, and acid precipitated (AA) conditioned to 12% moisture content.

A denaturation temperature of ~94 ºC has been previously reported by Paredes-López et al. (1991) for faba bean micellar PIs, by Nivala et al. (2017) for AA-PIs, and by Bühler et al. (2020) for dry heat treated (100 ºC) and non-dry heated faba bean PCs. The thermograms associated with the denaturation/melting of faba bean proteins confirm that the W_{35}-PIs were in their native state following the extraction steps. The difference in free energy between the native and denatured (or unfolded) forms of the protein molecules serves as a measure of the stability of the native protein structure (Damodaran, 2017). The amount of energy required to unfold a protein from its native state to its denatured state is referred to here as the endothermic enthalpy ($\triangle H_{d1,\ d2}$). After being heat denatured in the first heating step of the DSC analysis, proteins exhibit a typical glass transition in the second heating step (Fig. 7.8c). This behaviour in samples of lower protein content (i.e. S_{23}-D-PCs) suggests a plasticization role of the other components present in the protein sample. However, unlike water, the presence of polysaccharides has minimal impact on the denaturation temperature of the proteins (Alpizar-Ryes et al., 2018).

The T_g values of the W_{35}-PIs were higher followed by AA-PCs and S_{23}-D-PCs, which were positively correlated to their purity ($r = 0.8019$, $P < 0.05$) according to Pearson's correlation analysis. Similarly, the findings of Ricci et al. (2018) confirm that both T_g as well as the thermal stability of legume proteins were positively correlated to their purity whereas the denaturation temperature was not. The purity values of W_{35}-D-PIs, W_{35}-M-PIs, S_{23}-D-PCs and AA-PCs reported in Chapter 6 (Tables 6.2-6.4) for LT were 93.23 ± 0.11, 99.18 ± 0.10, 79.14 ± 0.13 and 82.26 ± 0.24%, and HT were 96.43 ± 0.29, 98.73 ± 0.22, 81.58 ± 0.50 and 87.49 ± 1.06%, respectively. These values were positively correlated ($r = 0.8917$, $P < 0.05$) to $\triangle H_{d1}$ but not to T_{d1}. Similarly, the findings of Ricci et al. (2018) confirmed that both T_g and

thermal stability of legume proteins were positively correlated to their purity whereas the denaturation temperature was not.

Apart from the first endothermic peak, corresponding to the denaturation of the proteins, additional endothermic peaks were observed during the first heating cycle of the low-moisture samples (Fig.7.8a and Table 7.4), which could be associated with the different stages of protein denaturation. Even though the amounts of tannins were greatly reduced by the wet fractionation of the proteins, any tannins remaining after extraction can cross-link with the proteins and influence the denaturation process. According to the findings of Lisperguer et al. (2016), tannins showed greater thermal resistance with a Tg of 116.8 °C and only decomposition of 3.7% at 196.91°C and maximum degradation was reached at 257.8 °C. Even though the amounts of tannins were greatly reduced by the wet extraction of the proteins, any tannins remaining after extraction can cross-link with the proteins and influence the denaturation process.

The endothermic peak at 53-69 ºC could be associated with starch gelatinisation, which is comparable to the gelatinisation temperature (60-76 ºC) of faba bean native starch as reported by Dong et al. (2022). However, this peak was missing for W_{35}-M-PIs due to their higher protein purity, and for AA-PCs as the harsh extraction conditions could have altered the native properties of the starch. The endothermic peak T_{dg} at ~212-220 ºC could be mainly associated with the melting/degradation of the proteins. The maximum temperature peak found between 183 to 211 ºC was identified as the denaturation peak by Ricci et al. (2018), which did not depend on the legume variety or purity. The transition from the native to the denatured state of the proteins is expressed as the enthalpy change, which reflects the extent of ordered structure remaining after the wet fractionation process (Paredes-López et al., 1991). Large differences exist between the heat capacity profiles of pulse protein endothermic denaturation transitions

reported in the literature. The ΔH_{d1} and ΔH_{d2} values were lowest for the AA-PCs and S_{23}-D-PC as compared to the W_{35}-PIs, which confirm that these proteins are not in their native state and a significant level of denaturation has taken place during the extraction procedure.

Overall, the faba bean PCs and PIs with a $T_g<T_{d1}$ are suitable to be used as food ingredients in extrusion processing, which requires a viscous mixture to be processed above its softening point (Ricci et al., 2018). Thus, the glass transition temperature can be used as a parameter for establishing physical or structural stability conditions. Very high melting temperatures (>200 °C) from calorimetric tests suggest that the materials evaluated here are thermally stable for high-temperature extrusion in food applications.

7.4. CONCLUSIONS

Physicochemical properties affect the functional properties of protein ingredients and ultimately the final food product. Therefore, it is pivotal to understand the changes associated with the physicochemical properties of the proteins following their extraction from faba bean cultivars of different origins. The mild wet extraction methods employing water and $NaCl_{(aq)}$ solution did not significantly influence the size distribution of the particles in their water suspensions and a substantial portion were small and easily dispersible particles, yet less uniform as compared to the larger particles of alkali-extracted and acid-precipitated protein concentrates, with more than 90% of the dispersed particles were less than 268 μm for LT and 167 μm for HT AA-PCs. The XRD patterns of faba bean PCs/PIs confirmed the pre-dominantly amorphous nature of the faba bean proteins, which was complementary to the DSC glass transition results. The XRD results revealed a small intensity peak at ~11° and a high-intensity peak at ~23-24° 2θ corresponding to α-helix and β-sheet structures. Both FTIR and CD results

confirmed that β-sheet structures were the most abundant secondary structures of the faba bean PCs/PIs and the mild fractionation method employing water had minimal impact on their secondary structure conformation. According to CD results, the unordered structures were the second most prominent structures in contrast to the FTIR and XRD results. However, differences in the results can be attributed to the approaches and the principles behind the analytical method.

As the functional properties are largely dependent on the structural conformation, the selection of appropriate processing conditions during the application of these proteins is important. The findings demonstrated that the mild fractionation methods have less impact on the physicochemical characteristics of the protein secondary structure conformation. Further studies are required to establish the correlation between the functional properties and the physicochemical characteristics of the proteins resulting from the mild extraction treatments. An association between the protein purity level, the glass transition temperature and endothermic enthalpy of the denaturation peak was observed for the W_{35}-PIs, S_{23}-D-PCs, and AA-PCs regardless of the cultivar. A high protein melting/degradation temperature above the glass transition temperature confirms the suitability of the faba bean protein concentrates and isolates in extrusion processing.

CHAPTER 8: Impact of mild extraction of faba bean (*Vicia faba* L.) proteins against conventional methods on their physicochemical and functional characteristics[7]

[7]A version of this chapter will be published: Jeganathan, B., Vasanthan, T., & Temelli, F. Impact of mild extraction of faba bean (*Vicia faba* L.) proteins against conventional methods on their physicochemical and functional characteristics.

8.1. INTRODUCTION

Faba bean is a protein-rich, underutilized pulse with great potential to supplement the fast-growing global demand for protein. Food industries prefer pulse protein concentrates (>50%, db) or isolates (>80%, db) for use in their formulations (Boye et al., 2010; Multari et al., 2015). The major drawbacks of the conventional wet fractionation methods are the large consumption of water and energy as compared to hybrid fractionation and dry fractionation, the partial loss of the native functionality of the proteins (Assatory et al., 2019; Berghout et al., 2015; Schutyser et al., 2015; Sun & Arntfield, 2010), and the formation of the undesirable amino acid derivative lysinoalanine (Gould & MacGregor, 1977).

Functional properties are defined as the physical and chemical properties, which affect the behaviour of proteins in food systems during processing, storage, preparation and consumption (Kinsella, 1981). Functional properties displayed by food proteins are dictated by their amino acid composition, protein structure and conformation (e.g., surface hydrophobicity), as well as the processing conditions they are exposed to such as pH, temperature, and the interactions that occur between proteins and other food components (e.g., salts, lipids, carbohydrates and phenolics). Although much is known about the physicochemical properties of several food proteins, the prediction of functional properties from their molecular properties has not been successful (Boye et al., 2010; Damodaran, 2017).

Compared to the conventional pH-shift method, mild fractionation approaches to extract legume proteins are proven to be more resource efficient by using less water and energy and no chemicals (Geerts et al., 2018). Proteins obtained in this manner retain their native properties and exhibit better solubility (Pelgrom et al., 2015). Aqueous salt ($salt_{(aq)}$) extraction is a relatively common mild extraction method employed to isolate pulse proteins based on the

solubility behaviour of the major storage proteins (Wang et al., 2010). Depending on the method of extraction and the conditions used to isolate the proteins, their functionality may vary due to the changes in their compositional and physicochemical characteristics and the magnitude to which these characteristics are affected (Jarpa-Parra et al., 2015; Singhal et al., 2016). Thus, following the separation of high-value protein isolates from two Canadian faba bean cvs. Snowbird and Athena by mild wet fractionation methods in comparison to the conventional pH-shift method as described in previous chapters (Chapters 4-6), the overarching goal of this study was to comparatively explore the molecular (zeta potential, surface hydrophobicity) and functional (colour, foaming, solubility, emulsification and hydration) attributes of the protein concentrates (PCs) or protein isolates (PIs) obtained for potential food applications.

8.2. MATERIALS AND METHODS

8.2.1. Materials

The low tannin (LT) faba bean cv. Snowbird and high tannin (HT) cv. Athena were chosen for this study. Protein concentrates (PCs) resulting from alkali extraction and acid precipitation (AA), and $NaCl_{(aq)}$ solution (1% (w/v)) extraction at 23 °C (S_{23}) followed by dialysis at 4 °C (D) using a 6-8 kDa molecular weight cut-off (MWCO) dialysis tubing and protein isolates (PIs) from water extraction at 35 °C (W_{35}) followed by either dialysis at 4 °C (D) or micellization (M), as described in Chapter 6 (Section 6.2.3), were selected for this study. Bovine serum albumin (BSA) standard solution ampules of 2 mg/mL concentration were purchased from Thermo Scientific (Rockford, IL, USA). Sigma-Aldrich Canada Co. (Saint Louis, MO, USA) provided the remaining chemicals.

8.2.2. Characterisation of molecular structure and functional properties

8.2.2.1. Zeta potential

Zeta potential (ζ) values of faba bean protein dispersions of 0.05% concentration in Milli-Q water prepared as a function of pH (3-10) adjusted with 0.1 M HCl or 0.1 M NaOH were estimated using a Zetasizer nano-Z (Malvern Instruments Ltd., Worcestershire, England, UK). Samples were hydrated for 16 h at 4 °C and then hand shaken in a Wrist Action Shaker (Burrell, 75–765 BT, Pittsburgh, PA, USA) at the high setting (10) for 1 h at 23 °C. To remove any insoluble material, the samples were centrifuged (Eppendorf accuSpin Micro, Osterode, Germany) for 10 min at 3000 ×g. A refractive index of 1.33 and an absorption index of 0.001 were used. Measurement of the ζ- potential was based on automatic voltage selection and calculations were done based on the Smoluchowski model (Vogelsang-O'Dwyer et al., 2020).

8.2.2.2. Solubility

Solubility values of the proteins present in LT and HT PCs/PIs were determined according to the methods of Morr et al. (1985) and Wang et al. (2010) with minor modifications. Protein dispersions of 1% (w/v) concentration were prepared in Milli-Q water by homogenising (Heidolph DIAX 900, Instruments GmbH & Co. KG, Schwabach, Germany) with the 6G-coarse shaft (P/N 596-06000-00) at 14,000 rpm for 1 min, adjusting to different pH values (2-12) with 0.1 M HCl or 0.1 M NaOH, and allowing to hydrate at 4 °C overnight. Thereafter, the samples were shaken in a hand shaker (Burrell Wrist Action Shaker, 75–765 BT, Pittsburgh, PA, USA) at the high setting (10) for 1 h at 23 °C similar to the protocol reported in Chapter 6 (Section 6.2.4.2.4) and the remaining steps were followed accordingly. The precipitates were freeze-dried following the removal of the supernatants by centrifugation

at 3000 ×g for 20 min at 23 °C. The protein contents of the precipitates were measured using a LECO elemental analyser (N × 6.25) as indicated in Chapter 4 (Section 4.2.4.2.1) and the protein solubility was expressed as the % of protein remaining in the supernatant relative to the original samples according to Eq. (6.3) expressed in Chapter 6 (Section 6.2.4.2.4).

8.2.2.3. Surface hydrophobicity

The average surface hydrophobicity values of the faba bean PCs/PIs were determined by fluorescence measurements in a SpectraMax® M3 Multi-Mode Microplate Reader (Molecular Devices LLC, San Jose, CA, USA) at an excitation wavelength of 390 nm and an emission wavelength of 470 nm with 1 nm slit width according to the modified method described by Karaca et al. (2011) originally derived from the method of Kato and Nakai (1980). Serial dilutions of 0.001%, 0.002%, 0.004%, 0.006%, 0.008% and 0.01% concentrations were prepared from protein stock solutions (0.02%, w/v) in 0.01 M sodium phosphate buffer (pH 7). Protein solution (4 mL) was vortexed for 10 s with 8 mM ANS (20 μL) in 10 mM sodium phosphate buffer and was added as a hydrophobic fluorescence probe. The fluorescent intensity was measured after holding for 15 min in the dark. The fluorescent intensity of the diluted protein blanks without the ANS probe and the ANS blank were deducted from the readings of the protein solutions with ANS. The slope of the plot of corrected fluorescence intensity against protein concentration was calculated by linear regression analysis and used as an index of protein surface hydrophobicity (Karaca et al., 2011).

8.2.2.4. Hydration capacity

The hydration (water holding or water absorption) capacities of the LT and HT PC/PI (1%, w/v) dispersions in Milli-Q water were measured at room temperature (23 °C). The samples were centrifuged at 2000 xg for 20 min following homogenization at 14,000 rpm for 1 min to ensure the uniform distribution of water. Hydration capacity was calculated as the difference between the hydrated weight and original dry weight relative to the original protein dry weight and expressed in grams of water absorbed per gram of protein dry weight.

8.2.2.5. Emulsifying capacity and emulsion stability index

The emulsifying capacities of LT and HT faba bean PCs/PIs were determined by the method described by Pearce and Kinsella (1978) with a few modifications. Protein dispersions (1% (w/v)) were prepared in 10 mM phosphate buffer containing 0.1% sodium dodecyl sulphate (SDS) in a hand shaker (Burrell Wrist Action Shaker, 75–765 BT, Pittsburgh, PA, USA) at high setting (10) for 30 min at 23 °C. Then, canola oil was added at a 1:1 (v/v) ratio and homogenised (Polytron, PTMR 2100, Kinematica AG, Malters, Switzerland) at 15,000 rpm for 2 min. The emulsion volume and the total volume were measured. Emulsifying capacity (EC) was expressed as the percentage of the emulsion formed relative to the total volume.

An aliquot (50 µL) of the homogenate was mixed with 7.5 mL of phosphate buffer (Karaca et al., 2011). The absorbance was read at 500 nm in a Genesys 10 UV–visible spectrophotometer (Thermo Scientific, Madison, WI, USA) against phosphate buffer immediately after homogenisation (A0) and after 10 min (A10) to estimate the emulsion stability index (ESI) using Eq. (8.1).

$$ESI = \frac{A_0}{A_{10} - A_0} \times 10 \quad (8.1)$$

8.2.2.6. Foaming capacity and foam stability

Foaming properties were assessed according to Alonso-Miravalles et al. (2019). Protein dispersions with a protein concentration of 1% (w/v) in Milli-Q water (20 mL) in 50 mL centrifuge tubes were frothed using (Polytron, PTMR 2100, Kinematica AG, Malters, Switzerland) at 15,000 rpm for 30 s. The height of the sample (liquid and foam phase) was measured immediately and after 60 min. Foaming capacity (FC) was taken as the sample expansion percentage at 0 min (Eq. (8.2)), while foam stability (FS) was the percentage of sample expansion at 0 min relative to the sample expansion at 0 min.

$$FC = \frac{Sample\ height\ after\ foaming - Initial\ sample\ height}{Initial\ sample\ height} \times 100 \quad (8.2)$$

8.2.2.7. Colour

Colour measurements of the faba bean PCs/PIs were obtained based on Hunter *L*, *a* and *b* colour scales (Hunter & Harold, 1987) using a CR-410, Konica Minolta colourimeter (Sakai Osaka, Japan). The colourimeter was calibrated against a white standard tile (y = 94.4, x = 0.3133, z = 0.3190) and then the ground samples were packed into plastic sample holders and examined for colour. The lightness of the product was assessed using *L* values (0-50 for darkness and 51-100 for whiteness). Positive *a* values symbolise the redness level, while negative *a* values relate to the greenness level. While positive *b* values signify yellowness, the negative *b* values represent blueness (Ratnaningsih & Songsermpong, 2021).

8.2.3. Statistical analysis

All the results expressed as mean ± SD on a dry weight (dw) basis were based on the significant differences ($P < 0.05$) between samples and were tested by analysis of variance

(ANOVA) followed by mean separation using Duncan's multiple range test ($P < 0.05$). Any comparisons between two sets of data were tested at a 95% confidence level according to the two-sample t-test. Additionally, Pearson's correlation analysis was performed ($P < 0.05$) to understand the correlation between the L/V ratio and any functional properties. All these statistical analyses were carried out using SAS Studio University Version for Windows (SAS Institute Inc., Cary, NC, USA).

8.3. RESULTS AND DISCUSSION

8.3.1. Surface charge of the protein fractions

Zeta potential is an index that can be used to predict the stability of a colloidal system as it reflects the magnitude of interactions between colloidal particles. One of the key elements affecting a sample's ζ-potential in an aqueous medium is its pH, followed by conductivity and the concentration of the protein (Hunter, 1981). The changes in ζ-potential values as a function of pH for LT and HT PCs/PIs presented in Fig. 8.1 show a similar tendency. A suspended particle with a negative ζ-potential tends to become increasingly negatively charged with the addition of alkali. However, if acid is introduced into the suspension, a point identified as the isoelectric point will be reached where the net surface charge will be zero, and introducing more acid may result in a buildup of positive charge (Hunter, 1981).

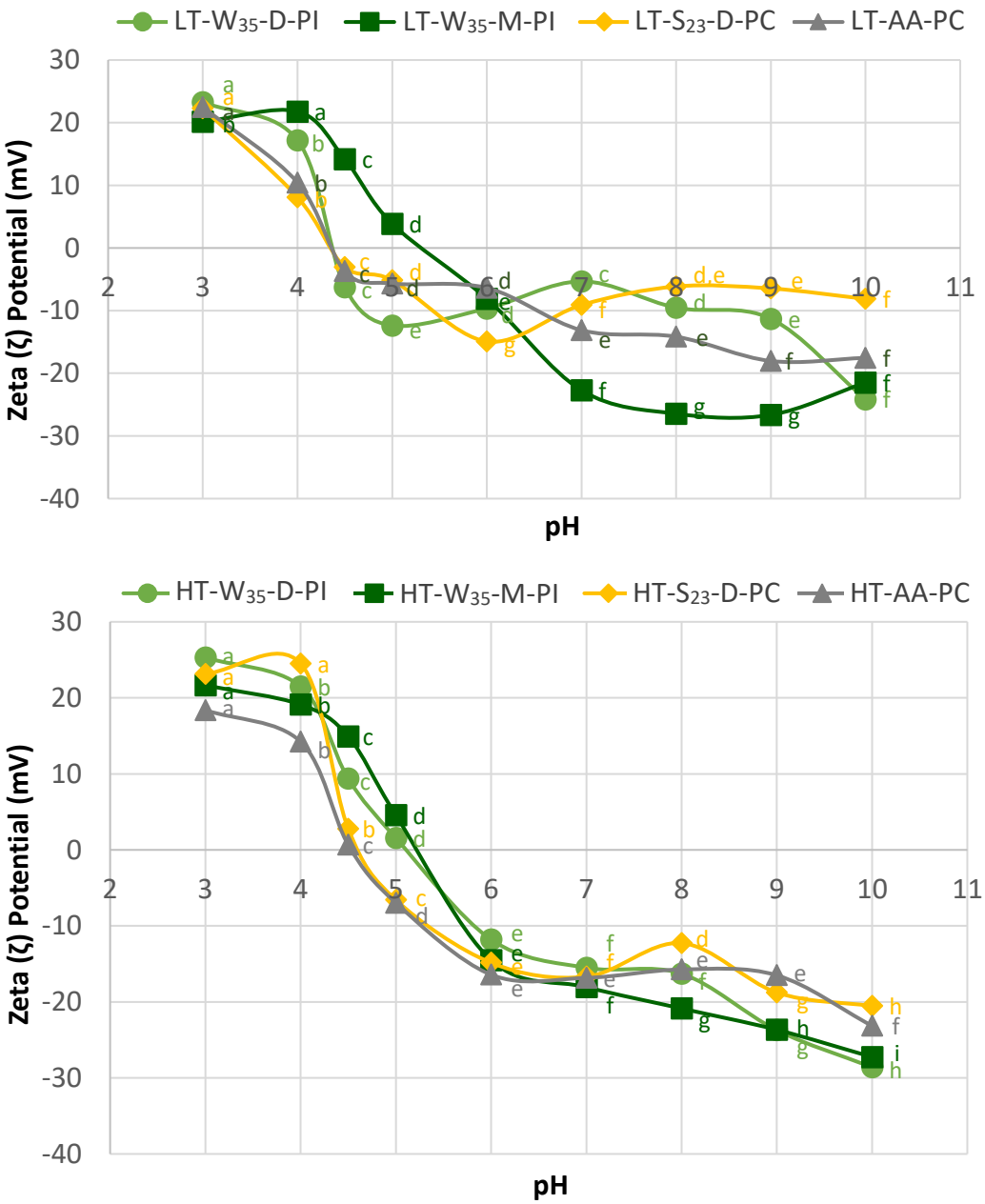


Fig. 8.1. Zeta potential of Snowbird (LT) and Athena (HT) faba bean protein concentrates/isolates (PCs/PIs).

[a-i]Mean (n = 6) ± SD values for a particular extraction method followed by different letters are significantly different ($P < 0.05$), according to Duncan's multiple range test.

Water extracted at 35 °C followed by either dialysis at 4 °C (W_{35}-D) or micellization (W_{35}-M); or extracted with 1% $NaCl_{(aq)}$ solution at 23 °C followed by dialysis at 4 °C (S_{23}-D); or alkali extracted, and acid precipitated (AA).

All the samples had a positive surface charge at pH 3 and 4 and negative ζ-potential from pH 6-10. The net surface charge was zero between pH 4.5 and 5.5 for all the samples, which was the isoelectric point with the highest dispersion instability. However, compared to HT (4.6-5.2), the LT PCs/PIs showed a slightly wider isoelectric point (4.5-5.3) range, which confirms that the cultivar is a more significant determinant of the surface charge as a function of pH as compared to the extraction method. According to Kosińska et al. (2011), the affinity of tannins towards proteins decreased along with the decreasing molecular weight of faba bean proteins. The 11S legumins are the most abundant proteins present in faba bean PCs/PIs (Chapter 6, Section 6.3.4) so they could have exhibited comparatively higher affinity towards tannins. Despite the significant reduction of tannins in the HT PIs as compared to the raw material (Chapter 6, Table 6.4), the levels were substantially higher in HT-AA-PCs as compared to LT. Therefore, the tannins present in HT could have a significant impact on the surface charge of the proteins, which could be overshadowing the impact of the extraction method on the ζ-potential of the proteins.

The highest ζ-potential values were observed for HT samples (W_{35}-D-PI: -28.6 mV; W_{35}-M-PI: -27 mV; S_{23}-D-PC: -21 mV; and AA-PC: -23 mV) at pH 10. At neutral pH, LT W_{35}-D-PI (-5 mV) had the lowest electrophoretic mobility followed by S_{23}-D-PC (-9 mV) and AA-PC (-13 mV) but W_{35}-D-PI (-23 mV) had a relatively higher surface charge. A similar trend with higher values was observed for HT PCs/PIs at pH 7. The highest ζ-potential values for alfalfa protein suspensions were observed at pH 7 in the absence of salt followed by pH 3 (Nissen et al., 2021). In general, the AA method led to slightly higher ζ-potential in alfalfa protein except for pH 7 (Nissen et al., 2021). Karaca et al. (2011) reported -23 mV and -18 mV ζ-potential for faba bean PIs, resulting from alkali extraction (pH 9.5) followed by acid

precipitation (pH 4.5), and salt extraction (5% $K_2SO_{4(aq)}$ solution), respectively. These differences in ζ-potential values could be due to the differences in the extraction conditions used (e.g. solvent type, solvent-to-feed ratio, pH, and temperature), which could greatly influence the physicochemical characteristics of the protein obtained. A reduction in the surface charge of field pea PIs with the increasing concentration of $NaCl_{(aq)}$ was reported by Zhang et al. (2021). The salt-extracted field pea proteins had an overall lower surface charge across the different pH ranges as compared to the acid-precipitated proteins (Zhang et al., 2021). In the present study, it was also confirmed that both S_{23}-D-PCs and AA-PCs showed a similar trend but had an overall lower surface charge compared to the water-extracted proteins. This is possibly due to the extreme pH conditions of the AA method and the shielding effect of the salt deposits still present on the surface of the S_{23}-D-PC after dialysis due to the salting out effect as discussed in Chapter 6 (Section 6.3). When present in high concentrations, several salts have been found to have a relatively high affinity for proteins. These salts could block the charged sites of the proteins and alter their electrical properties in a food system (Xu et al., 2017; Zhang et al., 2021). The increase in net charge caused by ion binding can enhance repulsive forces and cause instability in the proteins (Paredes-López et al., 1991).

8.3.2. Protein solubility

Protein solubility plays an important role in many food systems as it impacts foaming, emulsification and gelation properties (Boye et al., 2010; Karaca et al., 2011). Protein-protein and protein-water interactions play an important role in protein solubility, which are dictated by surface charge (Boye et al., 2010). Thus, the solubility of pulse proteins is significantly influenced by the pH of the media. Pulse proteins of different origins generally demonstrated

better solubility at high alkaline and low acidic pH values but were least soluble near their pI, which for most pulse proteins typically varied between pH 4.0 and 5.5 (Joshi et al. 2017; Karaca et al. 2011; Shevkani et al. 2015) and more specifically between pH 4.5 and 5.5 for the faba proteins under investigation here (Fig. 8.1). Under alkaline pH conditions, there is more negative charge, and the charged particles repel each other limiting protein-protein interactions, but favouring the protein-water interactions; thus, allowing the proteins to be more dispersed (Hwang & Damodaran, 1996). However, at their isoelectric point, proteins have no net charge and no repulsion between charged molecules, and therefore the protein-protein interactions can encourage the aggregation of the proteins (Karaca et al., 2011).

The relationships between surface charge and protein solubility against pH can be established based on Figs. 8.1 and 8.2. According to Fig. 8.2, both LT and HT PIs are best soluble at low acidic (3-4) and high alkaline (8-12) pH levels as their surface charge were positively and negatively higher under both conditions (Fig. 8.1), respectively. Although the overall protein solubility pattern was similar for both PIs regardless of the cultivar, the protein solubility was lower for HT-PIs as compared to LT-PIs in the pH range of 4 to 8. This is plausibly due to the affinity of the remaining tannins in HT towards the formation of insoluble protein-tannin complexes that can hinder protein solubility. According to Fig. 8.2, between pH 4 and 6, the protein solubility sharply declined and the lowest protein solubility values for LT-PIs were observed at pH 4.5 and close to pH 5 for HT-PIs, which were the isoelectric points of the corresponding protein fractions (Fig. 8.1). The isoelectric points of chickpea and lentil albumins are 3.9 and 3.7, whereas it is 4.2 and 4.3 for lentil globulins, respectively (Torki et al., 1987), comparable to the results of this study.

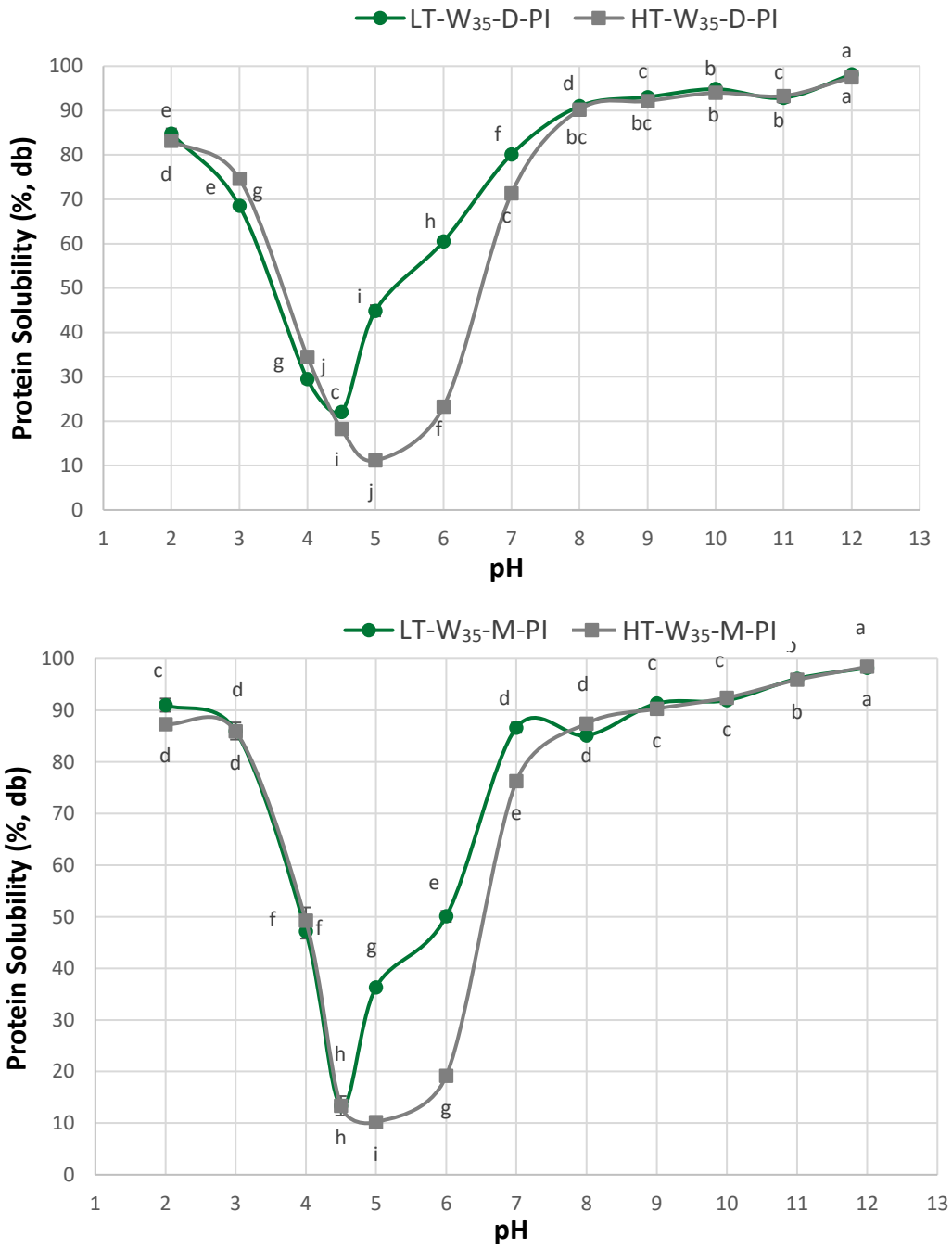


Fig. 8.2. Solubility of Snowbird (LT) and Athena (HT) faba bean protein isolates (PIs) extracted with water at 35 °C followed by dialysis (W_{35}-D) or micellization (W_{35}-M) as a function of pH.

[a-i]Mean (n = 6) ± SD values for a particular extraction method followed by different letters are significantly different ($P < 0.05$), according to Duncan's multiple range test.

According to the findings of Chapter 6 (Section 6.3.5), faba bean PCs/PIs contain a significant amount of polar and hydrophilic amino acid residues such as aspartic acid, arginine, glutamic acid and lysine, which contribute to favourable hydrophilic interactions between protein molecules and water, such as hydrogen bonds and electrostatic interactions. Although faba bean proteins are also rich in hydrophobic amino acids, like many other globulin proteins most of these hydrophobic moieties are probably buried inside the core with the hydrophilic residues located on the external surface similar to the findings on lentil proteins (Jarpa-parra et al., 2014).

When proteins are in their native form or have undergone a minimal level of denaturation, they should have better protein solubility in theory as compared to the AA-PCs, which have undergone partial denaturation as discussed in Chapter 7 (Sections 7.3.2. and 7.3.4). Thermodynamically, the most stable state is the native form of the proteins, which can undergo conformational adaptability as a result of the delicate changes in structure or can transform into an ill-defined final state due to major changes in the secondary, tertiary and quaternary structures without the cleavage of the backbone peptides, also known as denaturation (Damodaran, 2017). The level of denaturation that can take place during the extraction and isolation of AA-PCs may be responsible for their significantly ($P < 0.05$) lower protein solubility as compared to the rest at pH 7 regardless of the type of cultivar (Table 8.1). Protein molecules can also form macromolecular aggregates through non-covalent interactions, resulting in reduced solubility (Martínez-Velasco et al., 2018). Comparatively large aggregates were formed in AA-PC suspensions (Chapter 7, Section 7.3.1), which could lead to reduced solubility (Ercili-Cura et al., 2015).

Table 8.1. Physicochemical properties of faba bean PCs/PIs from four different wet fractionation methods.

Sample	Surface hydrophobicity at pH 7	Colour			Hydration capacity (g/g protein)	Protein solubility (%) at pH 7
		L	*a*	*b*		
LT-W_{35}-D-PI	25499 ± 1286^{a}	77.76 ± 0.07^{a}	0.81 ± 0.01^{c}	12.60 ± 0.03^{c}	2.10 ± 0.14^{c}	80.12 ±0.69^{b}
LT-W_{35}-M-PI	26965 ± 2438^{a}	76.61 ± 0.05^{b}	0.65 ± 0.01^{d}	14.34 ± 0.04^{b}	2.47 ± 0.07^{b}	86.62 ± 0.98^{a}
LT-S_{23}-D-PC	12398 ± 1175^{b}	76.42 ± 0.07^{b}	1.33 ± 0.03^{b}	15.26 ± 0.10^{a}	1.94 ± 0.15^{c}	86.69 ± 0.29^{a}
LT-AA-PC	6032 ± 1258^{c}	57.64 ± 0.35^{c}	1.83 ± 0.03^{a}	8.66 ± 0.02^{d}	7.25 ± 0.08^{a}	50.75 ± 0.44^{c}
HT-W_{35}-D-PI	22236 ± 517^{b}	78.76 ± 0.08^{a}	0.49 ± 0.01^{d}	13.63 ± 0.03^{b}	1.92 ± 0.08^{c}	71.31 ± 1.49^{c}
HT-W_{35}-M-PI	26124 ± 2283^{a}	75.49 ± 0.14^{b}	0.77 ± 0.03^{c}	12.88 ± 0.02^{c}	2.60 ± 0.37^{b}	76.26 ± 0.27^{b}
HT-S_{23}-D-PC	25992 ± 4442^{a}	73.78 ± 0.13^{c}	3.19 ± 0.05^{a}	17.63 ± 0.04^{a}	2.35 ± 0.16^{bc}	86.38 ± 0.58^{a}
HT-AA-PC	9927 ± 2773^{c}	51.14 ± 0.04^{d}	1.61 ± 0.01^{b}	4.45 ± 0.02^{d}	6.12 ± 0.66^{a}	69.81 ± 1.05^{c}

$^{a\text{-}d}$Mean (n = 3) ± SD values for a particular extraction method within the cultivar followed by different letters are significantly different ($P < 0.05$), according to Duncan's multiple range test.

Faba bean protein concentrates/isolates (PCs/PIs) of cultivars Snowbird (LT) and Athena (HT) extracted with water at 35 °C followed by either dialysis (W_{35}-D) or micellization (W_{35}-M), or alkali extracted, and acid precipitated (AA), or treated with 1% $NaCl_{(aq)}$ solution at 23 °C followed by dialysis at 23 °C (S_{23}-D) as a function of pH.

Faba bean proteins extracted by the AA method had ~66% protein solubility at both pH 3 and 7 (Vose, 1980) compared to chickpea proteins with 60.4% at pH 7 (Paredes-López et al., 1991). These findings were comparable to the findings of the present study as both LT and HT AA-PCs at pH 7 had significantly ($P < 0.05$) lower protein solubility as compared to the rest of the protein fractions. Although the surface charge of HT-S_{23}-D-PC was overall higher than LT-S_{23}-D-PC (Fig. 8.1) and the surface charge favours protein solubility (Damodaran, 2017), both LT and HT had a similar protein solubility at pH 7 (86.4-86.7%) regardless of the cultivar. This could mean that the salts remaining after dialysis could be influencing the protein solubility through salting in effect as discussed in Chapter 6 (Section 6.3.2). On the other hand, Fernandez-Quintela et al. (1997) reported the solubility of faba bean PIs as 81.2% at pH 7 and Paredes-López et al. (1991) reported the protein solubility of chickpea protein fractions isolated by micellization as 72.5% at pH 7. Relatively, the protein solubility of W_{35}-D-PIs and the W_{35}-M-PIs at pH 7 were significantly higher, which makes them suitable for high-protein dairy milk substitute formulations where good solubility is deemed favourable. No significant correlation ($P > 0.05$) was observed between the L/V ratio and the solubility of PCs/PIs at pH 7.

8.3.3. Hydration capacity and surface hydrophobicity of the protein fractions

The hydration capacity of proteins is an important functional property to hold water within the food system to improve the sensory profile (texture, flavour and mouthfeel) of food products with low or intermediate moisture content such as minced meat, meat mimics, soups and bakery products (Alonso-Miravalles et al., 2019; Shevkani et al., 2015). Based on the hydration capacity of faba bean PCs/PIs listed in Table 8.1, it can be concluded that the AA-PCs had the highest hydration capacity as compared to the rest of the PCs/PIs regardless of the cultivar. Hydration

capacity, more commonly known as the water holding capacity is the measurement of grams of water absorbed per gram of dry protein equilibrated with water at 90-95% relative humidity (Damodaran, 2017). Water plays a key role as a plasticizer and can bind to charged groups (ion-dipole interactions); backbone peptide groups; the amide groups of asparagine, glycine; hydroxyl groups of serine, threonine, and tyrosine residues (all dipole-dipole interactions); and nonpolar residues (dipole–induced dipole interaction and hydrophobic hydration) (Damodaran, 2017; Mallamace et al., 2015; Shevkani et al., 2015). Although the hydrophilic/hydrophobic balance, amino acid content, and charge distribution on the protein surface are the major factors that have an impact on the ability of a protein matrix to hold water, the physical entrapment mechanism also plays an equally important role (Damodaran, 2017).

Although partial denaturation of AA-PCs can expose the hydrophobic amino acid residues hidden inside the protein moiety, more hydrophilic groups can also be exposed, which are a significant component of faba bean proteins according to the amino acid profile discussed in Chapter 6 (Table 6.5). Unlike PIs, which had more than 90% protein content, the PCs also had other macromolecules, such as starch and dietary fibre, which could have contributed to the hydration capacities measured. Although the hydration capacities of W_{35}-D-PIs, W_{35}-M-PIs and S_{23}-D-PCs given in Table 8.1 are significantly ($P < 0.05$) lower than those of AA-PCs, these values are comparable to or higher (0.5-5.0 g/g) than the literature values for legume proteins (Boye et al., 2010; Martinez et al., 2016; Sosulski & McCurdy, 1987). Additionally, no significant correlation ($P > 0.05$) was observed between the L/V ratio and hydration capacity possibly due to the influence of other hydrocolloids. The protein-protein interactions are favoured during the micellization or dialysis at 4 ºC due to the reduction of the ionic strength of the media as discussed in Chapter 4 (Section 4.3.3). This could be the reason that the PIs had lower hydration capacities

compared to the rest. The ability of the proteins to bind and retain water against gravitational separation within the food matrix prevents the loss of quality during processing and storage (Alonso-Miravalles et al., 2019).

The AA method primarily precipitates globulin and glutelins at their isoelectric point (Johnston et al., 2015), whereas the products of the W_{35} and S_{23} methods are a mixture of both globulins and albumins. The surface hydrophobicity values of the AA-PCs were significantly ($P < 0.05$) lower than the rest (Table 8.1). This could be associated with their substantially ($P < 0.05$) higher hydration capacities as compared to the rest of the PCs/PIs regardless of the cultivar (Table 8.1), likely due to the partial hydrolysis of proteins at alkaline pH, which could increase terminal residues, hence charged groups for interactions with the aqueous medium (Jarpa-Parra et al., 2014). The alkaline extraction of globulin proteins (e.g., legumin (11S, hexamer) or vicilin (7S, trimer)) can also lead to their disassociation into subunits, and thus resulting in increased surface hydrophobicity due to the exposure of the originally buried hydrophobic side-chain groups (Alonso-Miravalles et al., 2019). However, the AA-PCs had relatively low surface hydrophobicity and solubility at pH 7 (Table 8.1). Hayakawaand and Nakai (1985) showed through regression analysis that hydrophobicity can be negatively correlated to the insolubility of the proteins, which was significant and raw material dependent. Nevertheless, according to Table 8.1, the hydrophobicity values were more positively correlated to high solubility. Other macromolecules present in PCs in particular starch and dietary fibre may influence the functional properties.

Surface hydrophobicity is also an indicator of protein conformation changes (Murray et al., 2002). Protein conformational changes are more likely to occur at the quaternary and tertiary levels as the FTIR results confirmed minimal impact on the protein secondary structure conformation (Chapter 7, Section 7.3.2). Legumins (11S) have a dense structure due to the hydrophobic

interactions resulting from the relatively more hydrophobic basic polypeptide (Yang et al., 2018). It is possible that the dialysis/micellization steps leading to amphiphilic micelle arrangements at 4 °C could be exposing the aromatic amino acid residues hidden inside the moiety resulting in an increased protein surface hydrophobicity. It is suggested that a combination of the aliphatic and aromatic hydrophobicities may be important in understanding the impact of surface hydrophobicity on the solubility of proteins (Hayakawaand & Nakai, 1985).

8.3.4. Colour parameters of the protein fractions

Seed colour is an important sensory attribute of faba bean for food applications. Colour indices *L*, *a* and *b* corresponding to lightness, redness and yellowness of LT and HT PCs/PIs are reported in Table 8.1. The *L*, *a*, and *b* values showed statistically significant ($P < 0.05$) differences among the PCs/PIs resulting from the different extraction methods tested. The AA-PCs had the darkest tone, and the W_{35}-D-PIs had the lightest tone as compared to the rest regardless of the cultivar. The positive *a* values corresponding to redness were significantly ($P < 0.05$) lower for PIs as opposed to the PCs. The positive *b* values corresponding to yellowness were significantly ($P < 0.05$) lower for AA-PCs as compared to the others. The extreme pH conditions of the AA method could have oxidised the colour pigments of faba beans leading to a darker colour. Wet fractionation of faba bean proteins increased their orangish tinge when compared to the *L* (LT: 87.77 ± 0.04 and HT: 87.86 ± 0.09), *a* (LT: -0.93 ± 0.01 and HT: -0.38 ± 0.01) and *b* (LT: 12.45 ± 0.03 and HT: 11.40 ± 0.12) values of the dehulled faba bean flours, which had a light green colour. AA proteins subjected to freeze-drying can lead to dark colour as compared to spray drying them (Sosulski & McCurdy, 1987) although Maillard browning reactions can take place during thermal treatment, due to the reaction between amino acid and/or peptides with carbohydrates (Sharan et al., 2021).

Chickpea protein fractions resulting from M and AA techniques were significantly ($P < 0.05$) darker, redder and yellower compared to the defatted flour according to Paredes-López et al. (1991). Similar to the findings of the present study, these authors found the M-PI to be similar in overall colour to the raw material.

The presence of pigments in faba bean seeds and the changes upon exposure to extraction conditions can hinder the utilisation of faba bean PCs/PIs as functional food ingredients in various food applications (Nasar-Abbas et al., 2009). Faba beans are also a good source of total polyphenols as mentioned in Chapter 6 (Table 6.5). Although total tannin content was significantly ($P < 0.05$) lower in the PCs/PIs as compared to their corresponding parent flours, the HT protein fractions had more total tannins as compared to LT. High tannins correlate to more proanthocyanidins, which can lead to dark discolouration in the presence of air (Nasar-Abbas et al., 2009) or get oxidized through phenolic reactions (Sharan et al., 2021).

8.3.5. Emulsifying properties of the protein fractions

Plant proteins can serve as emulsifiers and improve the thermodynamically unstable nature of dispersions by lowering the interfacial tension, preventing coalescence and enhancing emulsion stability (Dickinson 2010; McClements, 2015b). The emulsifying properties (EC, ESI) of PCs/PIs with canola oil are presented in Fig. 8.3. The ECs of AA-PCs were significantly ($P < 0.05$) higher than the rest of the samples regardless of the cultivar except for LT-W_{35}-D-PI. HT protein fractions had higher EC than LT except for W_{35}-D-PI.

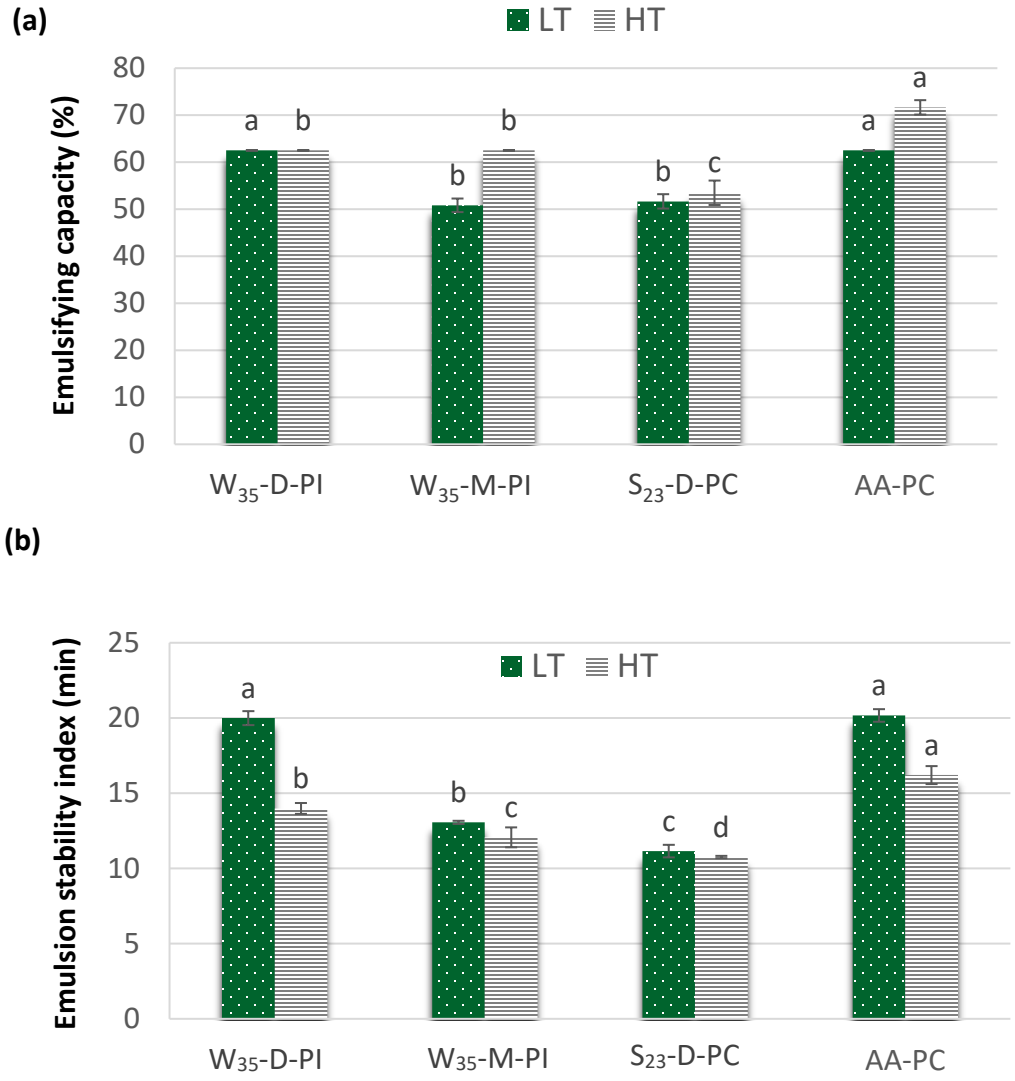


Fig. 8.3. Emulsifying capacity (%) and emulsifying stability index of faba bean proteins at pH 7.

[a-d]Mean (n = 3) ± SD values within a cultivar followed by different letters are significantly different ($P < 0.05$), according to Duncan's multiple range test.
Faba bean protein concentrates/isolates (PCs/PIs) of cultivars Snowbird (LT) and Athena (HT) treated with water at 35 °C followed by either dialysis (W_{35}-D) or micellization (W_{35}-M), or alkali extracted, and acid precipitated (AA), or treated with 1% $NaCl_{(aq)}$ solution at 23 °C followed by dialysis (S_{23}-D). (a) emulsifying Capacity (EC); and (b) emulsifying stability index (ESI).

In general, EC increased as the isolate surface charge and solubility increased, and surface hydrophobicity decreased (Karaca et al., 2011). The AA-PCs also had the highest hydration capacity as well as the lowest surface hydrophobicity but also the lowest protein solubility, which could be synergistically correlating to their high ECs. Interestingly, LT-W_{35}-D-PI also had a higher EC (Fig. 8.3a), which was not significantly different ($P > 0.05$) from LT-AA-PC.

According to the ESI values presented in Fig. 8.3b, overall LT cv. had better ESI as compared to HT, whereas the EC values were higher for HT as compared to LT in general. This pattern can be correlated to the emulsifying capacity of the residual tannins present in the HT fractions due to their amphiphilic nature (Figueroa-Espinoza et al., 2015). However, they might not be contributing to the ESI due to their affinity to protein to form complex, which could have hindered the consistency of the interfacial film over time.

The 7S globulins have been reported to show better emulsifying characteristics than the corresponding 11S globulins mainly due to their high solubility and surface hydrophobicity (Boye et al., 2010; Day, 2013). Although less surface-active, the 11S globulins with highly ordered native structures can form strong and unified interfacial films and stabilize the emulsions more efficiently. Similarly, albumins also show better emulsifying properties due to fewer structural constraints (Karaca et al., 2011). The PIs, in particular, contain both globulins and albumins, resulting from the accumulation of the amphiphilic proteins, which grow in size and number (Murray et al., 1979) and thus can lead to enhanced emulsifying properties.

The legumin/vicilin (L/V) ratio can impact the effectiveness of plant protein isolates as emulsifying agents. Shi and Nickerson (2022) reported LT-AA-PIs to have a high L/V ratio corresponding to better emulsion stability while negatively correlating to the emulsifying activity. The highest ESI values were observed for both LT-AA-PC and LT-W_{35}-D-PI and these values

were comparable to the values reported for LT-AA-PI at pH 3 and 7 by Shi and Nickerson (2022). According to Chapter 6, the S_{23}-D-PCs had the lowest L/V ratio (Appendix B: Table B.2) and exhibited both the lowest EC and ESI values. It is important to emphasize that both W_{35}-D-PIs and W_{35}-M-PIs were >90% pure PIs. Therefore their EC and ESI values should correlate to the proteins, whereas the purity of the S_{23}-D-PCs (70-82%) and AA-PCs (86-87%) were low (Chapter 6, Section 6.3.3) and other macro molecules present could have an impact on the EC and ESI values. Apart from the purity of the proteins, EC and ESI also depend on the protein/oil ratio (Nivala et al., 2017).

8.3.6. Foaming properties of the protein fractions

The foaming capacities of different faba bean protein fractions are presented in Fig. 8.4. As the FC of LT-S_{23}-D-PC was not significantly ($P > 0.05$) different from LT-W_{35}-M-PI, which was comparable to that of LT-W_{35}-D-PI and LT-AA-PC. All four protein fractions exhibited comparable FC values, however, HT-AA-PC had the lowest FC compared to the rest. The HT-W_{35}-PIs had superior FCs as compared to HT-S_{23}-D-PC and HT-AA-PC. The presence of tannins and other macromolecules in the PCs could alter FC values. The FS values were significantly ($P < 0.05$) higher for the AA-PCs regardless of the cultivar. The W_{35}-PIs had comparable FS and the significantly lowest FS was observed for S_{35}-D-PCs of both cultivars.

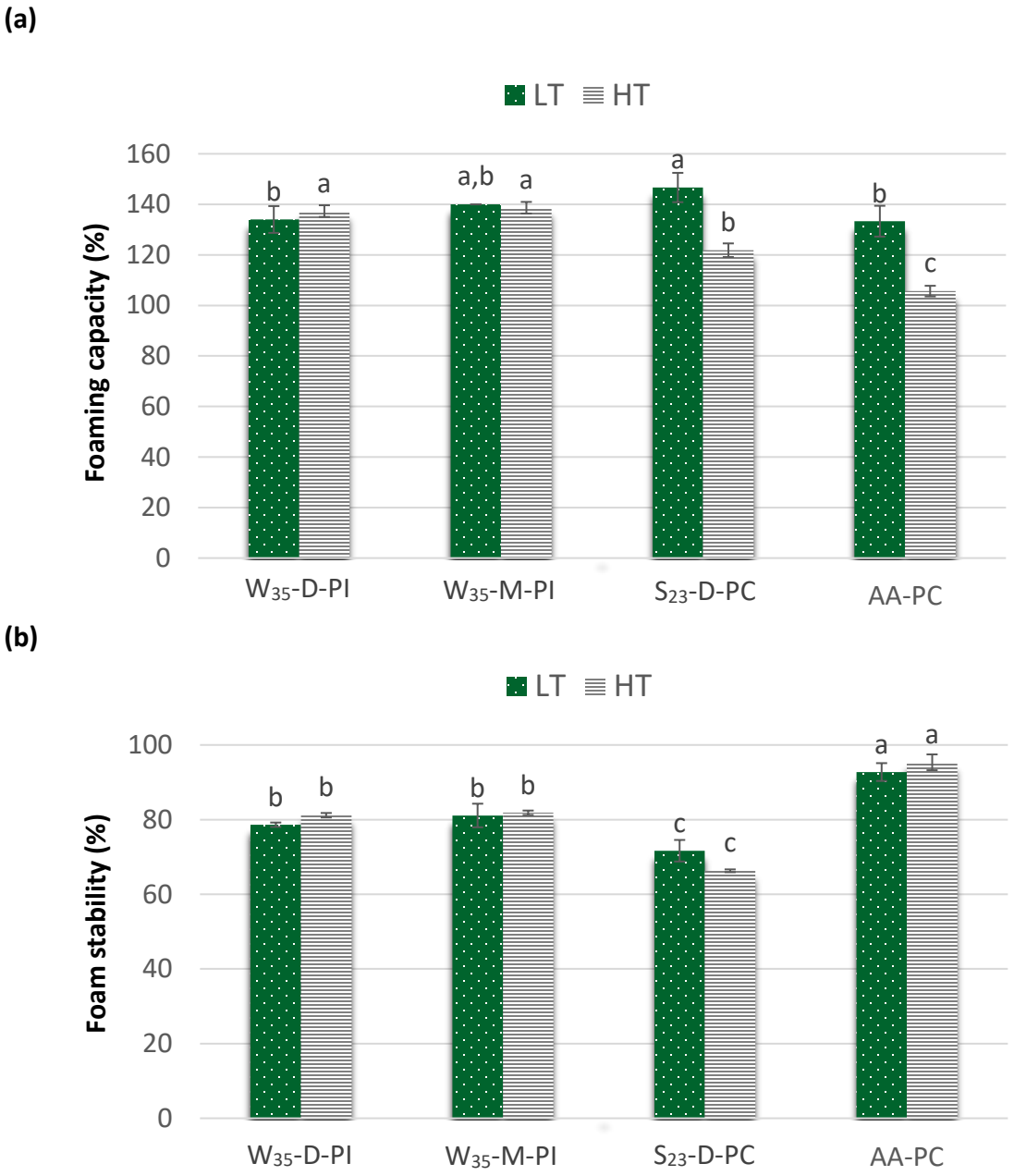


Fig. 8.4. (a) Foaming capacity and (b) stability (%) of Snowbird (LT) and Athena (HT) faba bean protein concentrates/isolates (PCs/PIs).

[a-d]Mean (n = 3) ± SD values within a cultivar followed by different letters are significantly different ($P < 0.05$), according to Duncan's multiple range test.

Faba bean protein concentrates/isolates (PCs/PIs) of cultivars Snowbird (LT) and Athena (HT) extracted with water at 35 °C followed by either dialysis at 4 °C (W_{35}-D) or micellization (W_{35}-M), or alkali extracted, and acid precipitated (AA), or treated with 1% $NaCl_{(aq)}$ solution at 23 °C followed by dialysis at 23 °C (S_{23}-D).

The PIs had albumins as well as globulins according to their SDS-PAGE profiles discussed in Chapter 6 (Section 6.3.4). It has been previously reported that albumins, were highly soluble and had improved ability to foam due to enhanced protein unfolding, but globulins were found to have lesser foaming capability due to their reduced ability to unfold/reorient at the interface, which restricts encapsulation of air bubbles (Mundi and Aluko 2012; Shevkani et al., 2019). Vose (1980) reported that faba bean PIs resulting from UF had superior foaming properties in comparison to soy and pea PIs, wheat flour as well as skim milk powder. According to Shevkani et al. (2015), increased protein surface area weakened hydrophobic interactions, increased protein solubility, and increased protein flexibility, allowing for faster protein spreading over the interface and faster encapsulation of air particles.

The principles behind foaming and emulsification are similar, however, the molecular requirements for protein activity at these interfaces are different. Thus, a protein fraction that is effective at emulsifying may not be effective at foaming (Damodaran, 2017). Foams primarily consist of dispersed air bubbles in the continuous water phase, which can separate in the absence of a surfactant (Jarpa-Parra, 2018). FC is an indicator of the increase in volume after whipping. It depends on the ability of proteins to diffuse to the interface, reorient, and form a viscous film without excessive aggregation, whereas FS reflects the ability of proteins to maintain the foam.

Surface hydrophobicity can impact the affinity of the PCs/PIs to the interfacial space and the adsorption process (Jarpa-Parra et al., 2016). Structural flexibility is also significant in surface denaturation and contact with other proteins or non-protein molecules upon film formation (Jarpa-Parra et al., 2014). These intrinsic characteristics are correlated to the suitability of a protein as a foaming agent. The factors that influence FC do not necessarily influence FS and should be considered separately. Small and flexible proteins present in egg and whey 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 the coalescence of air bubbles.

8.4. CONCLUSIONS

The findings of this study demonstrate the suitability of faba bean proteins as functional food ingredients. The functionality of proteins is of great importance in food formulations, as they are associated with the sensory, physicochemical, and mechanical characteristics of the final 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.

The W_{35}-PIs had superior solubility, colour parameters and foam stability as compared to AA-PCs, which make them a suitable ingredient in potential dairy-based alternative products. Emulsion stability and foaming and emulsifying capacities of the W_{35}-PIs were comparable to those of AA-PCs. The S_{23}-PCs had relatively limited properties probably due to the interference caused by the salt deposits, resulting in either salting in or salting out effect depending on the concentration of the suspension. Although AA-PCs had superior hydration capacities and low surface hydrophobicity, these properties cannot be exclusively attributed to the proteins as other macromolecules like starch and dietary fibre were also present in considerable amounts. Overall, PIs extracted with water and isolated by dialysis or micellization demonstrated superior physicochemical and functional properties compared to salt-extracted protein concentrates and are

chemical-free sustainable alternatives to alkali-extracted and acid-precipitated protein concentrates for value-added food applications.

CHAPTER 9: General conclusions and future research considerations

9.1. GENERAL CONCLUSIONS

The fast-increasing demand for sustainable protein ingredients, driven by growing consumer awareness of the environmental impacts associated with traditional animal protein production (Boland et al., 2013; Henchion et al., 2017), has led to the development of many fractionation and refining operations for pulses worldwide (Boye et al., 2010; Multari et al., 2015). Legumes are a valuable source of proteins and contain bioactive compounds, some of which can also act as anti-nutritional factors that interfere with nutrient absorption and bioavailability (Kumar et al., 2015; Mayer Labba et al 2021; Yegrem, 2021). One of the main challenges associated with sustainable protein ingredient production is the processing required to eliminate these anti-nutritional factors from the plant-based ingredients while isolating proteins, as processing methods such as soaking, germination, fermentation and cooking can only reduce their levels to a certain extent (Boukid & Castellari, 2022; Gemede & Ratta, 2014; Multari et al., 2015).

Currently, protein extraction technologies involve different dry and wet fractionation methods. Dry fractionation uses milling and air classification to concentrate the proteins (Pelgrom et al., 2015). While wet fractionation technologies use alkaline- or salt-based extraction followed by either isoelectric precipitation, ultrafiltration/diafiltration, or heat treatment to isolate the proteins (Langton et al., 2020; Singhal et al., 2016). These dry and wet methods are being explored individually or in combination as hybrid fractionation methods to extract pulse proteins (Dumoulin et al., 2021; Ruiz et al., 2016). However, these conventional protein extraction techniques have several limitations that need to be addressed, including lower yield, energy and resource intensive, negative impacts on the techno-functional properties of the proteins, reduced

nutritional quality, and undesirable colour and flavour (Chemat et al., 2019; Zhang et al., 2015). Therefore, it is essential to develop eco-friendly and innovative extraction technologies that can overcome these technological obstacles associated with sustainable protein ingredient development.

Micromorphological and elemental information on pulse cotyledon topographies is an essential aspect in identifying grain physiological and structural interactions at cotyledon-seed coat (abaxial) and cotyledon-cotyledon (adaxial) interfaces, which can influence grain quality and processing behaviour in food industry applications. The objective of the first study (**Chapter 3**) was to characterise the topographies of the abaxial and adaxial surfaces of two cultivars each of faba beans in comparison to chickpeas, field peas, and lentils via micromorphological and elemental analyses. Scanning electron microscopic imaging of the abaxial and adaxial cotyledon surfaces showed gentle undulation of either tabular- or polygonal-shaped epidermal cells that were projected as flat or rugose. They were covered by epicuticular wax films, which had smooth, striate, or granular micromorphology. Elemental results confirmed the presence of C, O, K, Ca, and Mg as the key elements on both cotyledon's abaxial and adaxial surfaces. Abaxial and adaxial cotyledon topographies of all the cultivars confirmed varietal differences associated with their micromorphological characteristics. It can be concluded that both abaxial and adaxial surfaces are cemented by a complex cuticle layer of similar elemental composition with a few significant ($P < 0.05$) variations. The ease of milling/pearling of pulses could be directly associated with the legume species, cultivar, architecture, micromorphology, and elemental composition of the pulse cotyledon abaxial and adaxial surfaces. These findings will not only assist in interpreting the differences observed in the processing behaviour of different pulse species and cultivars but will

also enhance our understanding of the process design, targeting pearling of pulses for value-added food applications.

In **Chapter 4**, the compositional analyses of sequentially pearled fractions of high-tannin (HT, Athena) and low-tannin (LT, Snowbird) faba bean (*Vicia faba* L.) cultivars demonstrated that the decreasing trend of protein, ash and total dietary fibre (TDF) contents from outer to inner layers was offset by starch contents. LT and HT proteins were positively correlated ($r = 0.9$, $P < 0.0001$) with ash and total dietary fibre contents while negatively correlated ($r = -0.9$, $P < 0.0001$) with starch contents with the increasing degree of pearling. The 55-57% single-step pearling flours had higher ($P < 0.05$) protein contents (LT: 39.07 ± 0.06% and HT: 37.37 ± 0.03%, N × 6.25) as compared to whole beans (LT: 30.51 ± 0.39% and HT: 29.63 ± 0.20%). The starch contents of the pearled beans were significantly higher ($P < 0.05$) than those of whole beans. These single-step pearling flour fractions subjected to aqueous fractionation to isolate proteins resulted in starch isolates and dietary fibre concentrates as co-products. Hybrid fractionation had minimal impact on the native protein secondary structures where β-sheets were dominant. This study suggests that the inclusion of pearling as an upstream processing step prior to wet fractionation of both LT and HT faba beans has the potential to generate novel "clean label" ingredients for the food industry. In the conventional protein, starch and dietary fibre separation processes, the dehulled bean by itself is utilised. By understanding the compositional distribution across the bean and by employing simple techniques, such as tangential abrasive dehulling (TAD), new dimensions can be opened for the value-added utilisation of pulse fractions. Most of the pulse flour-related product applications are prepared with highly refined flours that are lacking in health-promoting compounds. If the end-product focus is on minimally processed food ingredients, pearling can be used to preserve the native functionality of the proteins and not compromise their nutritional

properties. It is noteworthy that the underutilised high tannin faba bean cvs. can be better utilized by pearling, which can significantly reduce the tannin content while preserving the total polyphenol content. The hybrid fractionated protein isolates will be of great value if the end-use focuses more on the functional aspects of pure protein fractions. Overall, the study demonstrates a robust chemical-free mild approach in line with clean label and sustainability trends to add value to the faba bean processing industry to introduce clean-label plant proteins into the food chain.

In **Chapter 5**, the impact of air-currents-assisted particle separation (ACAPS) of faba beans prior to wet separation was evaluated on chemical composition, yield and recovery of protein, starch and dietary fibre, and protein secondary structure conformational changes. The protein and dietary fibre contents of faba bean cultivars LT and HT were found to be positively correlated ($r = 0.9$, $P < 0.0001$) with the increasing particle size, which was offset by the starch contents. ACAPS treatment of faba bean dehulled bean flours resulted in both protein- and dietary fibre-rich coarse fractions (75-500 μm). Subjecting the ACAPS-treated protein-rich (LT: 38.96 ± 0.33% and HT: 37.54 ± 0.20%) coarse fractions (250-500 μm) to water extraction followed by micellization resulted in protein isolates (LT: 97.74 ± 0.18% and HT: 94.30 ± 0.29%). This method also resulted in starch isolates (LT: 94.05 ± 1.67% and HT: 94.33 ± 0.53%) and dietary fibre concentrates (LT: 54.41 ± 1.87% and HT: 44.89 ± 1.40%) as co-products, which were "clean label" ingredients. Similar to the findings of Chapter 3, the FTIR data confirmed that hybrid fractionation had minimal impact on the protein secondary structure conformational changes with β-sheets as the major structures.

In **Chapter 6**, the impact of different chemical-free wet fractionation conditions on yield, purity and recovery of faba bean protein concentrates/isolates (PCs/PIs) was established. Although salt-soluble globulins (74-75%) were the primary proteins found in faba beans, PIs resulting from

water extraction at 35 °C (W_{35}) at a solvent/feed (S/F) ratio of 2 followed by dialysis at 4 °C (D) or micellization (M) had higher protein contents (93.2 ± 0.1% and 99.2 ± 0.1%, respectively, for LT and 96.4 ± 0.3% and 98.7 ± 0.2%, respectively, for HT). In comparison, the PCs resulting from $NaCl_{(aq)}$ solution (1% (w/v)) extraction at 23 ºC (S_{23}) followed by D (LT: 79.1 ± 0.1% and HT: 81.6 ± 0.5%) or alkali extraction followed by acid precipitation (LT: 87.1 ± 1.4% and HT: 86.3 ± 0.2%) had lower protein contents. These differences in protein extractability of W_{35} and S_{23} methods could be attributed to the high levels of naturally present minerals. Although methionine and cysteine were limiting, the W_{35} methods resulted in high-quality PIs compared to the rest due to the presence of albumins as confirmed by SDS-PAGE. The W_{35}-PIs were low in raffinose family oligosaccharides, tannins and trypsin inhibitory activity and yielded starch isolates and dietary fibre concentrates as co-products.

The utilisation of the Canadian high tannin faba bean cultivar on par with the low tannin cultivar in food applications requires an in-depth understanding of the impact of different wet fractionation methods on the physiochemical attributes as they can ultimately influence their functional properties. The study reported in **Chapter 7** was designed to provide insight into the physicochemical attributes of the PCs/PIs resulting from mild fractionation methods: W_{35}-D, W_{35}-M, and S_{23}-D in comparison to the AA method. As the cultivar and environmental factors also play a crucial role in the composition and structure of the proteins and the interaction with other macromolecules (Multari et al., 2015), PCs/PIs obtained from both LT and HT cultivars were selected for this study. The objective was to characterie the protein extracts for their secondary structural conformational changes using Fourier transform infrared spectroscopy (FTIR) and circular dichroism (CD), thermal property changes using differential scanning calorimetry (DSC), particle size distribution, and X-ray diffraction (XRD) patterns.

The mild extraction methods employing water and $NaCl_{(aq)}$ solution did not significantly influence the size distribution of the particles in their water suspensions and a substantial portion were small and easily dispersible particles, yet less uniform as compared to the larger AA-PC particles. The XRD patterns of faba bean PCs/PIs confirmed a pre-dominantly amorphous nature of the faba bean proteins. Based on XRD, FTIR and CD analyses, β-sheet structures were the most abundant secondary structures of faba bean proteins. The FTIR results demonstrated that the mild fractionation methods have less impact on the physicochemical characteristics of the protein secondary structure conformation. As the functional properties are largely dependent on the structural conformation, the selection of appropriate processing conditions during the application of these proteins is important. An association between the protein level, the glass transition temperature and the endothermic enthalpy of the denaturation peak obtained by DSC were observed among the PCs/PIs resulting from different extraction techniques. A high protein melting/degradation temperature of over 200 ºC regardless of the extraction method and cultivar suggests that the materials are thermally stable for high-temperature extrusion for food applications.

Depending on the method of extraction and the conditions used to isolate the proteins, their functionality may vary due to changes in their compositional and physicochemical characteristics and the magnitude to which these characteristics are affected (Jarpa-Parra et al., 2015; Singhal et al., 2016). Zeta potential, surface hydrophobicity, colour, foaming, solubility, emulsification and hydration attributes of the PC/PIs resulting from the extraction of LT and HT by mild wet fractionation methods in comparison to the conventional pH-shift method were investigated in **Chapter 8**. The outcome of this study demonstrates the suitability of faba bean as an alternative protein source to meet the nutritional requirements of the growing world population due to the

applicability of different mild fractionation technologies in developing functional food ingredients. The functionality of proteins is of great importance in food formulations, as they are associated with 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. The findings of this study showed that the W_{35}-PIs had superior solubility, colour parameters and foam stability as compared to the AA-PCs, which makes them a suitable ingredient in potential dairy-based alternative products. Emulsion stability and foaming and emulsifying capacities of the W_{35}-PIs were comparable to those of AA-PCs. The S_{23}-PCs had relatively limited properties probably due to the interference caused by the salt deposits, leading to either salting in or salting out effect depending on the concentration of the suspension. Although AA-PCs had superior hydration capacities and low surface hydrophobicity, these properties cannot be exclusively attributed to the proteins as starch and dietary fibre were also present in considerable amounts. Overall, W_{35}-PIs resulting from water extraction followed by dialysis or micellization demonstrated superior physicochemical and functional properties compared to S_{23}-D-PCs and are chemical-free sustainable alternatives to AA-PCs for value-added food applications.

Gelation is the most fundamental characteristic of proteins for functional food applications. Konjac glucomannan (KGM) is a water-soluble, neutral, high molecular-weight polysaccharide, with texture-enhancing and gelling properties (Zhou et al., 2014). Therefore, additional work (**Appendix C**) was carried out to evaluate the impact of protein extraction methods and KGM addition on the rheological and microstructural properties of faba bean protein gels. The gelation of faba bean protein was temperature-dependent, and primarily influenced by the degree of protein denaturation. Gels formed with both W_{35}-D-PIs and AA-PCs had dense and

fine network structures. LT-W_{35}-D-PI had the highest stress and strain at fracture at the minimum gelling concentration and formed the strongest gel due to the lower degree of denaturation during extraction. Protein gels enriched with 0.5% KGM promoted protein aggregation and water binding to form more stable complexes and the stability of the protein network structure by strengthening the hydrophobic interactions.

In summary, the hybrid-fractionation approach, involving single-step pearling by employing less-sophisticated techniques, such as TAD or the patented ACAPS high throughput sieve separation technique followed by subsequent aqueous fractionation, was a promising method for extracting dietary fibre concentrates alongside the high purity protein and starch isolates with large yields. The proposed hybrid fractionation approaches had minimal impact on protein secondary structure and can be utilized as native functional food ingredients in food applications. Furthermore, these novel mild fractionation approaches are flexible so that processing steps can be added or removed depending on the desired final ingredient composition and functionality. The findings suggested that the inclusion of pearling and ACAPS as upstream processing steps prior to wet fractionation of faba beans have the potential to create new markets for the underutilised high tannin faba bean cvs. and to generate novel "clean label" ingredients for the food industry. In the recent past, these research activities have attracted the attention of several organizations and companies in North America.

Although dry fractionation of faba bean protein is a sustainable alternative to energy-intensive wet fractionation approaches, it can only lead to relatively modest enrichment in protein content. Although salt-soluble globulins were the primary proteins found in faba beans, PIs resulting from water extraction followed by D and M had significantly higher protein purity as compared to protein fractions resulting from salt extraction. The proposed mild extraction method

(W_{35}) involving a low S/F ratio of 2 over the conventional S/F ratios of 10 to 20 often adopted by the industry was advantageous as the water usage was 21 kg/kg PI, which can be recycled.

Furthermore, solubility, foaming and emulsification properties, and hydration capacities of water-extracted protein isolates were superior or comparable to the conventional alkali-extracted and acid-precipitated PIs and salt-extracted PIs. The gelation and textural properties of the water-extracted protein gels were comparable to those of whole egg gels (Appendix C). Overall, these studies demonstrate a robust chemical-free mild approach in line with clean label and sustainability trends to add value to the faba bean processing industry. These outputs will be invaluable to the Canadian agri-food industry in developing strategies to produce superior protein ingredients, thereby increasing their competitiveness. From a consumer standpoint, these outcomes will meet their increasing demand for plant proteins.

9.2. FUTURE RESEARCH CONSIDERATIONS

There are several limitations associated with sustainable protein ingredient processing from faba beans. Depending on the processing method used, the protein yield can be low, leading to high production costs. In addition, the processing of faba bean proteins can generate significant amounts of starch isolate and dietary fibre concentrate as valuable co-products. Starch isolates subjected to modification can be used as thickeners, stabilizers, and gelling agents in a range of food applications (Ratnayake & Naguleswaran, 2021). Starch can also be converted into sweeteners such as glucose, fructose, and maltose, which are used in a variety of food and beverage products. Additionally, starch-based bioplastics have been developed as a sustainable alternative to conventional plastics. Starch co-products can be processed to produce biofuel, which is a renewable energy source that can be used as an alternative to fossil fuels (Lu et al.,

2020). The biofuel industry is growing rapidly, and the use of starch co-products as a feedstock for biofuel production can provide an additional revenue stream for plant protein manufacturers. Additionally, the high-fibre co-product is a valuable ingredient for the production of dietary fibre-based products such as prebiotics, which promote gut health, and fibre-enriched food products. Additionally, dietary fibre can be processed into a range of non-food products, including paper, textiles, and pharmaceuticals. Both starch and dietary fibre co-products can also be used as animal feed, providing a sustainable source of nutrition for livestock. For example, starch co-products can be used to create high-energy animal feed, while dietary fibre co-products can be used as a source of fibre for ruminant animals such as cows (Ratnayake & Naguleswaran, 2021). Overall, the co-products resulting from the plant protein industry, including starch and dietary fibre, offer a range of value-added opportunities that can contribute to the sustainability and profitability of the industry. However, further work is needed focusing on the performance of such faba bean co-products in specific applications and their scale-up. By utilizing these co-products, the faba bean protein industry can create a more circular economy, reducing waste and creating new products with multiple applications.

Faba bean protein ingredients can have a strong flavour and gritty texture that may be unappealing to some consumers. This can limit their use in certain food applications, particularly those that require a neutral taste and smooth texture. Further research is needed in developing processing methods that can improve the flavour and texture of faba bean PCs/PIs. Sour and stale notes have been previously reported in isolated faba proteins (Hinchcliffe et al., 1977). The beany flavour of faba bean protein isolates reduced by solvent defatting and acid washing indicates that lipid-associated flavour compounds may be responsible for the off-flavour (Güzel and Saya, 2012). Recent studies have revealed that lipid removal by supercritical carbon dioxide (SC-CO_2)

enabled the concentration of the main components of oats into specific fractions (Sibakov et al., 2011; Temelli, 2009) and facilitated the removal of beany flavour components in soy (Maheshwari et al., 1995). Based on the lipid class profile of faba bean established by Yoshida et al. (2008), the extraction can be done with SC-CO_2 alone followed by ethanol introduced as a co-solvent into SC-CO_2 to recover neutral and polar lipids, respectively. Headspace-solid phase microextraction followed by gas chromatography-mass spectrometry can be used before and after lipid removal to evaluate the impact of lipid removal on the flavour profile. The defatted meal can also be subjected to dry fractionation including ACAPS to explore the impact of defatting on particle size-based protein separation efficiency.

Faba beans are a substantial source of the pyrimidine glycosides vicine and convicine, which can cause favism in individuals lacking glucose-6-phosphate dehydrogenase (Khazaei et al., 2019). Their levels, need to be measured in the protein ingredients in comparison to the raw materials in addition to the micronutrients. Although there has been significant advancement in the processing of sustainable protein ingredients from legumes, there is still a need for further research and development to improve the nutritional value and digestibility of these protein sources. Additional work is essential for product development utilising the PCs/PIs obtained and assessing their sensory quality.

Studies have revealed that both physical and non-thermal technologies can effectively decrease the levels of antinutritional components in legumes and oilseeds, ultimately elevating protein digestibility (Luo et al., 2009; Patterson et al., 2017). Nonetheless, the precise mechanisms behind this enhanced protein digestibility and functionality have yet to be comprehensively understood (Shi et al., 2022; Shi & Nickerson, 2022). The environmental repercussions of traditional extraction technologies continue to be a pressing concern in the protein industry (Boer

and Aiking, 2011). In recent years, innovative eco-friendly extraction methods involving treatment with cold plasma, deep eutectic solvents (Yu et al., 2021), enzymes (Nivala et al., 2017; Wei et al., 2018), microwave (Jiang et al., 2016), pulse electric field (Li et al., 2007) reverse micelles (Zhao et al., 2018), subcritical water (Zhang et al., 2018) and ultrasonication (Martínez-Velasco et al., 2018) have shown promising results in enhancing the protein yield and its nutritional, physicochemical and techno-functional properties in addition to being clean-label, and sustainable. The potential of the above techniques in faba bean protein extraction needs further investigation.

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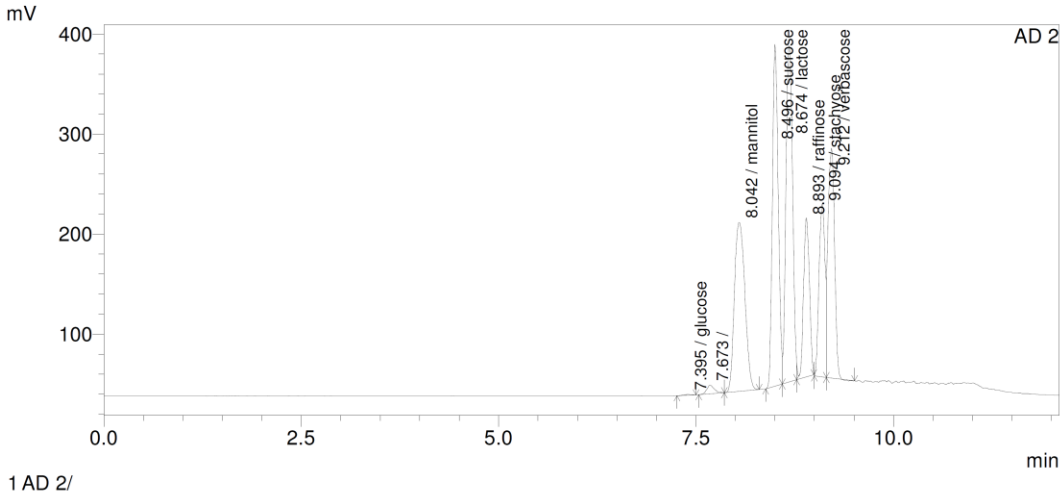
APPENDICES

Appendix A **(Supplementary Information - CHAPTER 4)**



PeakTable

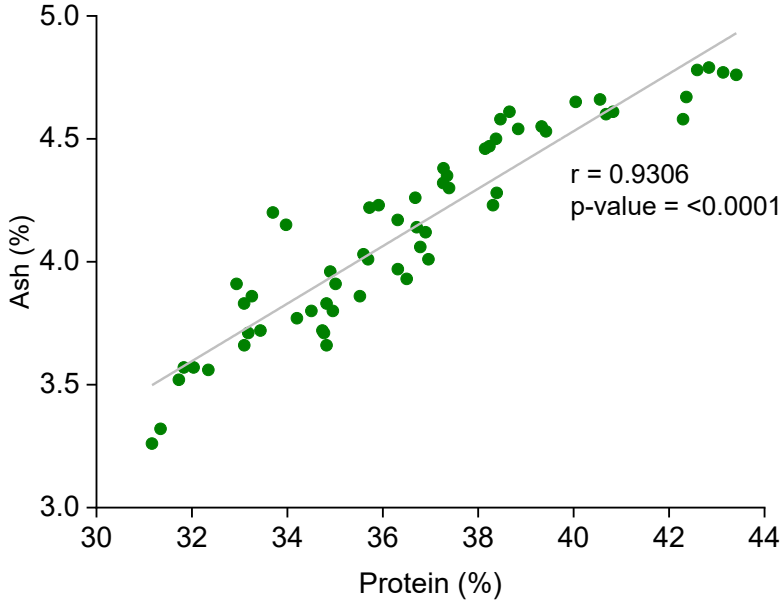
AD2 Ch1

Peak#	Ret. Time	Area	Height	Area %	Height %	Name
1	7.395	6214	854	0.074	0.060	glucose
2	7.673	58902	8731	0.701	0.615	
3	8.042	1558234	168876	18.541	11.899	mannitol
4	8.496	1754234	341684	20.873	24.075	sucrose
5	8.674	1957434	336221	23.290	23.690	lactose
6	8.893	831878	159186	9.898	11.216	raffinose
7	9.094	927939	173909	11.041	12.254	stachyose
8	9.212	1309621	229777	15.582	16.190	verbascose
Total		8404456	1419238	100.000	100.000	

Fig. A.1. HPLC chromatogram of a standard solution containing glucose, mannitol, sucrose, lactose (internal standard), raffinose, stachyose and verbascose.

HPLC peaks are identified in the chromatogram as well as the peak table.

(a1)


5.0
4.5
4.0
3.5
3.0
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40
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Protein (%)
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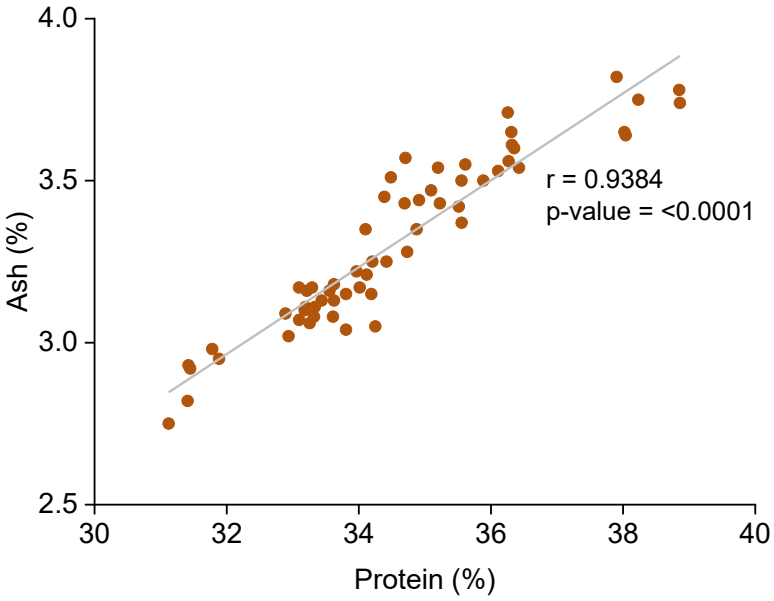
p-value = <0.0001

(a2)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Protein (%)
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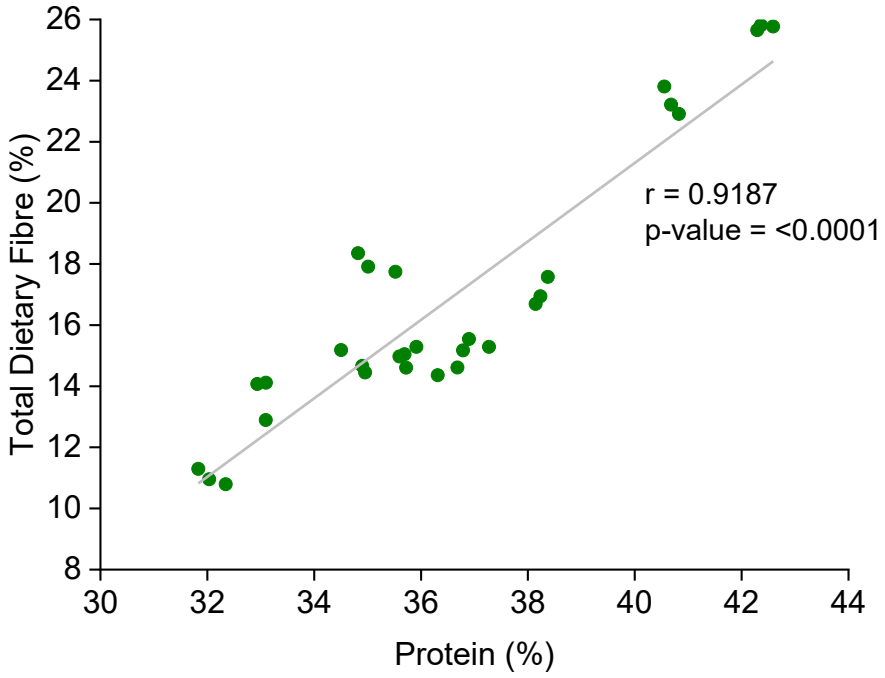
(b1)


Total Dietary Fibre (%)
Protein (%)
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p-value = <0.0001
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18
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10
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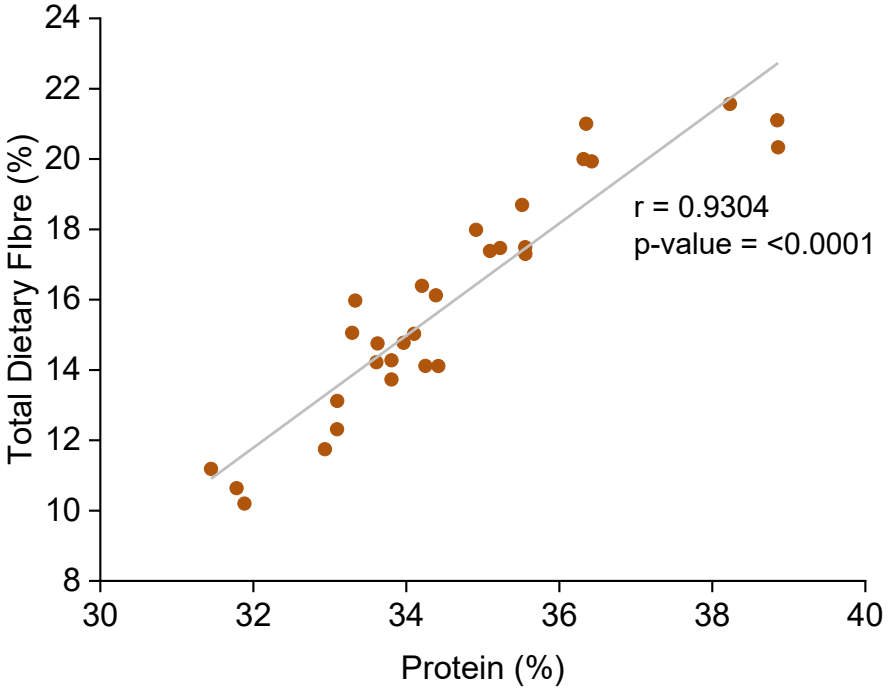
30
32
34
36
38
40
42
44

(b2)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Protein (%)
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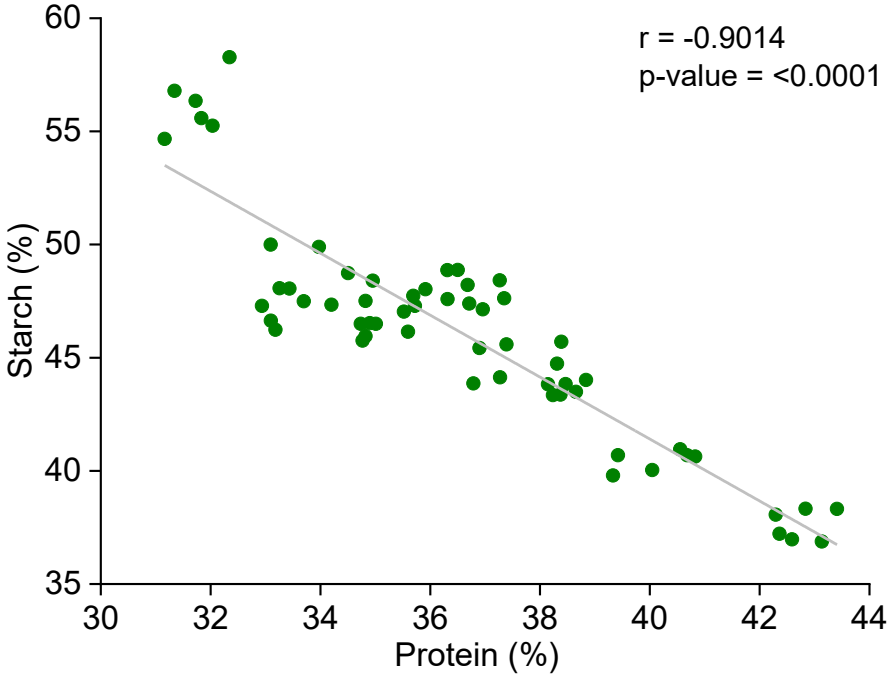
36
38
40

(c1)



(c2)

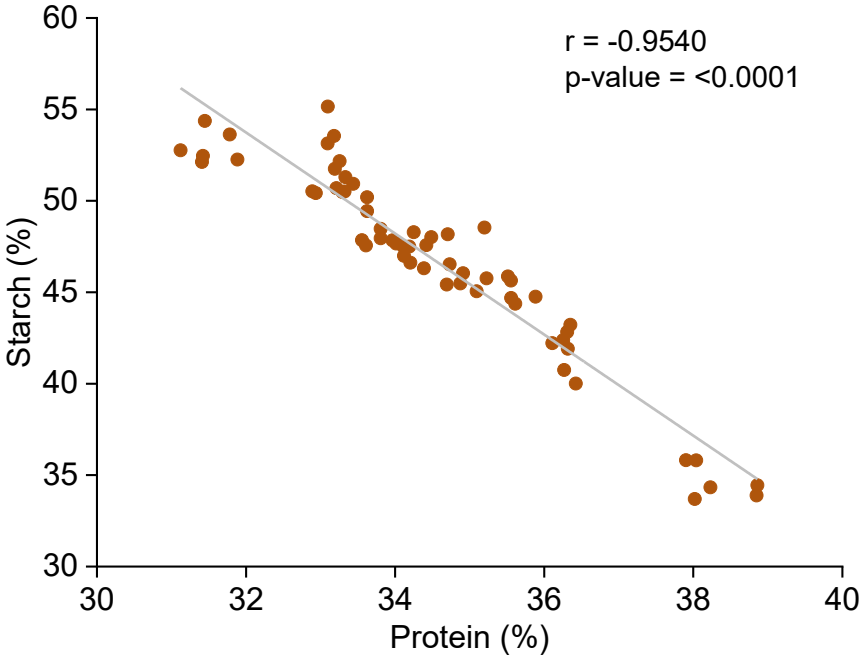


Fig. A.2. Linear correlation analysis of faba bean protein, starch and total dietary fibre distribution.

a) protein and ash, **(b)** protein and total dietary fibre and **(c)** protein and total starch content of Snowbird **(1)** and Athena **(2)** cultivars between different faba bean pearling fractions. Pearson's correlation coefficient (r) with significance ($P < 0.05$) for the different macronutrients between the different pearling flour fractions (2-10) and pearls is given (n = 60 for a and b and n = 30 for c)**.**

Appendix B **(Supplementary Information – CHAPTER 6)**

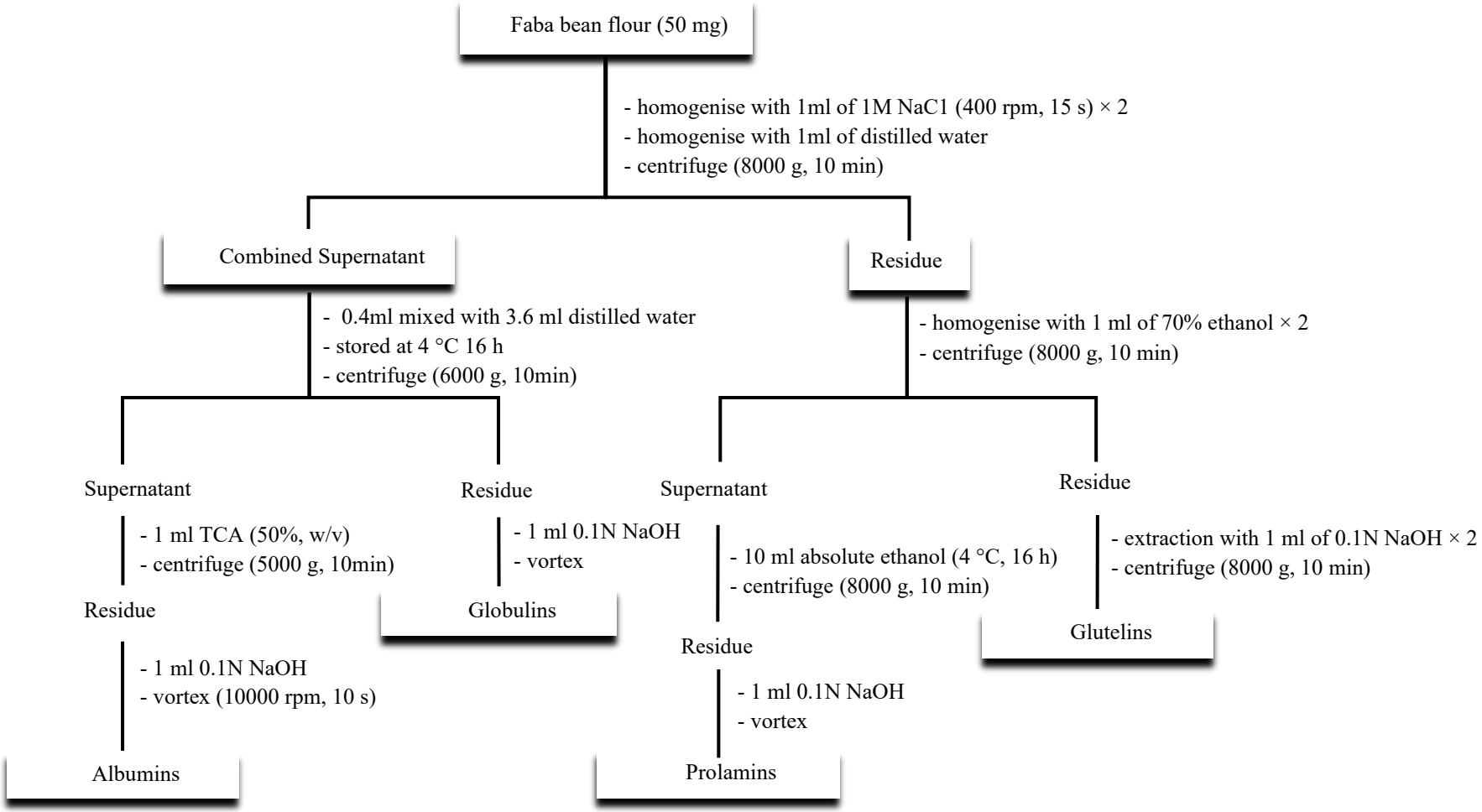


Fig. B.1. Flow diagram of Osborne's solubility-based extraction of faba bean proteins (adapted from Peterson & Smith (1976)).

Table B.1. Osborne's solubility-based classification of faba bean proteins on a dry basis (%)

Cultivar	Globulins	Albumins	Prolamins	Glutelins
Snowbird (LT)	74.81 ± 1.70	22.29 ± 1.87	2.20 ± 0.01	0.19 ± 0.04
Athena (HT)	74.45 ± 1.71	22.77 ± 1.82	2.20 ± 0.06	0.19 ± 0.02

Mean (n = 6) ± SD values expressed as a percentage of the total protein content.
Values in the same column are not significantly different at $P > 0.05$ according to the two-sample t-test

Table B.2. Legumin/Vicilin (L/V) ratio of faba bean protein fractions

Cultivar	Protein Fraction	L/V ratio
Snowbird (LT)	W_{35}-D	3.55 ± 0.22^{ab}
	W_{35}-M	3.76 ± 0.02^{ab}
	S_{23}-D	2.73 ± 0.13^{d}
	AA	3.44 ± 0.20^{ab}
Athena (HT)	W_{35}-D	3.39 ± 0.26^{ab}
	W_{35}-M	3.23 ± 0.11^{bc}
	S_{23}-D	3.01 ± 0.09^{cd}
	AA	3.63 ± 0.20^{ab}

$^{a\text{-}d}$Mean (n = 3) ± SD values within the column followed by different letters are significantly different ($P < 0.05$), according to Duncan's multiple range test.

Snowbird (LT) and Athena (HT) faba bean cultivars treated with water at 35 °C (W_{35}) or salt at 23 °C (S_{23}) followed by either dialysis (D) or micellization (M)

Appendix C **(Supplementary Information – CHAPTER 8)**

Impact of protein extraction methods and konjac glucomannan on the gelling, textural and microstructural characteristics of faba bean proteins[8]

[8]A version of Annex C will be published: Jeganathan, B., Vasanthan, T., & Temelli, F. Impact of protein extraction methods and konjac glucomannan on the rheological and textural and microstructural characteristics of faba bean (*Vicia faba* L.) proteins.

C.1. INTRODUCTION

Gelation behaviour is one of the most important fundamental characteristics of plant proteins for functional food applications (Shevkani et al., 2019). However, rheological attributes of plant proteins have been less explored as compared to synthetic polymers or animal proteins (van Vliet et al., 2002). Amongst the plant proteins, soy proteins have emerged as a prominent functional and nutritional ingredient as well as a plant-based substitute for milk, meat and egg protein (Hettiarachchy & Kalapathy, 1997; Sun et al., 2011). However, with the increasing demand for planet-friendly proteins, a recent trend to explore the potential of under-utilised alternative protein sources with similar functional and nutritional properties has emerged (Day, 2013; Henchion et al., 2017). Faba bean (*Vicia faba* L.) is a gluten-free pulse that is abundant in proteins, predominantly legumins and vicilin, which are the major globular proteins found in soy (Nivala et al., 2017). These proteins, akin to soy protein, play a vital role in conferring functional properties (Multari et al., 2015). There is potential for faba bean proteins to be an alternative to soy, although limited studies have explored their gelation properties (Johansson et al., 2022; Nivala et al., 2021; Vogelsang-O'Dwyer et al., 2020), which is needed to recognize their full potential.

The conventional methods used for extracting pea proteins can lead to extensive protein denaturation and diminished gelation ability primarily due to extreme pH conditions or excessive heating of proteins at high temperatures (Joshi et al. 2017). The gelation attributes of pea protein isolate extracted by a mild salt-extraction method resulting in a lower level of denaturation exhibited improved gelation properties as compared to conventional pea protein isolates extracted by the pH shift method (Sun & Arntfield, 2010 & 2011). With the increasing demand for mildly treated proteins free of chemicals, there is a need to explore the gelation properties of the mildly extracted faba bean proteins in comparison to the proteins extracted by the conventional methods.

In addition, it is essential to demonstrate interactions between faba bean protein, polysaccharides, and oil considering potential food applications.

Amorphophallus konjac tubers are the source of konjac glucomannan (KGM), a neutral water-soluble high molecular weight (200-2000 kDa) polysaccharide (Ji et al., 2017; Zhang et al., 2023). According to the findings of Kato and Matsuda (1969), it is made up of β-1,4-linked D-glucose and D-mannose residues with a molar ratio of 1:1.6. KGM has anti-obesity, anti-diabetic, anti-inflammatory and laxative properties and is utilised for drug delivery in the pharmaceutical industry due to its ability to biodegrade and form gels (Devaraj et al., 2019). In addition, it has a track record of being employed as a thickener, attributed to its capability to enhance texture and form gels (Zhang et al., 2023; Zhou et al., 2014). KGM has been documented as having the ability to facilitate protein aggregation and strengthen the resilience of the gluten network structure in the process of making bread. This is primarily attributed to its remarkable hydrophobic interactions and the superior capacity to bind water, which result in the formation of more robust complexes (Wang et al., 2017; Zhang et al., 2023; Zhao et al., 2017). On the other hand, alternative studies have indicated that KGM can potentially diminish the available water for gluten network formation during dough making, which in turn can decrease the number of disulphide linkages within the dough subsequently preventing the aggregation of glutenin (Zhou et al., 2014). Despite the valuable insights provided by these studies regarding the positive impact of KGM on bread quality, limited information exists regarding the influence of KGM on the aggregation behaviour and gelation properties of pulse proteins.

Eggs are known to play a significant role as a food ingredient in food formulations owing to their gelling, foaming and emulsification properties (Boukid & Gagaoua, 2022). Eggs are also one of the most widely consumed animal-based food products, which are also high in cholesterol

(Xia et al., 2020). With the increasing demand for vegan diets, there is an increasing demand for vegan egg substitutes (Alcorta et al., 2021). Manufacturers have developed vegan egg substitutes utilising different plant-based substances (such as proteins, and polysaccharide hydrocolloids) to satisfy the growing vegan and health-conscious markets (Boukid & Gagaoua, 2022). Considering this new prospect, the objective of the present study was to understand the impact of different extraction methods (mild and pH-shift) as well as the addition of konjac glucomannan as well as canola oil on the rheological, textural and microstructural characteristics of faba bean protein gels in comparison to fresh whole eggs. Selected faba bean protein fractions from Chapter 6 (Section 6.2.3) were studied to evaluate these essential attributes for formulating egg analogues involving gel network structures, incorporating varying levels of glucomannan and canola oil.

C.2. MATERIALS AND METHODS

C.2.1. Materials

Faba bean Snowbird (LT) and Athena (HT) protein isolates (PIs) extracted with water (W_{23}) at 35 °C followed by either dialysis (D) or micellization (M), and protein concentrates (PCs) extracted with alkali and precipitated with acid (AA) and extracted with salt at 23 °C followed by dialysis using a 6-8 kDa molecular weight cut-off (MWCO) dialysis tubing (S_{23}-D) described in Chapter 6 (Section 6.2.3) were selected for this study. Konjac root glucomannan was obtained from Now Foods (Guelph, ON, Canada). Organic canola oil was obtained from Highwood Crossing Foods Ltd. (High River, AB, Canada). Commercial brown shell eggs (Grade AA) were obtained from Walmart (Edmonton, AB, Canada).

C.2.2. Characterisation of rheological and textural properties

C.2.2.1. Gel formation and minimum gelling concentration

Faba bean protein dispersions of 6-22% (w/v) concentration prepared with PCs/PIs in demineralised water by vortexing for 6 s and macerating in a homogeniser (Polytron, PTMR 2100, Kinematica AG, Malters, Switzerland) at 15,000 rpm for 30 s were hydrated overnight at 4 °C. The tubes were equilibrated to room temperature (23 °C) in a hand shaker (Burrell Wrist Action Shaker, 75–765 BT, Pittsburgh, PA, USA) at a high setting (10) for 1 h. The tubes were heated at 90 °C in a water bath for 30 min, cooled under running tap water, and were allowed to stand overnight at 4 °C. Minimum gelling protein concentration was taken as the concentration at which there was no liquid flow in the inverted tubes (Vogelsang-O'Dwyer et al., 2020).

C.2.2.2. Rheological measurements of small deformation

Rheological properties of 3% glucomannan, fresh whole eggs, faba bean PCs/PIs (at their minimum gelling concentration), PC/PI (20%) + glucomannan (0.2%) and PC/PI (18%) + glucomannan (0.5%) + canola oil (9%) in water suspensions and LT-S_{23}-D-PC and LT-W_{35}-D-PI suspensions with thermo stable α-amylase were tested in a small oscillatory rheometer (HR-3, TA Instrument, New Castle, DE, USA) with a plate geometry (diameter: 40 mm; truncation gap 50 μm). Fresh whole eggs were homogenised (Heidolph DIAX 900, Instruments GmbH &Co. KG, Schwabach, Germany) with the 6G-coarse shaft (P/N 596-06000-00) at 14,000 rpm for 30 s at 25 °C and faba bean PC/PI suspensions were freshly prepared and hydrated at 4 ºC according to Section C.2.2.1, equilibrated at 25 °C for 30 min prior to each test and ~ 1 mL of the suspension was loaded onto the lower plate and the parallel plate was lowered to a gap width of 1.00 mm. A thermally stable environment was created with a solvent trap cover and a thin layer of light

mineral oil to prevent moisture loss from the samples during heating. Samples were initially equilibrated at 25°C for 2 min and then the temperature ramp tests were carried out from 25 °C to 90 °C to investigate the relationship between viscoelastic moduli and temperature (Vogelsang-O'Dwyer et al., 2020)

The tests were performed at an oscillating strain of 1%, a frequency of 1 Hz, and a heating rate of 2 °C/min. Samples were held at 90 °C for 30 min and cooled to 25 °C at a cooling rate of 2 °C/min and conditioned at 25 °C for 10 min. The storage modulus (G') and loss modulus (G") were determined as a function of temperature for each sample. The loss tangent or tan delta (tan δ = G"/G'), a measure of the energy lost due to viscous flow compared to the energy stored due to elastic deformation in a single deformation cycle, was also calculated (Sun & Arntfield, 2011).

C.2.2.3. Rheological measurements of large deformation

Compression tests were performed using an Instron 5967 universal testing machine (Instron Corp., Norwood, MA, USA) equipped with a 500 N load cell and a 50 mm cylindrical aluminium probe. Gels from fresh whole eggs, faba bean PCs/PIs (at their minimum gelling concentration), and PC/PI (20%) + glucomannan (0.2%) and PC/PI (18%) + glucomannan (0.5%) + canola oil (9%) in water suspensions were prepared using the formulations provided under Section C.2.2.2 and were sliced into cylindrical pieces with 13 mm diameter and 10 mm height using a cutting die. Egg protein gels were formed by holding at both 90 ºC and 75 ºC for 30 min. The samples were compressed to 70% of the original height at a rate of 1 mm/s. Young's modulus, compressive stress at 40% strain as well as compressive stress and strain at fracture were established. The fracture was identified either as the first peak in the stress-strain graph or as the beginning of the first plateau, when a distinct peak was not discernible (Langton et al., 2020).

C.2.2.4. Microstructural characterisation

Gels prepared according to Section C.2.2.1 were sliced using a jigsaw to examine the internal microstructure and were freeze-dried (Labconco 401904, Kansas City, MO, USA) for 24 h followed by imaging using a variable pressure-field emission SEM (VP-FESEM, Zeiss Sigma 300, Oberkochen, BW, Germany) according to Chapter 3 (Section 3.2.3).

C.2.3. Statistical analysis

All the experiments were run at least thrice (n=3) unless specified otherwise. Duncan's multiple range test was used as the mean separation technique ($P < 0.05$). Significant differences ($P < 0.05$) between samples were tested according to the two-sample t-test All these statistical analyses were carried out using SAS Studio University Version for Windows (SAS Institute Inc., Cary, NC, USA).

C.3. RESULTS AND DISCUSSION (SELECTED)

C.3.1. Minimum gelling concentration

Gelation is an important aspect of various food applications, and the minimum gelling concentration is an essential measure to know the minimum concentration of the protein sample required to form a stable gel. The minimum gelling concentration of W_{35}-D-PIs, W_{35}-M-PIs, S_{23}-D-PCs and AA-PCs were 13%, 13%, 22% and 11%, for LT and 12%, 13%, 23% and 12% for HT, respectively. Although the PCs were less pure compared to the PIs and contained other macromolecules which could contribute to their minimum gelling concentration, except for S_{23}-D-PCs, the rest had a very similar minimum gelling concentration (12-13%). This could mean

that proteins played a significant role as compared to other macromolecules (starch and dietary fibre) in the formation of the heat-induced PI gels.

The minimum gelling concentrations of faba bean PCs resulting from air classification and PI resulting from pH-shift methods were reported as 7% and 12%, respectively (Vogelsang-O'Dwyer et al., 2020). The minimum gelling concentrations of the water-extracted PIs and AA-PCs in the present study were comparable to these findings regardless of the cultivar. These results confirm that the purity of the protein plays a major role as compared to the cultivar in the formation of heat-induced gels. On the other hand, the presence of the hydrocolloids starch and dietary fibre could positively contribute to the formation of heat-induced gels even though the proteins in the AA-PCs could have been negatively influenced by the extraction conditions, as the minimum gelling concentrations were 11 and 12% in the present study and 7% according to the findings of Vogelsang-O'Dwyer et al. (2020).

On the contrary, salt deposits on the surface of S_{23}-D-PCs as shown in Chapter 6 (Section 6.3.3, Fig. 6.2) SEM images at a low solvent/feed ratio (S/F), could have interfered with their gelation properties. According to Sun and Arntfield (2011), pea PI suspension at 14.5% concentration formed stronger heat-induced gels. However, in their study, a S/F ratio of 3 was used with 0.3 M $NaCl_{(aq)}$ solution and the extract was dialysed for 72 h; whereas, in the present study, a S/F ratio of 2 was used with 1% $NaCl_{(aq)}$ solution followed by dialysis for only 48 h. In addition to the differences associated with the extraction method, the faba bean flour samples had higher mineral content, which could have led to the salting out effect as discussed in Chapter 6 (Section 6.3.1).

C.3.2. Rheological characteristics

Rheological measurements can indicate the relation between the small/ large deformation (strain) of a material and the stress employed to attain such deformation as a function of time. At small deformation, the measurement is performed without damaging the microstructure of the gel so that the obtained modulus is independent of the applied strain particularly in the linear regime. However, with large deformation measurements, the gels are mostly deformed until fracture takes place.

C.3.2.1. Rheological characteristics at small deformation

The typical trends and differences in the gelling behaviours of PCs/PIs at their minimum gelling concentration were reflected in the rheological heating/cooling cycle (Fig. C.1). The heat-induced gelation of globular proteins undergoes denaturation during the heating cycle, aggregation during the holding cycle, and the formation of a continuous three-dimensional protein network during the cooling cycle, which can lead to important functional characteristics for food applications (van Vliet et al., 2002; Vogelsang-O'Dwyer et al., 2020). Non-covalent (hydrogen bonds and electrostatic and hydrophobic interactions) and covalent (disulfide bonds) bonds may be involved in gelation (Langton et al., 2020; Vogelsang-O'Dwyer et al., 2020). Small oscillatory (dynamic) testing is more sensitive to changes in the chemical composition and physical structure of the sample (Sun et al., 2010). According to Fig. C.1, the onset of G' increase occurred earlier, at lower temperatures, for all the mild-extracted protein gels. A similar early increase may exist for the S_{23}-D-PCs, but maybe the rheometer was not sensitive enough to detect it, or the salt deposits present (Chapter 6, Fig. 6.2.) could be interfering.

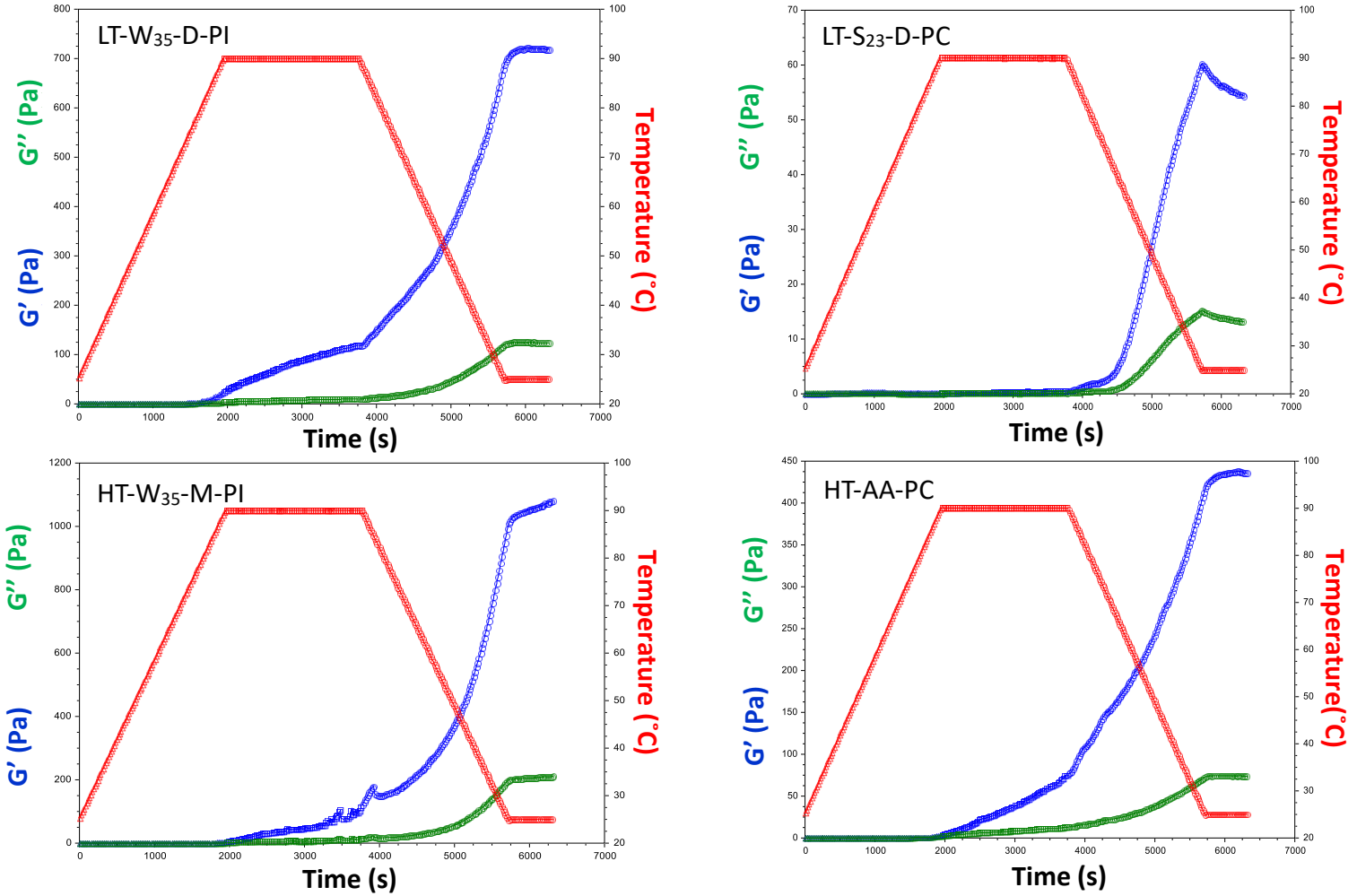


Fig. C.1. Typical patterns of the changes in the storage modulus (G') and loss modulus (G") of faba bean protein concentrates/isolates (PCs/PIs) during temperature-induced gelation in a rheometer.

Snowbird (LT) and Athena (HT) faba bean proteins extracted with water at 35 °C followed by either dialysis at 4 °C (W_{35}-D) or micellization (W_{35}-M), or alkali-extracted and acid precipitated (AA) or extracted with 1% $NaCl_{(aq)}$ solution at 23 °C followed by dialysis (S_{23}-D).

The final values of G' for W_{35}-D-PIs and W_{35}-M-PIs were greater than those of PCs indicative of the formation of a stronger gel network. The final tan δ values of the PCs/PIs were <0.26 relating to the elastic nature of the structures formed after the heating and cooling cycles. These results were in agreement with the findings of Vogelsang-O'Dwyer et al. (2020). According to the findings of Langton et al. (2020), the effect of both pH and NaCl on the final G' value of faba bean protein gels was significant, with an increase in either pH or NaCl leading to a decrease in the final G' value. The G' values showed a drastic increase during the cooling cycle as compared to the increase during the heating cycle for all the PCs/PIs in the present study regardless of the cultivar, which corresponds to the increasing gel strength during the cooling cycle. The final values of G' for W_{35}-D-PIs and W_{35}-M-PIs were greater than those of PCs indicative of a stronger gel network.

The final tan δ values of the PCs/PIs were <0.26 relating to the elastic nature of the structures formed after the heating and cooling cycles. These results were in agreement with the findings of Vogelsang-O'Dwyer et al. (2020). According to the findings of Langton et al. (2020), the effect of both pH and NaCl on the final G' value of faba bean protein gels was significant, with an increase in either pH or NaCl leading to a decrease in the final G' value. The G' values showed a drastic increase during the cooling cycle for all the PCs/PIs in the present study regardless of the cultivar, which corresponds to the increasing gel strength.

Dehulled faba beans contain considerable levels of carbohydrates, in particular, total starch (~48%) and dietary fibre (~11%), in addition to protein (Chapter 4, Table 4.2). The role of starch in mixed gels formed by combining protein and starch from various plant sources can vary depending on whether the starch gels before or after the protein (Johansson et al., 2022). Since faba bean PCs/PIs contained different levels of starch (Chapter 6, Tables 6.2-6.4), it was

important to determine the contribution of starch in the formation of protein gels (Fig. C.2). Experimental trials were conducted in the presence of thermostable α-amylase to hydrolyse starch to dextran in the starch-rich LT-S_{23}-D-PC and low-starch LT-W_{35}-D-PI samples (Fig. C.2). These trails confirmed that although the gelation patterns observed in Fig. C.1 are resulting primarily from the proteins, starch was also playing a contributing role as the final G' values have significantly dropped (Fig. C.2) following thermostable α-amylase treatment.

When the starch has a higher gelatinisation temperature and the protein undergoes gelation first, diffusion and network formation by amylose will be hindered, whereby starch might function primarily as a filler than actively participating in gel formation (Olsson et al., 2002). Whereas, when starch gelatinises before proteins in a protein/starch mixture, it will form an independent network that does not interact specifically with the protein (Johansson et al., 2022). The denaturation temperature of faba bean PIs at a high moisture content (75%) was ~95 °C (Chapter 7, Section 7.3.4), whereas the gelatinisation temperature of faba bean native starch was reported between 60-76 ºC (Dong et al., 2022). Therefore, it possible that the starch formed a gel first, which had minimal interactions with the protein gel.

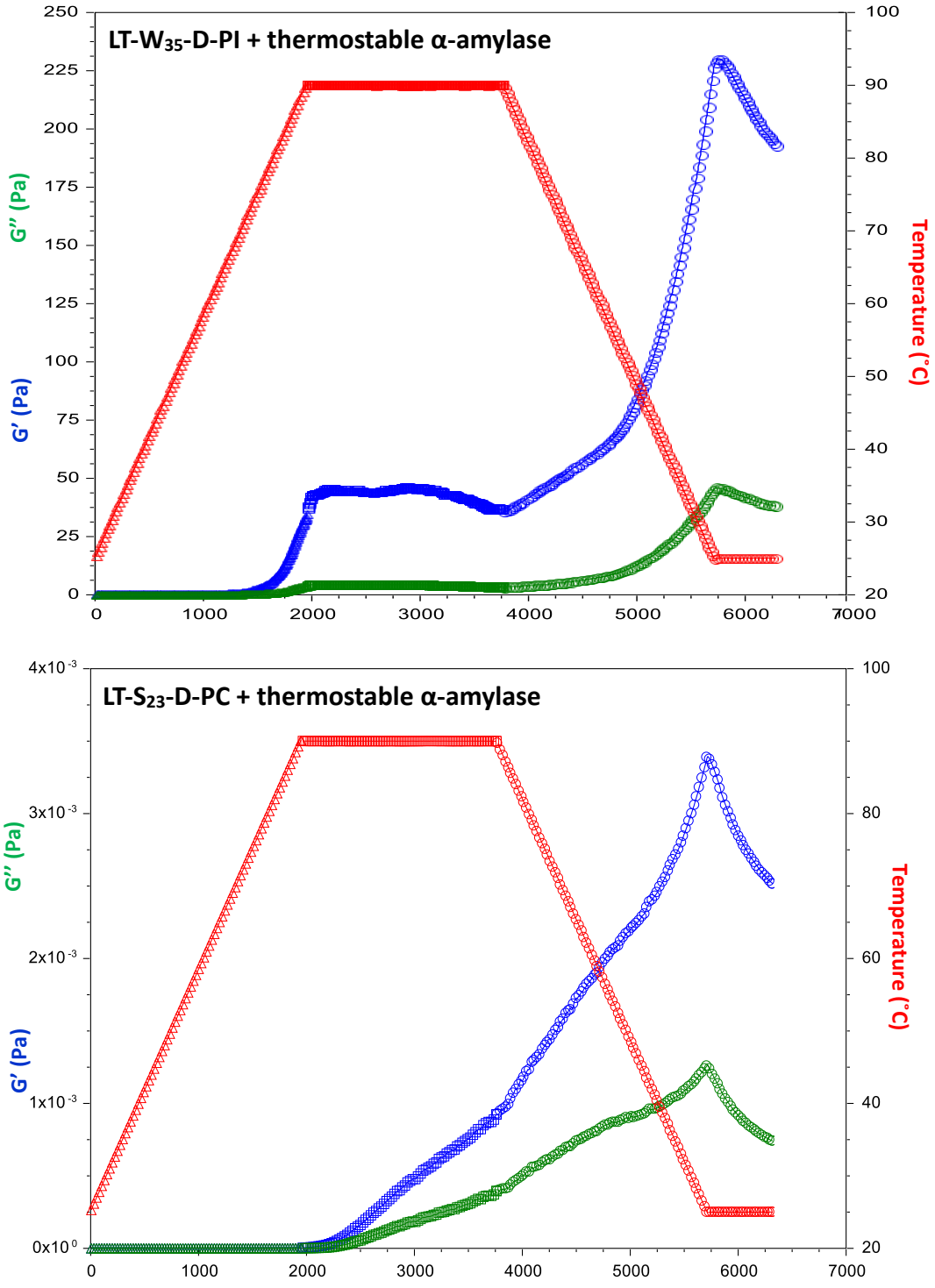


Fig. C.2. Typical patterns of the changes in the storage modulus (G') and loss modulus (G") of faba bean protein isolates (PIs) in the presence of thermostable alpha-amylase.

Snowbird (LT) faba bean proteins were extracted with water at 35 °C followed by dialysis (W_{35}-D) and 1% $NaCl_{(aq)}$ solution at 23 °C followed by dialysis (S_{23}-D).

C.3.3. *Rheological properties of large deformation*

The mechanical properties of the faba bean protein gels were investigated using large deformation tests. Comparisons of compression test results are shown in Fig. C.3 for the protein gels and Fig. C.4 for protein + KGM + canola oil gels. Among the replicates one curve with the middle fracture value was chosen for the respective samples to depict the stress-strain curves obtained from compression tests. Water-extracted protein isolates resulted in relatively firm gels (Figs. C.2) and the partial denaturation of proteins taking place due to the pH-shift method could have weakened the gel structures. Small deformation stress-controlled rheometers are more sensitive to chemical and physical changes happening during the heat-induced gelation, which happens in a more controlled environment as compared to preparing the gels in a water bath, which was the case for compression testing.

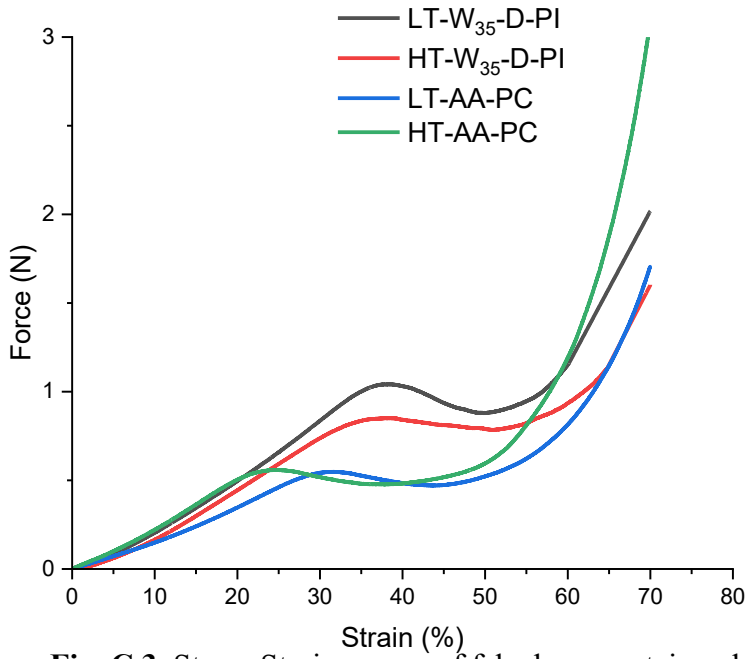


Fig. C.3. Stress-Strain curves of faba bean protein gels.

Snowbird (LT) and Athena (HT) faba bean proteins extracted with water at 35 °C followed by either dialysis at 4 °C (W_{35}-D) or micellization (W_{35}-M), or alkali-extracted and acid precipitated (AA) or extracted with 1% $NaCl_{(aq)}$ solution at 23 °C followed by dialysis (S_{23}-D).

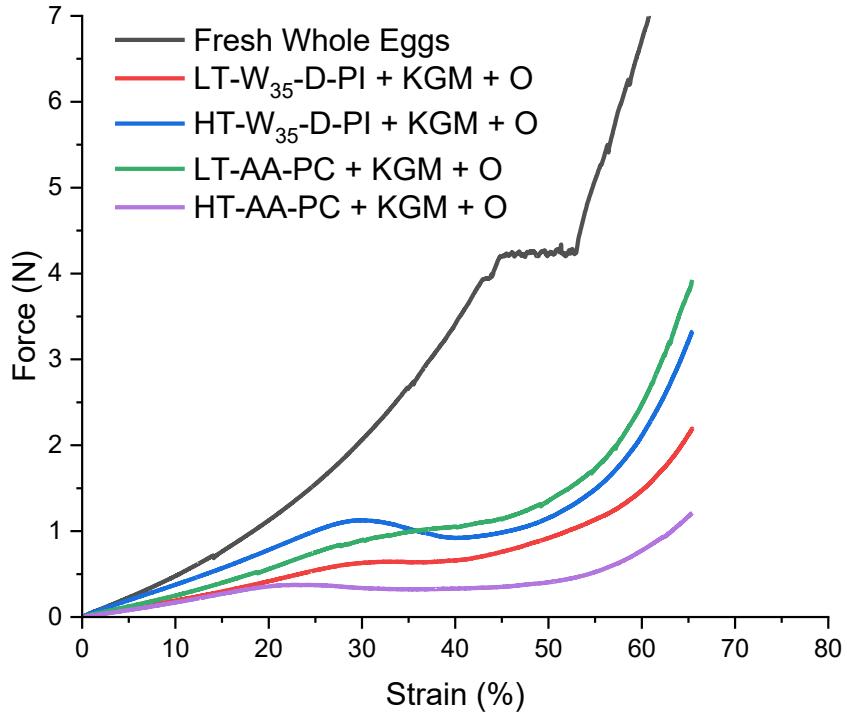


Fig. C.4. Stress-Strain curves of faba bean protein gels formed with konjac glucomannan (KGM) and canola oil in comparison to fresh eggs gels.

Snowbird (LT) and Athena (HT) faba bean proteins extracted with water at 35 °C followed by either dialysis at 4 °C (W_{35}-D) or micellization (W_{35}-M), or alkali-extracted and acid precipitated (AA) or extracted with 1% $NaCl_{(aq)}$ solution at 23 °C followed by dialysis (S_{23}-D). G-Konjac glucomannan.

Amongst the different protein gels, the HT-W_{35}-D-PI resulted in the strongest gels in the presence of KGM and canola oil, since it had the highest stress at fracture in comparison to the rest of the protein gels. However, these gels (Fig. C.4) were softer than the protein only gels (Fig. C.3) and egg gels (Fig. C.4). According to the compressive stress at fracture values, it is interesting to note that the incorporation of KGM and canola oil did not positively influence the mechanical strength of the HT-AA-PC gels while they enhanced that of LT-AA-PC gels. However, the LT-W_{35}-D-PI gel strength was not substantially influenced by the addition of KGM and canola oil.

C.3.4. Microstructure

The morphology observed under SEM shows the impact of KGM and canola oil on the microstructures of LT (Fig. C.5) and HT (Fig. C.6) protein gels in comparison to fresh egg and KGM alone (Fig. C.7). All the protein gels showed compact fine gel structures (Figs. C.5-C.6), comprising of fine globular structures. The AA-PC gels appeared denser as compared to the W-D-PI gels. A dense homogeneous protein microstructure has previously been observed for alkali extracted faba bean protein gels at pH 7 (Langton et al., 2020). The KGM gels appeared less dense, more hollow and more complex as compared to the rest. Some major differences in the protein gel networks were observed in the SEM micrographs after the addition of KGM (Fig. C.5-C.6), probably due to its ability to absorb more water, which could have altered the protein concentration in the continuous phase (Ji et al., 2017). The addition of KGM appeared to yield a more open structure (more heterogeneous) as compared to using the faba bean proteins alone. The egg gels also comprised fine and dense globular structures (Fig. C.7). Protein gels formed along with KGM, and canola oil had the denser structure in comparison to protein gels and protein + KGM gels.

Amylose leached from the less than 1% and 5% starch present in W-D_{35}-PIs and AA-PCs, respectively, during the gelatinisation process should be present as aggregates inside the spherical pores (approximately 1 μm in diameter) according to the findings of Johansson et al. (2022). However, it was challenging to distinguish them from the protein aggregates through SEM imaging. Confocal laser scanning microscopy of the colour-stained gels could provide additional information on that note. No or few such cavities were observed in the protein-only gels. Both starch and dietary fibre can affect protein networks formed during heat-induced gelation (Johansson et al., 2022).

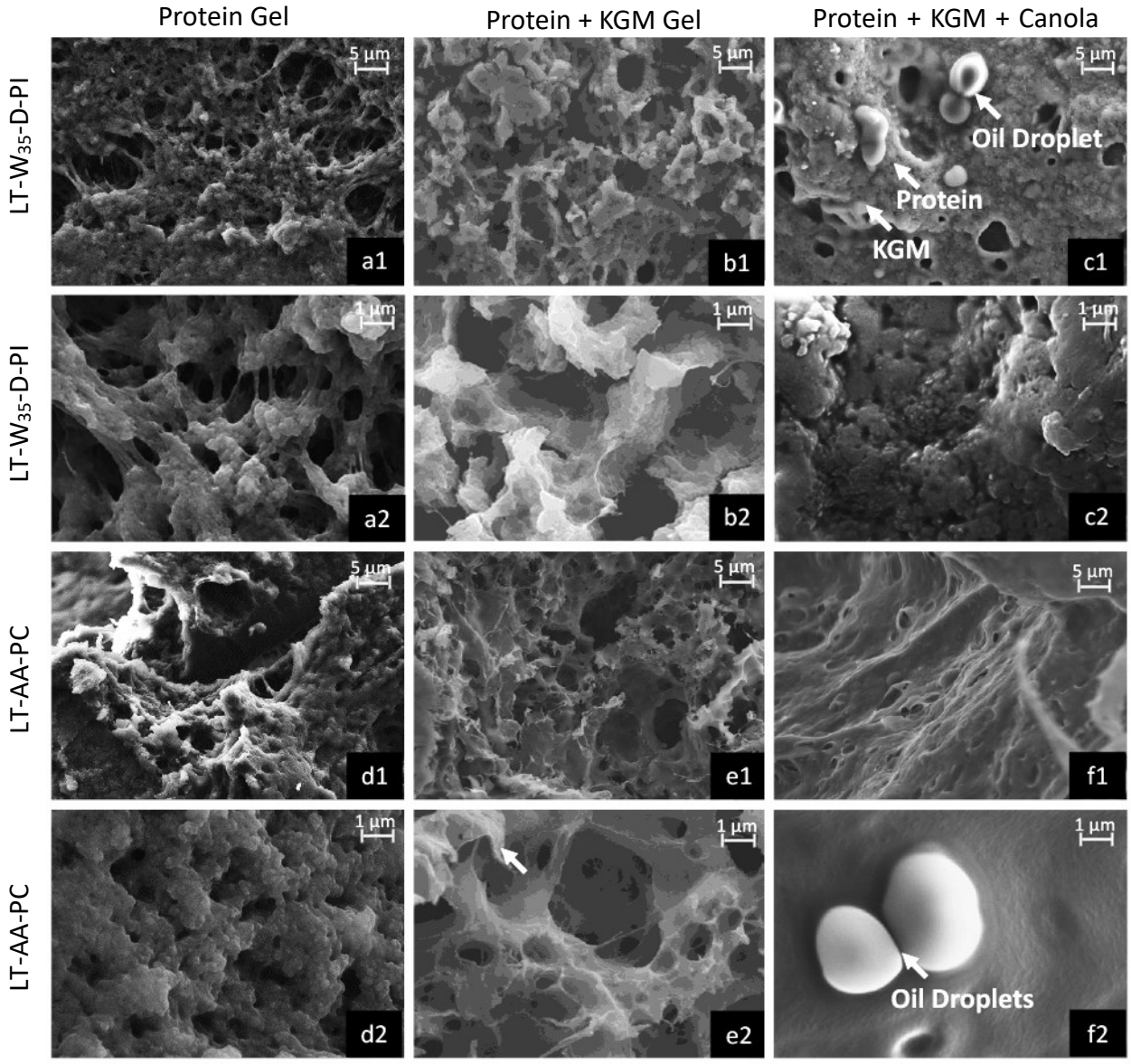


Fig. C.5. Impact of konjac glucomannan (KGM) and canola oil on the microstructure of Snowbird (LT) protein concentrate/isolate (PC/PI) gels.

1: ×2,000 and 2: ×10,000 magnification.
Water extracted at 35 °C followed by either dialysis at 4 °C (W_{35}-D) and alkali-extracted and acid precipitated (AA).

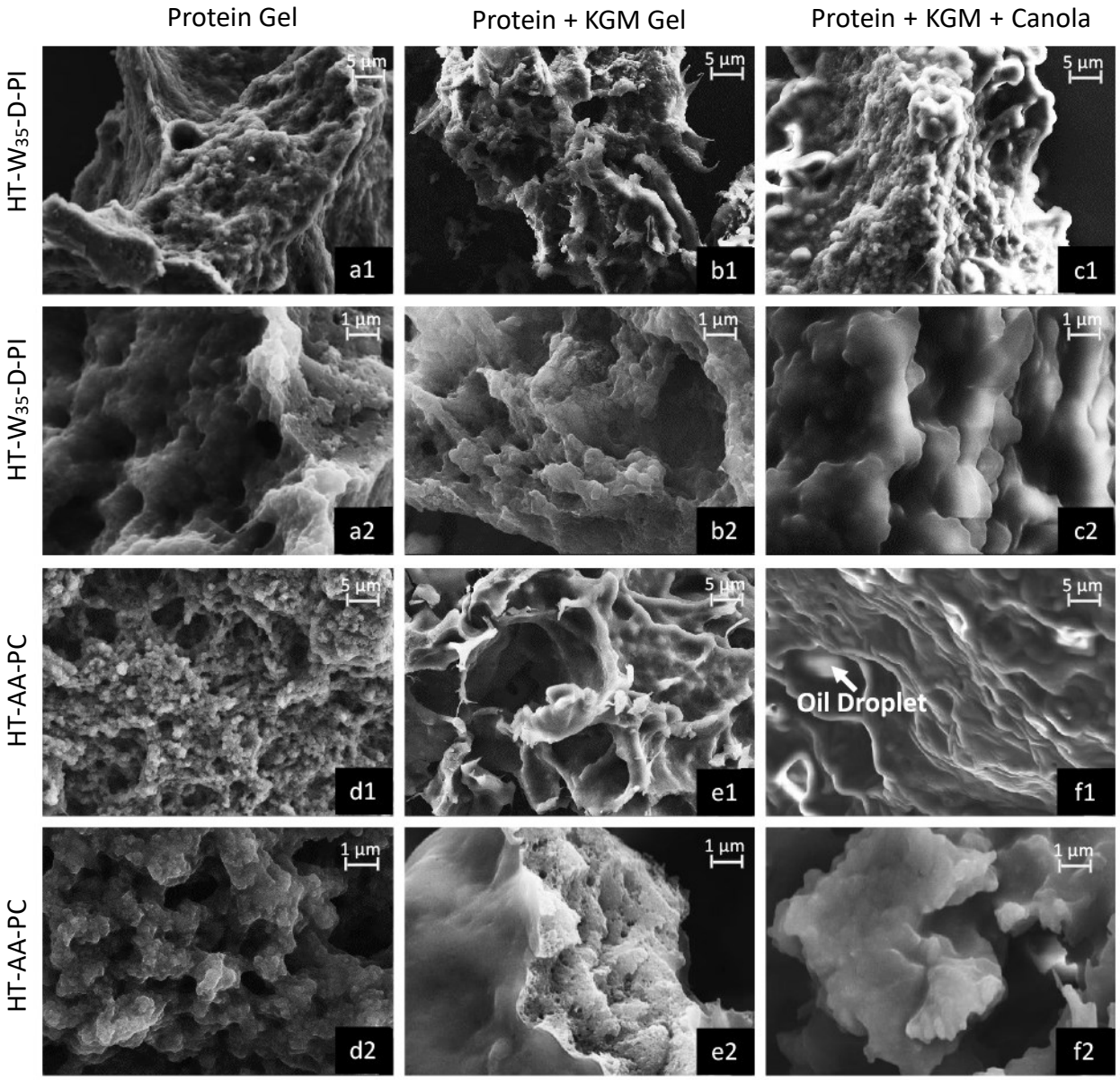


Fig. C.6. Impact of konjac glucomannan (KGM) and canola oil on the microstructure of Athena (HT) protein concentrate/isolate (PC/PI) gels.

1: ×2,000 and 2: ×10,000 magnification.
Water extracted at 35 °C followed by either dialysis at 4 °C (W_{35}-D) and alkali-extracted and acid precipitated (AA).

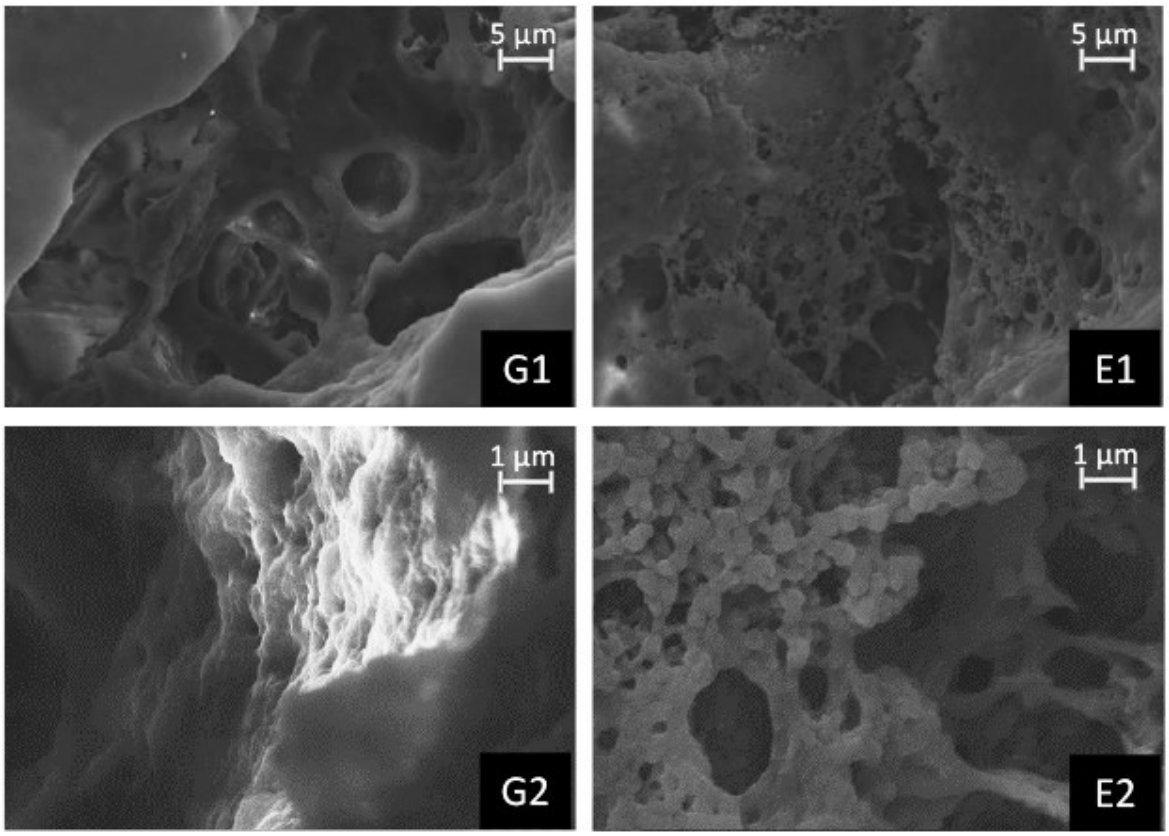


Fig. C.7. Microstructure of konjac glucomannan (KGM) and fresh liquid egg (E) gel microstructure under SEM.

1: ×2,000 and 2: ×10,000 magnification.

C.4. CONCLUSIONS

The minimum gelling concentration for all the PC/PIs was 12-13% except for the salt-extracted protein fractions (-22-23%), which could have been due to the interference caused by the salt deposits remaining on the protein surface. Different responses were obtained when using large and small deformation tests. Faba bean proteins mild extracted with water followed by dialysis resulted in the formation of relatively stronger gels as compared to the gels formed with alkali-extraction followed by acid precipitation, due to minimal changes in their protein secondary structure conformation. Faba bean protein concentration affects the gelation properties, generally,

higher protein concentrations produced stronger gels. However, the addition of KGM and canola oil increased the softness and the porous structure of the gels. The aggregation behaviour and structural properties of mild extracted PI gels were altered favourably by the addition of KGM and canola oil and were comparable to that of egg gels. Overall, this study provides valuable insights into the gelation behavior, rheological characteristics, and microstructures of faba bean protein fractions. Understanding these properties is crucial for the development of food applications that require gel networks, and it highlights the potential of using faba bean proteins as an ingredient in the formulation of vegan egg analogues.

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