IMPACT OF DIFFERENT PROCESSING METHODS ON ANTI-NUTRITIONAL FACTORS, *IN VITRO* PROTEIN QUALITY, AND REACTIVE LYSINE OF YELLOW FIELD PEAS AND THEIR DERIVATIVES

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GENERAL ABSTRACT

Background: Yellow field peas (*Pisum sativum* L.) are a highly promising source of plant-based proteins due to their affordability, high nutritional profile, low-fat content, lack of major allergens and environmental sustainability among pulses. However, their utilization in food products is constrained by anti-nutritional factors (ANFs), which, while beneficial for plant defense, impair the nutritional value, protein digestibility, and overall protein quality of plant-based food products. Processing methods may provide a means to reduce ANFs and improve protein quality, but lysine, an indispensable amino acid (IAA), remains highly susceptible to chemical modifications during processing, further diminishing the nutritional value of the processed products.

Methods: This study examined the effects of thermal (boiling, autoclaving, micronization, and baking) and non-thermal (high-hydrostatic pressure (HHP)) treatments on yellow field pea flour, as well as the impact of baking and HHP on pea protein derivatives (protein concentrate, 80% protein isolate, and 90% protein isolate). A starch-rich byproduct (coarse fraction) separated through air classification along with the protein-enriched fine fraction (protein concentrate) was also included in this study for evaluation. The focus was on assessing ANF contents including trypsin inhibitor activity (TIA), lectins, phytic acid (PA), polyphenols, and condensed tannins (CT), as well as amino acid profiles, *in vitro* protein digestibility, *in vitro* protein quality (via *in vitro* Protein Digestibility Corrected Amino Acid Score, *in vitro* PDCAAS), and reactive lysine content (using an *ortho*-phthaldialdehyde (OPA) fluorometric assay).

Results: In terms of ANFs, thermal treatments (particularly boiling and baking) significantly reduced TIA, lectin, PA, and polyphenols, with boiling proving the most effective, decreasing lectin content by 99.88% and TIA by over 88.60% in the flour. HHP demonstrated moderate ANF reductions but led to substantial increases in lectin and CT contents, with the flour showing a

substantial increase in CT of up to 521.57%. With respect to protein quality, tryptophan with amino acid scores (AAS) of 0.91 – 0.97, was the first limiting amino acid in untreated and treated flours and protein concentrates, as well as the coarse fraction, whereas sulfur-containing amino acids (AAS of 0.84 – 0.91) were most limiting in untreated and treated protein isolates and baked protein concentrate. The *in vitro* protein digestibility (determined by the true fecal protein digestibility, TPD1) of both protein isolate samples (95.77% – 97.33%) was higher than that of coarse fraction, flour, and protein concentrate samples (88.90% – 94.60%). Only boiled flour (91.57%) showed a significant increase in *in vitro* PDCAAS, while other processing methods gave no improvement or a decrease for yellow field pea samples. Furthermore, reactive lysine content was comparable to total lysine in untreated flour and protein concentrate, but decreased significantly following boiling and baking, with boiling causing a decrease of over 70% in the reactive lysine content of the flour. In contrast, HHP treatment preserved reactive lysine better than other treatments, with reductions of only up to 4.73% in both flour and 90% pea protein isolate.

Conclusion: In conclusion, boiling was the most effective method for reducing ANFs and improving the *in vitro* PDCAAS in yellow field pea flour. HHP-treated samples retained reactive lysine but unexpectedly increased the concentrations of lectins and condensed tannins in most pea samples.

Significance and Novelty: This study highlights the differential effects of thermal and non-thermal processing on yellow field pea samples, emphasizing the need for tailored processing strategies to minimize ANFs while maximizing nutritional quality. It also demonstrates the importance of using specialized assays for accurately assessing reactive lysine to fully capture the true nutritional value and quality of processed products. The outcomes provide insights for researchers and food processors in optimizing processing methods to enhance the utilization of yellow field pea proteins and develop more nutritious, and sustainable plant-based products.

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DEDICATION

This thesis is specially dedicated to my supervisor, Dr. James House. “一朝沐杏雨, 一生念师恩”. Your guidance, wisdom, and encouragement have shaped not only my academic journey but also my personal growth. I will never forget you. Rest in Peace, Dr. House.

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LIST OF ABBREVIATIONS

AACC – American Association of Cereal Chemists

AAS – Amino acid score

AFPD – Apparent fecal protein digestibility

ALA – Alanine

ANF – Anti-nutritional factors

AOAC – Association of Official Agricultural Chemists

AOCS – American Oil Chemists' Society

ARG – Arginine

ASP – Aspartic acid

BBI – Bowman-Birk inhibitors

CE – Catechin equivalent

CP – Crude protein

CQM – Coning and quartering method

CT – Condensed tannin

CYS – Cysteine

DIAAS – Digestible Indispensable Amino Acid Score

DM – Dry matter

FAO – Food and Agriculture Organization

FDNB – Fluoro-di-nitro-benzene

GAE – Gallic acid equivalent

GLU – Glutamic acid

GLY – Glycine

HA – Hemagglutinating activity

HC – Health Canada

HHP – High-hydrostatic pressure

HIS – Histidine

HPLC – High-Performance Liquid Chromatography

HU – Hemagglutination units

IAA – Indispensable amino acid

ILE – Isoleucine

IP6 – *Myo*-inositol 1,2,3,4,5,6-hexakisphosphate

KTI – Kunitz trypsin inhibitors

LAA – Limiting amino acids

LEU – Leucine

LYS – Lysine

MET – Methionine

MS – Mass spectrometry

MW – Molecular weight

OPA – *ortho*-phthaldialdehyde

PA – Phytic acid

PD – Protein digestibility

PDCAAS – Protein Digestibility Corrected Amino Acid Score

PER – Protein Efficiency Ratio

PHE – Phenylalanine

pI – Isoelectric point

PRO – Proline

PSD – Particle size distribution

RBC – Red blood cell

SER – Serine

THR – Threonine

TI – Trypsin inhibition

TIA – Trypsin inhibitor activity

TIU – Trypsin inhibitor units

TPC – Total phenolic content

TPD – True fecal protein digestibility

TRP – Tryptophan

TYR – Tyrosine

UPLC – Ultra-Performance Liquid Chromatography

UV – Ultraviolet

VAL – Valine

WHO – World Health Organization

CHAPTER 1: GENERAL INTRODUCTION

Dietary proteins are a critical macronutrient in the human diet, essential for providing energy and supporting the growth, development, and maintenance of the body. To meet the expectations of today's consumers, food manufacturers are increasingly moving to the development of plant-based protein ingredients as alternative sources to minimize reliance on animal-derived proteins. This significant shift represents a response to contemporary health, environmental, and ethical considerations (Talwar et al., 2024; Hertzler et al., 2020). Plant-based protein sources are predominantly derived from legumes after cereals. Pea (*Pisum sativum* L.) seeds are a primary pulse crop characterized as a relatively high protein source, along with valuable dietary fibres, vitamins and minerals (Nosworthy et al., 2017a). Pea proteins are emerging as a promising ingredient of plant-based proteins to substitute for animal-based proteins in terms of nutritional benefits, cost-effectiveness, animal welfare considerations, health implications, environmental sustainability, and functional versatility in food products (Lu et al., 2019; Berrazaga et al., 2019). Commercially, pea protein ingredients have been processed into the forms of flours, protein concentrates, and protein isolates, which are widely utilized in the development of food and feed products due to their nutritional profile and functional properties (Bessada et al., 2019; Kumitch et al., 2019).

However, yellow field peas, a distinct variety characterized by yellow cotyledons, are under-utilized as a plant-based protein source primarily due to the presence of anti-nutritional factors (ANFs), incomplete amino acid profile, and lower protein digestibility when compared to animal protein sources (Yu et al., 2015; Nosworthy et al., 2017a; Herreman et al., 2020; Sá et al., 2019; Gilani et al., 2005). A variety of processing techniques, including dehulling, soaking, germination, boiling, fermentation, extrusion, and roasting, have been widely applied to alleviate

the limitations associated with yellow peas, particularly with respect to their ANFs and protein digestibility, as described by Ma et al. (2017), Çabuk et al. (2018), Khattab & Arntfield (2009), and Alonso et al. (1998). Among these methods, thermal treatments, especially boiling, have been recognized for their remarkable effectiveness in significantly inactivating or reducing ANFs such as protease inhibitors, phytic acid, and tannins, as well as improving protein digestibility. Nevertheless, high-hydrostatic pressure (HHP) is emerging as a prospective non-thermal technique for the reduction of ANFs in pulses, as well as for the preservation of thermosensitive beneficial compounds (Yamamoto, 2017; Gharibzahedi & Smith, 2021).

Plant-based protein sources, including yellow peas, inherently possess various ANFs, including protein-based and non-protein-based compounds (Drulyte & Orlien, 2019; Thakur et al., 2019; Bessada et al., 2019). Regarding protein-based ANFs, trypsin inhibitors interfere with digestive processes by inhibiting proteolytic enzymes (e.g., trypsin) to form insoluble trypsin-trypsin inhibitor complexes, thereby reducing enzyme activity and protein digestibility (Avilés-Gaxiola et al., 2018). Lectins, also known as hemagglutinins, can bind to carbohydrates on the surface of cells, resulting in the agglutination of red blood cells and impairing nutrient absorption (e.g., proteins, lipids, and vitamins) (Champ, 2002; Purohit et al., 2023; Petroski & Minich, 2020). On the other hand, non-protein-based compounds such as phytic acid, polyphenols, and condensed tannins pose distinct challenges. Phytic acid chelates essential minerals such as iron, zinc, and calcium, rendering their absorption and bioavailability (Purohit et al., 2023). Polyphenols and condensed tannins can form stable indigestible complexes with proteins, making them highly resistant to the proteolytic activity of enzymes, with condensed tannins, in particular, having the potential to precipitate proteins (Samtiya et al., 2020; Konieczny et al., 2020). These substances are of particular concern in plant-based proteins because they can induce a decrease in nutritional value and protein digestibility, negatively affecting overall protein quality when compared to

animal-based protein sources that are generally free of these inhibitors (Bessada et al., 2019). Therefore, it is imperative to determine the content of the ANFs in yellow field pea samples and control their levels using appropriate food processing techniques, particularly as the demand for plant-based proteins continues to rise.

With respect to the protein quality of food, two crucial parameters include the amino acid profile and protein digestibility. The Protein Digestibility Corrected Amino Acid Score (PDCAAS) is a widely recognized method for assessing the protein quality in foods, recently accepted in Canada and established as a standard approach in the United States (Health Canada, 2023; Wiggins et al., 2019; FAO/WHO, 2013). Furthermore, to address ethical concerns associated with bioassays and to enhance efficiency and cost-effectiveness, *in vitro* PDCAAS methods have been developed as alternatives to traditional *in vivo* assays (Prachansuwan et al., 2019; Mansilla et al., 2020).

A critical issue in food processing and prolonged storage is the susceptibility of lysine, an indispensable amino acid (IAA) essential for human nutrition, to chemical modifications, primarily through the Maillard reactions and oxidative processes (Rutherfurd, 2015; Van Lieshout et al., 2019; Moughan & Rutherfurd, 2008). Lysine is more likely to conjugate with other compounds present in food matrices, especially reducing sugars through the Maillard reaction, to generate modified lysine derivatives that are not nutritionally available in the body (Rutherfurd, 2015; Torbatinejad et al., 2005). Furthermore, a problem that has arisen when assessing the amino acid composition of processed foods is that continuously using standardized methods may lead to the overestimation of the nutritionally available lysine, as these methods typically measure total lysine without distinguishing it from the unmodified, nutritionally available form, known as reactive lysine (Rutherfurd, 2015; Van Lieshout et al., 2019). Therefore, specialized assays are needed to accurately measure the amounts of reactive lysine, which remains nutritionally available in food

sources as processing treatments are applied (Rutherfurd, 2015; Gilani et al., 2005; Hodgkinson et al., 2022).

Therefore, the overall purpose of this study is to examine the effect of various processing treatments including both thermal (boiling, autoclaving, micronization, and baking) and non-thermal (HHP) treatments on the concentrations of ANFs, including trypsin inhibitors, lectins, phytic acid, polyphenols, and condensed tannins in Study 1 (Chapter 4), as well as the effects on *in vitro* protein quality (i.e., amino acid score, *in vitro* protein digestibility, and *in vitro* PDCAAS) and reactive lysine content in Study 2 (Chapter 5), in yellow field peas and their derivative products (i.e., protein concentrate, coarse fraction and protein isolates).

CHAPTER 2: LITERATURE REVIEW

2.1 PLANT-BASED PROTEINS

2.1.1 Current Prospects and Trends in Plant-Based Proteins

With the ongoing challenges posed by climate change and a continuously expanding global population, ensuring adequate food supply for the entire human race remains a formidable task. Given that dietary protein is a fundamental macronutrient essential for human health and development, it is imperative to achieve sufficient protein intake through daily diets to meet both quantitative and qualitative nutritional requirements (Bessada et al., 2019; Hertzler et al., 2020). Sufficient protein intake not only implies getting enough protein to meet the Recommended Dietary Allowance (RDA) (0.8 grams of protein per kilogram of body weight per day for healthy adults, while other population groups such as infants, children, and athletes have increased protein requirements) but also ensuring that the protein consumed is of high nutritional quality to meet physiological needs (Wu, 2016; Campbell et al., 2007). Consumer awareness and preference for

plant-based protein sources are anticipated to grow significantly, with particular emphasis on protein-rich resources such as nuts, tofu, beans, peas, and pea proteins while lessening the reliance on animal-based proteins (MarketsandMarkets, 2023; Shahbandeh, M. 2022). According to Berrazaga et al. (2020), plant-based protein sources constitute a significant portion of dietary protein intake worldwide, accounting for approximately 60% of the total, which surpasses the 40% contribution from animal-based proteins. In comparison, a study by Fabek et al. (2021) revealed that Canadian adults have a different protein intake distribution, with an average of 63.3% derived from animal-based proteins and 36.7% from plant-based proteins. Plant-based proteins are increasingly favoured among consumers, food manufacturers, farmers, and policy makers for several reasons, including (1) cost-efficiency; (2) contributing to the creation of sustainable and environmentally friendly food systems by decreasing the use of energy, land, and water resources, as well as reducing greenhouse gas emissions associated with animal agriculture; (3) promoting soil health and quality through sustainable farming practices such as reduced fertilizer use, organic farming methods, and crop rotation, which contribute to long-term agricultural sustainability; (4) delivering health-promoting and nutritional benefits to humans, including providing antioxidant properties and lowing the risks of chronic diseases and cancers when compared to diets high in animal-based proteins; and (5) addressing growing concerns regarding animal welfare (Gharibzahedi & Smith, 2021; Hertzler et al., 2020; Meena & Lal, 2018; Bessada et al., 2019; Aschemann-Witzel et al., 2020). For those reasons, there is a rising trend in demand for plant-based protein products, as reflected by recent market analyses. According to a 2023 report by MarketsandMarkets (2023), the global market for plant-based proteins is projected to expand significantly, reaching an estimated value of 19.2 billion USD by 2028, up from its valuation of 13.3 billion USD in 2023. This growth reflects a Compound Annual Growth Rate (CAGR) of 7.7% over the forecast period. Furthermore, North America currently holds the largest share of the

worldwide plant-based protein market, which is estimated to reach a market value of approximately 6.14 billion USD by 2029 (Mordor Intelligence, 2024). Legumes such as peas, beans, lentils, and chickpeas, are among the most prevalent plant-based protein sources in the market (Gharibzahedi & Smith, 2021; Alcorta et al., 2021).

2.1.2 Overview of Legumes and Pulses

Legumes, which belong to the *Fabaceae* (or *Leguminosae)* family, are the most significant crops in the world besides cereals (Lu et al., 2019; Bessada et al., 2019). Within the legume family, pulses are a distinct sub-group consisting of the dry edible seeds of leguminous plants, differing from other legumes cultivated for oilseeds and fresh crops. Pulses are characterized by their low-fat content, making them distinct from high-fat leguminous dry seeds such as groundnut (*Arachis hypogaea*) and soybean (*Glycine max*), which are not classified as pulses. Similarly, fresh legumes such as green beans and green peas, are consumed as vegetables in their immature form and excluded from the pulse category (Tiwari et al., 2011; Lu et al., 2019). Pulses are widely recognized as an important source of plant-based protein in diets worldwide. The Food and Agriculture Organization (FAO) identifies 11 categories of consumable pulses, including dry beans, dry broad beans, dry peas, chickpeas, dry cowpeas, pigeon peas, lentils, Bambara beans, vetches, lupins and other minor pulses (Tiwari et al., 2011; Bessada et al., 2019). Pulses play a critical role in global food security and human nutrition, due to their high protein contents, along with indispensable amino acids, carbohydrates, dietary fibers, vitamins, and minerals (Bessada et al., 2019; Nosworthy et al., 2023).

2.2 PEAS AND PEA PROTEINS

2.2.1 Overview of Peas and Pea Nutritional Composition

Dry peas (*Pisum sativum* L.) are one of the most common and important pulses cultivated for both animal and human diets (Bessada et al., 2019; Lu et al., 2019). Peas are a rich source of protein, comprising approximately 21.2 – 32.9% protein on a dry matter (DM) basis, along with a relatively balanced profile of indispensable amino acids (IAAs) that are necessary for human health. Moreover, peas are abundant in carbohydrates (36.9 – 49.0%), total dietary fibers (14 – 26%), and essential micronutrients including the B vitamins, potassium, phosphorous, magnesium, calcium, iron, and selenium. Importantly, peas are low in fat, typically containing between 1.2% and 2.6% fat content (Pedrosa et al., 2020; Stone et al., 2019; Frias et al., 2011; Nosworthy et al., 2017a; Nosworthy et al., 2017c; Dahl et al., 2012; Shanthakumar et al., 2022). **Table 2.1** provides a summary of the proximate compositions of peas and their derivatives as reported in previous studies.

Canada, Russia, China, India, and the United States are the top five pea-producing countries globally, with Canada holding a prominent position as the leading producer and exporter of both green and yellow pea grains (Lu et al., 2019). According to the Government of Canada (2024), Canada allocated approximately 1.2 million hectares for pea cultivation in 2023, resulting in a production yield of approximately 2.6 million tonnes of pea grains. This represented a 23.8% decrease in pea production compared to the previous year, attributed to declines in both seeded and harvested areas. However, projections for the 2024 – 2025 period indicate a slight expansion in the seeded area compared to 2023 – 2024, driven by the favorable return on peas relative to other grains. Consequently, pea production is anticipated to rise to 3.16 million tonnes. Moreover, total exports of Canadian pea seeds reached 2.4 million tonnes from August to January during the

current crop year (2023-2024). However, this figure was lower than the amount exported during the same period in the previous year (2022-2023) (Government of Canada, 2024). Moreover, the market size of global pea protein is anticipated to reach 227.1 million USD by 2032, growing at a CAGR of 9.2% over the forecast period, compared to the market value of 94.6 million USD in 2022 (Allied Market Research, 2023).

Table 2.1. Proximate and amino acid compositions of yellow field pea seeds and their derivatives including protein concentrates and protein isolates.

Item	Yellow Field Peas[1] (g/100g on a DM basis)	Pea Protein Concentrates[2] (g/100g on an as is basis)	Pea Protein Isolates[3] (g/100g on an as is basis)
Moisture	8.70 – 12.62	7.45	–
Dry Matter	87.38 – 91.30	–	–
Crude Protein	21.20 – 32.90	46.50 – 59.20	82.60 – 86.9
Crude Fat	1.20 – 2.63	–	–
Crude Fiber	14.0 – 26.0	0.92 – 2.50	–
Starch	36.9 – 49.0	7.00 – 10.7	–
Ash	2.30 – 3.50	4.87 – 5.50	–
Indispensable Amino Acids			
Histidine	0.52 – 0.65	0.91 – 1.11	3.10
Threonine	0.84 – 0.96	1.55 – 1.73	3.30
Cysteine	0.25 – 0.31	0.47 – 0.58	0.60
Lysine	1.68 – 2.18	2.95 – 3.54	8.40
Tyrosine	0.59 – 0.73	1.52 – 1.54	3.80
Methionine	0.22 – 0.26	0.37 – 0.44	0.60
Valine	1.02 – 1.23	1.49 – 2.20	4.30
Isoleucine	0.87 – 1.09	1.45 – 1.98	4.10
Leucine	1.75 – 1.85	2.87 – 3.40	9.00
Phenylalanine	1.04 – 1.29	2.04 – 2.30	5.70
Tryptophan	0.20 – 0.52	0.41 – 0.46	1.00
Dispensable Amino Acids			
Serine	1.03 – 1.25	2.13 – 2.59	5.60
Arginine	1.49 – 2.02	3.62 – 3.87	11.1
Glycine	0.92 – 1.08	1.52 – 2.02	4.10
Aspartic acid	2.82 – 2.88	4.73 – 5.40	10.1
Glutamic acid	3.94 – 4.56	7.01 – 7.96	16.4
Alanine	0.83 – 1.13	1.55 – 1.97	4.40
Proline	0.85 – 1.04	1.76 – 2.03	5.40

[1] Stone et al. (2019); Frias et al. (2011); Nosworthy et al. (2017a); Nosworthy et al. (2017c); Jezierny et al. (2010); Dahl et al. (2012).
[2] Gunawardena et al. (2010); Konieczny et al. (2020); Nosworthy et al. (2017b); Çabuk et al. (2018).
[3] Shi (2022); Nosworthy et al. (2017b).

2.2.2 Pea Protein Composition

Pea proteins consist of four types of fractions: globulins (salt-soluble), albumins (water-soluble), prolamins (alcohol-soluble), and glutelins (insoluble). Among these, globulins and albumins are the predominant protein fractions in peas (Lu et al., 2019; Shanthakumar et al., 2022). Pea globulins, comprising 65 – 80% of the total protein content, serve as the primary storage proteins. They mainly consist of two major components: legumin (11S) and vicilin (7S), which are categorized according to their sedimentation coefficients (Shi, 2022; Shanthakumar et al., 2022). Legumin is featured by a compact quaternary structure with a molecular weight (MW) of 300 – 400 kDa. This complex structure is assembled from six monomers, each made up of an acidic – basic (α – β) polypeptide chain, which is covalently linked by disulfide bonds, to form a hexameric configuration (Lu et al., 2019; Lam et al., 2018). Legumin is characterized by a relatively high amount of the limiting of sulfur-containing amino acids (i.e., methionine and cysteine) (Shi, 2022; Boye et al., 2010). Vicilin exists in a trimeric form with a MW of 150 – 170 kDa. Each monomer of vicilin consists of three subunits, designated as α, β, and γ, which are bound together primarily through non-covalent hydrophobic interactions. This arrangement provides two potential cleavage sites between the α and β subunits or the β and γ subunits (Lam et al., 2018; Shi, 2022; Shanthakumar et al., 2022; Mertens et al., 2011; O'Kane et al., 2004). Unlike legumin, vicilin is distinguished by the absence of disulfide bonds and relatively lower contents of sulfur-containing amino acids and tryptophan. The lack of cysteine residues, in particular, limits the formation of disulfide bridges (Lam et al., 2018; Shi, 2022; Shanthakumar et al., 2022). Additionally, convicilin is the third type of globulin protein present in small quantities with a trimeric structure and a MW of approximately 210 – 290 kDa. It exhibits a distinctive amino acid profile, including sulfur amino acids as compared to vicilin, as well as the presence of highly charged N-terminal extensions (Lam et al., 2018; Shanthakumar et al., 2022). In peas, legumin predominates over vicilin, with a ratio

of vicilin to legumin ranging from 1:1.3 to 1:4.2 (Jezierny et al., 2010). The higher proportion of legumin suggests that pea seeds contain more sulfur-containing amino acids per unit of protein compared to vicilin. Such differences in content, structure, and composition of legumin and vicilin influence their nutritional value and functional properties (Lu et al., 2019). Pea albumins are water-soluble proteins with an estimated MW of 5 – 80 kDa, accounting for 10 – 20% of the total protein content in peas. They are classified into two major fractions: pea albumin 1 (PA1), with an approximate MW of 6 kDa, and pea albumin 2 (PA2), with an approximately MW of 25 kDa. Pea albumins serve both metabolic and enzymatic functions, playing crucial roles in seedling growth. They include a variety of molecules such as enzymes, protease inhibitors, amylase inhibitors, and lectins (Lam et al., 2018; Shanthakumar et al., 2022). Relative to globulins, which are predominantly rich in arginine, isoleucine, leucine, and phenylalanine, pea albumins contain high amounts of sulfur-containing amino acids, lysine, tryptophan, and threonine (Boye et al., 2010). Prolamins, present in a small amount of peas, are rich in glutamine and proline (Shanthakumar et al., 2022). Contrary to the previous findings, Stone et al. (2019) reported that yellow field pea proteins exhibited a high albumin content, consisting of 52.3% albumins, 41.9% globulins, and 5.8% prolamins. Given the diverse composition of yellow field pea proteins, consisting primarily of albumins, globulins, and prolamins, they offer a range of functional and nutritional properties to support their potential for further development and various applications.

2.2.3 Pea Protein Development and Applications

With the growing demand for plant-based proteins from consumers and food manufacturers, peas have emerged as a highly promising source due to their affordability, rich nutritional value, and environmental sustainability (Gharibzahedi & Smith, 2021; Hertzler et al., 2020; Bessada et

al., 2019; Lam et al., 2018). Pea protein is increasingly regarded as a viable alternative to other protein sources such as soy, wheat, and animal-based proteins, especially given its lower allergenic potential (Kumitch et al., 2019; Hertzler et al., 2020). This makes pea protein particularly appealing for individuals with dietary restrictions, further driving interest in its use as a substitute in allergy-causing products (Kumitch et al., 2019; Bessada et al., 2019; Lu et al., 2019; Hertzler et al., 2020). Furthermore, using pea protein in conjunction with other protein sources is beneficial for enriching the nutritional content and improving the overall amino acid profile of food items, as pea proteins are rich in lysine but tend to be deficient in sulfur-containing amino acids (i.e., methionine and cysteine) (Lu et al., 2019). One common way to complement the amino acid profile of pea proteins is to serve them with cereal-based products to create a more nutritionally complete protein source since cereal proteins are deficient in lysine content but are rich in sulfur-containing amino acids (Lam et al., 2018; Lu et al., 2019; Devi et al., 2020).

Peas seeds can be converted into a wide range of value-added products through various processing techniques, such as milling, fractionation, and extraction (Konieczny et al., 2020; Lam et al., 2018). These commercial products include pea flour (21.2 – 32.9% protein), protein concentrates (50 – 70% protein), protein isolates (70 – 95% protein), and protein hydrolysates (90 – 95% protein) (Dahl et al., 2012; Hertzler et al., 2020). Among these pea-derived products, protein concentrates and isolates are particularly noteworthy for their enriched protein content and functional properties. They are primarily processed by selectively removing other components from peas through wet fractionation or dry fractionation (Nosworthy et al., 2017b). Currently, the majority of commercially available pulse-derived proteins are produced through wet fractionations, with the predominant technique being the Alkaline Extraction-Isoelectric Precipitation (AE-IP) method (Sim et al., 2021; De Angelis et al., 2021). This process relies on the differential solubility of proteins in an alkaline environment to selectively extract them from other compounds.

Specifically, it involves dispersing pulse flour in water, then adjusting to a pH between 8 and 11 and incubating the mixture for 30 to 180 min at 25 to 65°C to maximize protein extraction and solubility (Nosworthy et al., 2017b; Shanthakumar et al., 2022; Boye et al., 2010). Following solubilization, purification techniques including centrifugation and/or filtration, are employed to remove any insoluble residues. The resulting clarified protein solution is then adjusted to the appropriate isoelectric point (pI) of the proteins, causing them to precipitate (Nosworthy et al., 2017b). The precipitated protein is then recovered through centrifugation, and further processed by washing to remove any residual contaminants, neutralizing, and drying (Boye et al., 2010). Protein isolates produced by this AE-IP method generally contain 75 to 90% protein, with pea protein isolates typically representing 90.1 to 90.8% protein (Neji et al., 2022; De Angelis et al., 2021; Nosworthy et al., 2017b). Similarly, Lam et al. (2018) stated that this technique can achieve a protein yield of approximately 80 – 94% in the production of protein isolates. The processing parameters including flour-to-water ratio, solubilization pH, extraction temperature, extraction time, particle size, and the type of solvent used, can significantly affect the yield, purity, and functionality of the resulting protein (Shanthakumar et al., 2022; Lam et al., 2018; Neji et al., 2022).

Alternatively, air classification is a widely used dry processing method for producing protein concentrates from seeds (Shanthakumar et al., 2022). This cost-efficient and more sustainable technique involves an initial pre-milling step to achieve optimal particle size reduction. Following this, the pre-milled flour undergoes air classification, where the particles are separated according to their size and density through controlled airflow (Rempel et al., 2019; Boye et al., 2010; De Angelis et al., 2021). This results in two fractions: a coarse fraction (starch-enriched fraction, heavier starch granules, 20 – 30 μm) and a fine fraction (protein-enriched fraction, lighter protein bodies, <10 μm) (De Angelis et al., 2021; Rempel et al., 2019; Carmo et al., 2020). This dry fractionation highlights the ability of air classification to enrich the protein content in the fine

fraction, making it an effective method for producing protein concentrates from various pulse samples (Nosworthy et al., 2017b). The paper by Rempel et al. (2019) reported protein contents of 42%, 42%, and 50% in pea protein concentrates derived from split green pea flour, split yellow pea flour, and whole green pea flour, respectively, utilizing air classification. Additionally, the process of re-milling and re-air classification under identical conditions was found to significantly improve the efficiency of protein concentration by further segregating the light protein bodies from the heavy starch granules, as observed by scanning electron microscopy. This resulted in a significant reduction of protein content in the coarse fractions, as more protein accumulated in the fine fraction, suggesting greater efficiency with repeated cycles (Rempel et al., 2019).

Dry fractionation techniques have emerged as a promising and sustainable alternative to wet fractionation methods for producing plant-based protein concentrates, addressing several key issues associated with wet fractionation, such as the extensive use of chemicals, loss of insoluble protein fractions, protein denaturation, high energy consumption, and substantial water requirements (Rempel et al., 2019). Moreover, dry fractionation techniques better preserve the native structure and functionality of the proteins (Asen et al., 2023; Assatory et al., 2019). However, the dry fractionation processes have limitations in achieving the same level of protein purity compared to wet fractionation methods. While dry fractionation by air classification typically produced protein concentrates of approximately 50% purity, wet fractionation by AE-IP resulted in protein isolates with purity as high as 95% (Asen et al., 2023; Assatory et al., 2019). To overcome the lower purity levels inherent in dry fractionation, strategies such as fraction recycling and the integration of multiple fractionation methods have been implemented (Rempel et al., 2019; Asen et al., 2023; Xing et al., 2020). For instance, combining air classification with advanced techniques such as electrostatic separation has been shown to yield pea protein concentrates with

a protein purity of 63.4 – 67.6%, significantly higher than the 57.1% purity achieved through air classification alone (Xing et al., 2020).

Both wet and dry fractionation techniques not only increase the protein content but also improve the nutritional and functional properties of food products by yielding high-protein ingredients with improved amino acid profiles and enhancing characteristics such as emulsification, gelling, foaming, and solubility, supporting food formulation and protein innovation (Lam et al., 2018; Lu et al., 2019; Shanthakumar et al., 2022; Asen et al., 2023). These enhancements make the proteins suitable for diverse food applications, across cereals, bakery items, snacks, and vegetarian food products (Bessada et al., 2019). For example, Jiang et al. (2024) demonstrated that a blend of pea protein (82.8% protein on a dry matter basis), chickpea protein (73% protein on a dry matter basis), and wheat gluten effectively improved the texture of the food products by promoting chewiness and a more fibrous structure, closely mimicking the mouthfeel of animal meat. These functional attributes make pea proteins highly suitable for the growing demand for plant-based, high-protein products in the food industry, thus contributing to the broader trend toward sustainable and health-conscious food options.

2.3 FOOD PROCESSING TREATMENTS

2.3.1 Overview of Processing Treatments

In general, pulses should not be consumed until they have been cooked properly (Drulyte & Orlien, 2019). Common processing methods such as boiling, baking, roasting, microwaving cooking, extrusion, and autoclaving are employed both domestically and industrially. These thermal treatments not only have diverse impacts on such pulse characteristics, including nitrogen solubility, gelling, binding capacity, and water-holding capacity but also impacts on the anti-

nutritional factors (ANFs), palatability, nutrient composition, digestibility and protein quality (Ma et al., 2017; Samadi & Yu, 2011; Khattab & Arntfield, 2009; Ma et al., 2011; Luo & Xie, 2013; Patterson et al., 2017). Recently emerging food technologies such as high-hydrostatic pressure (HHP), ultrasound, pulsed electric field, irradiation, and high-pressure carbon dioxide have gained attention for their potential benefits, including such energy efficiency, environmental sustainability, time savings, and nutrient retention (Khan et al., 2018). These technologies represent innovative approaches to food processing, offering alternatives to traditional thermal treatments such as boiling or roasting (Khan et al., 2018; Drulyte & Orlien, 2019). However, research on the specific impacts of these emerging technologies on ANFs and protein quality in pulses is currently limited.

It is essential to understand the significance of cooperative treatments, such as the assistance of pre-processing techniques including dehulling, soaking, germination, tempering and milling prior to cooking (Ma et al., 2017; EL-Suhaibani et al., 2020; Luo & Xie, 2013; Bai et al., 2018). For example, soaking serves as a prevalent pre-processing treatment designed for further cooking, particularly for dry seeds, aiming to hydrate them and soften their texture while shortening overall cooking time (Xu & Chang, 2008). According to Xu & Chang (2008), among chickpea, green pea, yellow pea and lentil seed samples, yellow peas exhibited a relatively high hydration level at the logarithmic phase of the water absorption curves. However, they entered a slow absorption phase after approximately 10 hr and eventually stabilized after 16 hr. Combining pre-processing treatments has been shown to enhance the overall nutritional quality of legumes and increase the efficiency of subsequent cooking methods (El-Suhaibani et al., 2020).

The main focus of the study is to assess the changes in the concentrations of the ANFs, reactive lysine content, and *in vitro* protein quality before and after applying different processing methods, including thermal treatments (i.e., boiling, autoclaving, baking and micronization), and non-thermal treatment (i.e., HHP).

2.3.2 Types of Processing Treatments

2.3.2.1 Thermal treatments

Boiling (or conventional cooking) is the most ordinary and straightforward thermal processing method used in home kitchens (Nosworthy et al., 2017a). Typically, pulse seeds that have been rinsed or soaked beforehand, are either submerged in water and gradually brought to a boil or directly added to boiling water in a pot. The cooking process continues until most of the seeds have softened (Nosworthy et al., 2017a; Ma et al., 2017). The time required for this process can vary significantly depending on the specific type of seeds used and any pre-processing methods applied, such as dehulling, drying or soaking prior to boiling (Drulyte & Orlien, 2019). For example, soaked yellow and green split peas, yellow field peas, and Canadian and Egyptian pea seeds are typically cooked to completion within 20 to 35 min (Nosworthy et al., 2017a; Ma et al., 2017; Khattab & Arntfield, 2009). Optimizing boiling conditions not only enhances the digestibility and palatability of pulses but also improves their overall nutritional quality by making the nutrients more bioavailable while mitigating the effects of ANFs such as trypsin inhibitors and phytic acid (Bessada et al., 2019).

Autoclaving (or pressure cooking) is a widely utilized hydrothermal processing commonly applied in industry, characterized by the application of high pressure and high temperature over a short period of time (Drulyte & Orlien, 2019). In this process, pulse seeds or flours are exposed to saturated steam within a pressurized chamber, causing the internal temperature of the autoclave to rise significantly above the ambient boiling point of water, typically exceeding 100°C (Drulyte & Orlien, 2019; Li et al., 2019; Thakur et al., 2019). Such excessive temperature conditions caused by autoclave may lead to a reduction of ANFs (including alkaloid, phytate, saponin, tannin, trypsin inhibitor, and oxalate), while also resulting in nutrient degradation, including vitamins (e.g.,

ascorbic acid, thiamine, niacin, vitamin A, and vitamin E) and certain IAAs (e.g., lysine, sulfur-containing amino acids, and tryptophan) (Ogechukwu et al., 2017; Gilani et al., 2005).

Micronization, also known as infrared (IR) heating, is a widely used thermal process that involves rapidly heating pulse seeds to high temperatures over a short period of time by directly transferring heat via infrared radiation emitted from IR heaters without involving water (Deepa & Hebbar, 2015; Wray, 1999). Rapid vibration can generate uniform internal heat within the seeds, ultimately leading to the expansion and rupture of the seed structures (Wray, 1999). The effectiveness of micronization is influenced by several critical factors, including the distance between the IR source and the seeds, the temperature of the IR source, and the duration of the heating process (Wray, 1999). However, the large size of the pulse seeds can potentially impede heat penetration. Despite this challenge, it is particularly effective for processing hard-to-cook pulse crops by greatly reducing their cooking time and enhancing production efficiency when served as a pre-treatment method (Sesikashvili et al., 2021; Patterson et al., 2017; Deepa & Hebbar, 2015).

Baking is a traditional culinary technique that involves processing foods using dry heat air, typically in an oven, under ambient pressure conditions (Drulyte & Orlien, 2019). A dough is made by combining pulse flour mixing with an appropriate amount of water, as described in Nosworthy et al. (2017a). The most critical parameters for baking include leavening time (typically around 30 min), baking time (approximately 30 min), and baking temperatures ranging from 180 to 220°C (Drulyte & Orlien, 2019). However, despite the widespread application of baking across various food products, there has been relatively limited research focusing on the impact of baking on the concentrations of ANFs in pulse-based products and even their derivatives.

These thermal treatments are responsible for developing the desirable flavor, aromas, and color of the final product through various mechanisms such as the Maillard reaction, lipid

oxidation, caramelization, and heat-induced degradation of food components. These activities involve complex chemical and biochemical reactions that occur during thermal treatments, alternating the sensory qualities of the foods (Akkad et al., 2023). Nonetheless, non-thermal processing could offer a less destructive approach when retaining nutritional integrity is a priority.

2.3.2.2 Nonthermal treatments

Unlike thermal treatments, the application of innovative non-thermal treatments such as high-hydrostatic pressure (HHP) can alleviate the difficulties caused by high temperatures better protecting thermosensitive beneficial compounds in pulses, improving sensory attributes, and enhancing product quality (Gharibzahedi & Smith, 2021; Lee et al., 2018; Dehnad et al., 2024). HHP, also known as high-pressure processing (HPP), is a novel processing method that applies extremely high pressure to foods for a short period of time, without the need for active heating (Muntean et al., 2016). During HHP processing, a packaged food product is loaded and evenly pressurized from all directions in a chamber filled with water that acts as a pressure transmission medium (Muntean et al., 2016). In the food processing industry, HHP equipment can generate intense pressure in the range of 400 to 600 MPa at room temperature (Yamamoto, 2017; Muntean et al., 2016). This method can cause either reversible or irreversible denaturation of proteins to varying degrees, depending on the processing parameters (e.g., pressure levels, temperature, and time) applied (Gharibzahedi & Smith, 2021; Yamamoto, 2017). HHP treatment induces protein denaturation by disrupting non-covalent interactions such as hydrogen bonds and hydrophobic interactions in proteins, leading to destabilization and unfolding, structural changes of proteins (Gharibzahedi & Smith, 2021; Aganovic et al., 2021; Dehnad et al., 2024; Sim et al., 2021). Unlike thermal treatments, which affect both covalent and non-covalent bonds, HHP predominantly

targets non-covalent interactions, resulting in significant alterations in the secondary, tertiary, and quaternary structures of proteins (Aganovic et al., 2021; Sim et al., 2021). Generally, the irreversible denaturation effects on proteins tend to occur at a pressure exceeding 500 MPa (Lim et al., 2008).

Currently, HHP is primarily used for food preservation, with the aim of retaining nutritional values, maintaining organoleptic properties, and prolonging the shelf life of food items (Khan et al., 2018; Muntean et al., 2016; Aganovic et al., 2021). HHP has been recently explored for the development of plant-based yogurts made from mung bean, chickpea, pea, lentil, and fava proteins. Instead of utilizing a typical fermentation process, the HHP (600 MPa for 5 min at 5°C) may be more adapted for improving the sensory characteristics (e.g., texture and flavor) of plant-based yogurts to a level comparable to commercial dairy yogurts, as well as enhancing process efficiency (Sim et al., 2020). However, there is limited research focused specifically on the effects of HHP treatment on ANFs and protein quality in pulses.

2.4 ANTI-NUTRITIONAL FACTORS

2.4.1 Overview of Anti-nutritional Factors

Pulses, including pea seeds, are known for their excellent nutritional profile, encompassing high levels of proteins, carbohydrates, dietary fibers, essential vitamins and minerals (Bessada et al., 2019). Additionally, they contain a variety of phytochemicals, which are secondary metabolites produced by plants. Importantly, these phytochemicals are not involved in the primary processes of cell growth and reproduction but rather contribute to the overall defense mechanisms of the plants (Makkar et al., 2007). These compounds help protect the plant against environmental threats

such as pests, pathogens, oxidative stress, light, metal ions, and free radicals (Makkar et al., 2007; Troszyńska & Ciska, 2011; Rahate et al., 2021).

The utilization of plant-based proteins is marked by complex benefits and challenges, particularly due to the presence of certain naturally occurring phytochemicals that are recognized as ANFs (Makkar et al., 2007; Zhou et al., 2024; Thakur et al., 2019; Konieczny et al., 2020). While these compounds play an essential role in plant survival, they have been extensively studied for their adverse effects on the nutritional value, amino acid bioavailability, protein digestibility, and overall protein quality of pulses, as described by Rahate et al. (2021), Avilés-Gaxiola et al. (2018), Konieczny et al. (2020), Bessada et al. (2019), and Ma et al. (2017). Consequently, the levels of ANFs have become a crucial indicator in assessing the nutritional quality of plant-based proteins (Rahate et al., 2021; Bessada et al., 2019). ANFs in pulses have been studied for their potential beneficial, deleterious, or both effects on human and animal health, as well as their impact on protein digestion and quality, and their mechanisms are included, as summarized in **Table 2.2**. Specifically, ANFs are broadly divided into two main groups based on their chemical structures: protein-based compounds such as protease inhibitors (trypsin and chymotrypsin inhibitors) and lectins, and non-protein-based compounds such as tannins, phytic acid, alkaloids, saponins, and polyphenols (Drulyte & Orlien, 2019; Thakur et al., 2019; Bessada et al., 2019). Fortunately, numerous scientific studies have reported that these ANFs in the pulses can be significantly inactivated through various processing treatments, including dehulling (Ma et al., 2017), soaking (Mubarak, 2005; EL-Suhaibani et al., 2020; Alonso et al., 1998), fermentation (Çabuk et al., 2018; Kumitch et al., 2019), germination (EL-Suhaibani et al., 2020; Alonso et al., 1998; Kalpanadevi & Mohan, 2013), infrared heating (Bai et al., 2018), boiling (Khattab et al., 2009), extrusion (Alonso et al., 1998), autoclaving (Khattab & Arntfield, 2009), and HHP (Linsberger-Martin et al., 2013). Alonso et al. (1998) and Khattab & Arntfield (2009) identified a variety of ANFs in peas

mainly including phytic acid, condensed tannins, polyphenols, trypsin inhibitors, chymotrypsin inhibitors, α-amylase inhibitors, lectins, and oligosaccharides. The concentrations of these ANFs can vary significantly among different types of pea products and even among different pea cultivars. Their studies demonstrated that a range of processing treatments including soaking, boiling, roasting, microwaving, autoclaving, fermentation, micronization, and extrusion resulted in a significant reduction in the concentration of these ANFs across different pea cultivars. Despite these findings, the presence of ANFs in pea protein concentrates and pea protein isolates has not been extensively explored.

Table 2.2. Mechanism, and beneficial and adverse effects of ANFs presented in pulses.

Compound	Mechanism	Beneficial effects	Adverse effects
Protease inhibitors[1]	Protease inhibitors can bind to pancreatic enzymes (e.g., trypsin and/or chymotrypsin) to form insoluble complexes.	Anti-carcinogenesis	Decreased proteolytic activity of pancreatic enzymes; Diminished protein digestion and absorption; Reduced amino acid bioavailability; Pancreatic hypertrophy and hyperplasia in animals; Inhibited animal growth.
Amylase inhibitors[2]	Amylase inhibitors can bind to amylase to form insoluble complexes.	Potentially therapeutic role in diabetes	Inhibited starch digestion.
Lectins[3]	Lectins are carbohydrate-binding proteins or glycoproteins by binding to specific carbohydrates on the cell surface (e.g., red blood cells, RBCs).	Antioxidative, antidiabetic, anti-obesity properties; suppress tumour growth; anti-microbial and immunomodulating agents.	Agglutinated RBCs; Metabolic toxicity; Interfered nutrient absorption (such as proteins, lipids, and vitamins); Growth retardation (in animals).
Phytic acid/phytate[2]	Phytic acid is a strong chelating agent in nature to form metal-phytic acid complexes by binding essential minerals such as calcium, iron, and zinc.	Prevent kidney stone formation; Antioxidant, anti-carcinogenic, and anti-diabetic properties	Inhibited mineral absorption and bioavailability, such as iron deficiency (i.e., anemia); Reduced protein solubility and digestion by binding to proteins or digestive enzymes.
Oxalates[2]	Oxalates can bind to calcium ions, forming insoluble calcium oxalate crystals.	N/A	Interferes with mineral absorption, in particular, calcium.

[1] Avilés-Gaxiola et al. (2018); Champ (2002); Clemente et al. (2005); Gilani et al. (2012).
[2] Champ (2002); Goertzen et al. (2020); Tiwari et al. (2011); Purohit et al. (2023); Bessada et al. (2019).
[3] Champ (2002); Mishra et al. (2019); Bessada et al. (2019); Purohit et al., 2023; Makkar et al. (2007)
[4] Champ (2002); De Camargo et al. (2019); Konieczny et al. (2020).
[5] Champ (2002); Smeriglio et al. (2017); Purohit et al. (2023).

Table 2.2. Continued.

Compound	Mechanism	Beneficial effects	Adverse effects
Total phenolic compounds[4]	Total phenolic compounds including condensed tannins are able to cross-link proteins to form protein-polyphenolic complexes.	Antioxidant properties; Reduced risk of cardiovascular diseases, diabetes, obesity, inflammation, and cancers	Interfered with digestion of proteins, lipids, and carbohydrates; Reduced the bioavailability of amino acids and minerals; Reduced food intake (in animals).
Condensed tannins[5]	Condensed tannins can connect with proteins to form protein-condensed tannin complexes.	Antioxidant, anticarcinogenic, antimicrobial, antidiabetic, and anti-inflammatory properties	Precipitated proteins; Interfered with protein digestion and quality; Provided astringent tastes; Reduced food intake (in animals).
Saponins[2]	Saponins can interact with cholesterol in the cell membranes.	Hypocholesterolasemic effect Anti-carcinogenic	Reduced nutrient absorption and utilization; Bitter taste; Reduced food intake (in animals).

[1] Avilés-Gaxiola et al. (2018); Champ (2002); Clemente et al. (2005); Gilani et al. (2012).
[2] Champ (2002); Goertzen et al. (2020); Tiwari et al. (2011); Purohit et al. (2023); Bessada et al. (2019).
[3] Champ (2002); Mishra et al. (2019); Bessada et al. (2019); Purohit et al., 2023; Makkar et al. (2007)
[4] Champ (2002); De Camargo et al. (2019); Konieczny et al. (2020).
[5] Champ (2002); Smeriglio et al. (2017); Purohit et al. (2023).

2.4.2 Types of Anti-Nutritive Factors

2.4.2.1 Trypsin inhibitors: Characteristics and measurements

Trypsin inhibitors are a class of anti-nutritional proteins commonly distributed in the seeds of soybeans and pulses such as chickpeas, lentils, fava beans, cowpeas, and peas (Bessada et al., 2019; Avilés-Gaxiola et al., 2018). These inhibitors are primarily concentrated in the seed cotyledons (Avilés-Gaxiola et al., 2018). Trypsin inhibitors are classified into two main protein-based protease inhibitors: Kunitz (KTI) and Bowman-Birk (BBI) inhibitors. The Kunitz type contains two disulphide linkages and primarily targets trypsin with high specificity at a single reactive site. In contrast, BBI contains seven disulphide bonds and can independently inhibit both trypsin and chymotrypsin at distinct reactive sites, allowing it to simultaneously affect multiple proteolytic enzymes (Gilani et al., 2012; Avilés-Gaxiola et al., 2018; Kong et al., 2022). The mechanism of action of trypsin inhibitors is to interfere with the proteolytic activity of pancreatic enzymes (e.g., trypsin) by binding to specific amino acid residues, such as serine, lysine, and arginine, in the active site of trypsin, forming a stable trypsin-trypsin inhibitor complex (Dantzger et al., 2015; Avilés-Gaxiola et al., 2018; Bai et al., 2018). This interaction hinders protein digestion and absorption, as well as reduces the amino acid bioavailability of pulses, particularly affecting sulfur-containing amino acids (i.e., methionine and cysteine), which are already present in low quantities in soybeans and most pulses (Bacon et al., 1995; Avilés-Gaxiola et al., 2018; Champ, 2002; Purohit et al., 2023).

Beyond their anti-nutritional effects, trypsin inhibitors, as members of protease inhibitors alongside chymotrypsin inhibitors, have been reported to possess bioactive properties, with potential anti-carcinogenic benefits (Champ, 2002). For example, pea protease inhibitors, which encompass both trypsin and chymotrypsin inhibitors, have demonstrated greater efficacy in

suppressing carcinogenic activity in the colon compared to BBI derived from soybeans, as reported by Clemente et al. (2005).

In general, pea cultivars contain between 1.84 and 15.25 trypsin inhibitor units (TIU) per mg of dry matter found in pea cultivars, as reported by Bacon et al. (1995), Shi et al. (2017), Frias et al. (2011), Pedrosa et al. (2020), and Liu et al. (2021). Trypsin inhibitors are heat-sensitive in nature and particularly vulnerable to hydrothermal treatments (Avilés-Gaxiola et al., 2018; Ma et al., 2017). Various processing methods such as boiling, microwave cooking, germination followed by boiling, and germination followed by roasting have been shown to significantly reduce trypsin inhibitor activity (TIA) in yellow field peas by as much as 83.1% – 87.2% (Ma et al., 2017). Moreover, complete removal of TIA has been achieved through boiling, roasting, microwaving, and autoclaving in different pulse varieties, including Canadian and Egyptian cowpeas, kidney beans, and peas (Khattab & Arntfield, 2009).

There are two official methods for the measurement of TIA, which are Method Ba 12-75, approved by the American Oil Chemists' Society (AOCS) and Method 22-40.01 endorsed by the American Association of Cereal Chemists (AACC, also known as the Cereals and Grains Association) (AOCS, 2017; AACC, 2009; Liu et al., 2021). Both official methods were adapted from the original approach developed by Kakade et al. (1974), which was primarily utilized to quantify TIA in raw and roasted soybean products. Moreover, these methods, as reported by the AOCS (2017), AACC (2009), Makkar et al. (2007), and Liu et al. (2021) utilized Nα-benzoyl-DL-arginine-p-nitroanilide (DL-BAPA) hydrochloride as a chromogenic synthetic substrate that can be cleaved by trypsin to produce a yellow-colored product, p-nitroanilide, which can be subsequently detected using a spectrophotometer at 410 nm. The presence of trypsin inhibitors can impede this reaction, resulting in a decrease in absorbance. Therefore, the TIA can be quantified based on the change in p-nitroanilide production (Kong et al., 2022). A recent study by Liu et al.

(2021) employed a variety of legume and cereal products including soybeans, barleys, navy beans, peas, and fava beans, as well as their protein concentrate and isolate forms, to conduct a comprehensive analysis of TIA. This analysis aimed to revise and improve the method as the AOCS Method Ba 12a – 2020. Results of the TIA analysis were reported in terms of either mg of pure trypsin inhibited (TId)/g sample, or trypsin inhibitor units (TIU, also known as trypsin units inhibited, TUI)/mg sample. One trypsin unit represents an increase of 0.02 absorbance units at 410 nm in a 5 mL total assay volume (Liu et al., 2021). Alternatively, a micro-assay utilizing a 96-well plate has been developed and widely adopted, enhancing the assay efficiency and greatly reducing reagent consumption (Bacon et al., 1995). Additionally, the AOCS method recommended employing an appropriate dilution factor for extracted samples by fulfilling a criterion, where 30 – 70% of trypsin inhibition (TI%) is produced in 1 mL of the diluted extracted samples. If this criterion is not met, the sample extract must be re-diluted and re-analyzed to fall within the desired range (Liu et al., 2021). When plotting a curve regarding trypsin activity (A410) against inhibitor levels, deviations from linearity can occur, particularly at higher inhibitor concentrations. Some studies have focused on using a narrower range of TI (e.g., 40 – 60%) (Çabuk et al., 2018; Makkar et al. 2007). However, employing a broader range of TI (e.g., 30 – 70%) offers an alternative approach to accommodate the variability observed across different sample types. This wider range is justified as a comparable relative standard deviation (RSD) observed between the narrower and wider TI ranges (Liu, 2019; Liu et al., 2021). In a separate study, Ma et al. (2011) described that the optimal dilution factors of raw and roasted yellow peas were 10 and 2, respectively.

2.4.2.2 Lectins: Characteristics and measurements

Lectins (also known as hemagglutinins) are a class of anti-nutritional proteins with MW ranging from 60 to 120 kDa (Bessada et al., 2019; Makkar et al., 2007; Samtiya et al., 2020). These proteins are widely distributed across various cereals, legumes, and tubers, with plant lectins mainly accumulating in the seed cotyledons (Boeck et al. 2021; De Azevedo Moreira et al., 1991). Structurally, legume lectins are often tetrameric proteins (MW of 100 – 120 kDa), which is recognized as a quaternary structure made up of four monomeric subunits (MW of 25 – 30 kDa) held together by non-covalent bonds, primarily ionic interactions (Shi et al., 2018; Tang et al., 2024; Van Damme, 2021). Each monomer subunit predominantly features a six- and seven-standardized antiparallel β-sheet arrangement (Tang et al., 2024; Rini, 1995). Additionally, dimeric structures with a MW of approximately 60 kDa have also been observed (Shi et al., 2018; Tang et al., 2024). Lectins play a significant role in the anti-nutritional effects, where they are presented in the form of carbohydrate-binding proteins or glycoproteins with a distinctive capacity to bind specific carbohydrates (Tang et al., 2024; Shi et al., 2018; Wang et al., 2024). For instance, pea lectins possess mannose- and glucose-binding sites that are essential for their interaction with specific carbohydrate structures (Rini, 1995; Mishra et al., 2019). This specificity allows lectins, through their multiple carbohydrate-binding sites, to reversibly bind glycoconjugate receptors present on the surface of cell membranes, particularly, on red blood cells (RBCs) or erythrocytes, leading to their agglutination or clumping upon absorption into the bloodstream, a process known as hemagglutination (Makkar et al., 2007; Samtiya et al., 2020; Boeck et al. 2021; Mishra et al., 2019; Petroski & Minich, 2020). Furthermore, lectins exert adverse effects on nutrient absorption (such as proteins, lipids, and vitamins) in the digestive system and can cause growth retardation in animals because their binding to intestinal epithelial cells damages the intestinal lining and disrupts nutrient transport mechanisms (Samtiya et al., 2020; Petroski & Minich, 2020). Lectins also

possess the potential to be toxic when ingested via raw and/or improperly cooked pulse seeds, with the capability to induce severe stomach and intestinal inflammation (Champ. 2002; Muramoto, 2017).

When utilized reasonably, lectins exhibit beneficial properties. Mishra et al. (2019) highlighted several specialized applications where lectins contribute significantly, serving as anti-viral, anti-fungal, anti-insect, anti-diabetic, anti-microbial, anti-parasitic, anti-cancer agents, as well as immunomodulators. In specialized contexts, lectins have demonstrated promising therapeutic potential. For instance, Kong et al. (2022) and Yau et al. (2015) have shown that lectins can induce apoptosis and autophagy in tumor cells, suggesting their potential use in the development of anti-cancer therapies.

According to Shi et al. (2018), lectin contents vary significantly among legume species, with soybeans containing the highest contents, up to 692.82 hemagglutination units (HU)/mg, followed by various beans (87.69 – 88.59 HU/mg), lentils (10.91 – 11.07 HU/mg), peas (5.53 – 5.68 HU/mg), fava beans (5.52 – 5.55 HU/mg), and chickpeas (2.73 – 2.74 HU/mg). Lectins also constitute approximately 2.5% of the total protein content in peas (Shanthakumar et al., 2022). However, lectins are known to be heat-labile substances, susceptible to degradation or elimination through thermal processing methods. Studies reported by Alonso et al. (2000), Umoren et al. (1997), Marquardt et al. (1974), Mubarak (2005), Shi et al. (2018), Kalpanadevi & Mohan (2013), and Luo & Xie (2013) have demonstrated that thermal treatments such as extrusion, boiling, and autoclaving can significantly reduce hemagglutinating activity (HA) by up to 100%, depending on the type of legumes and the processing conditions applied. For example, Alonso et al. (2000) reported that pre-processing methods, such as dehulling, soaking, and germination, had no effect on HA in fava and kidney bean seeds, with the HA remaining unchanged at 49.3 HU/mg DM for both unprocessed and pre-processed fava beans and 74.5 HU/mg DM for both unprocessed and

pre-processed kidney beans. In contrast, HA is extremely susceptible to hydrothermal processing methods, where extrusion drastically reduced it to as low as 0.2 HU/mg DM in both extruded fava and kidney beans.

The determination of lectin contents relies on its particular hemagglutination feature, which involves the agglutination of RBCs (Makkar et al., 2007). Lectins exhibit specificity towards different RBCs depending on their sources. Rabbit and cattle RBCs are commonly used for hemagglutinating assays of soybean and pulse samples (Shi et al., 2018; Makkar et al., 2007; Santos et al., 2014). In some cases, human RBCs, with their A, B, and O blood group variants, can be also employed for these assays, providing additional insights into lectin-binding specificities relevant to human health and dietary considerations (Makkar et al., 2007; Labba et al., 2021). For example, the HA observed in various fava bean cultivars ranged from 0.8 HU/mg to 3.2 HU/mg when measured with human RBC (Labba et al., 2021), but was significantly higher, achieving up to 49.3 HU/mg DM when assessed with trypsinized rabbit RBC (Alonso et al., 2000). Therefore, it highlights the importance of selecting the appropriate RBC type for hemagglutination assays based on the specific lectin source and the intended application. It is important to mention that treating RBC with trypsin contributes to improving assay efficiency by exposing sugar residues on the cell surface, facilitating stronger binding interactions between lectins and the exposed carbohydrates on the RBC surface (Hirabayashi, 2014; Alonso et al., 2000; Makkar et al., 1997; Makkar et al., 2007). Besides differences in blood cell types and sample species, other factors, such as the metabolic state of cells, assay parameters, and molecular characteristics of lectins, can also influence assay results (Labba et al., 2021; Lis & Sharon, 1986). Moreover, many current studies commonly used a twofold serial dilution method performed in a microplate (i.e., a 96-well microplate) adapted from the procedures of Liener & Hill (1953). The HA of pulse samples is typically expressed as HU/mg flour, where one HU is defined as the maximum dilution of the

lectin that results in the visible agglutination of RBCs (Liener & Hill, 1953; Labba et al., 2021; Shi et al., 2018; Liu et al., 2013). A positive agglutination result is observed and judged visually when at least five cells cluster together under a microscope (Makkar et al., 1997). In order to mitigate potential errors in visual judgment during hemagglutination assay, Liener (1954) reported an alternative method using a spectrophotometric technique by measuring the absorbance of the un-sedimented RBC layer after exposure to lectins, providing a quantitative measure of agglutination. Nonetheless, while this spectrophotometric method has the potential to improve accuracy and sensitivity in detecting hemagglutination, its use remains limited (Makkar et al., 2007).

2.4.2.3 Phytic acid: Characteristics and measurements

Phytic acid (PA, also known as phytate) is classified as an anti-nutritional chemical primarily found in plant-based sources such as seeds, grains, and nuts, typically occurring as globoid crystals within protein bodies and predominantly concentrated in the cotyledons of dicotyledonous seeds, including oilseeds and pulses (Drulyte & Orlien, 2019; Gilani et al., 2012; Bessada et al., 2019; Champ, 2002; Oomah et al., 2011; Shi et al., 2018). Importantly, these PA crystals function as storage reserves of phosphorus in seeds, constituting as much as 80% of the total phosphorus content, which is vital for supporting seed germination and development (Bohn et al., 2008; Dahiya et al., 2015). However, phosphorus availability is limited in monogastric animals when consuming phytate because their digestive systems often lack sufficient levels of the enzyme phytase to effectively break down phytate in the small intestine (Van Doorn et al., 2004; Boeck et al. 2021). PA, referred to as *myo*-inositol 1,2,3,4,5,6-hexakisphosphate (IP6), is a compound with six phosphate groups bound to an inositol ring that provides six potential binding

sites, and its salt form, known as phytate, is formed through interactions with minerals (Thakur et al., 2019; Dahiya et al., 2015; Brouns, 2022; Purohit et al., 2023). These highly negatively charged phytates have a strong affinity for chelating positively charged divalent and trivalent metal ions such as calcium, iron, magnesium, and zinc in nature, leading to the formation of insoluble metal-phytic acid complexes. Consequently, these complex formations can hinder their mineral absorption and bioavailability in the digestive tract (Brouns, 2022; Gilani et al., 2012; Purohit et al., 2023; Nikmaram et al., 2017; Boeck et al. 2021). Such mineral deficiencies potentially lead to conditions such as anemia (i.e., iron deficiency) (Shi et al., 2018; Nikmaram et al., 2017). Furthermore, PA can interact with digestive enzymes such as proteases or amylases to inhibit their catalytic activity, as well as bind to specific proteins and amino acids, leading to reduced protein solubility and impeding protein and carbohydrate digestion (Shi et al., 2018; Bessada et al., 2019; Gilani et al., 2012; Purohit et al., 2023). Therefore, PA (IP6) is generally considered an ANF.

However, it is important to recognize that not all forms of inositol phosphates exhibit the same anti-nutritional effects. Pedrosa et al. (2020) emphasized that the health benefits associated with PA are primarily attributed to its lower phosphorylated derivatives, such as inositol trisphosphate (IP3) to inositol pentakisphosphate (IP5). These lower phosphorylated forms are less likely to bind essential minerals, thereby mitigating the anti-nutritional effects. For instance, these forms have been shown to reduce the risk of kidney stone formation and have beneficial effects on type II diabetes, hypercholesterolemia, and irritable bowel syndrome (Pedrosa et al., 2020; Nikmaram et al., 2017). Moreover, the mineral-chelating characteristic of PA delivers beneficial health effects. For example, PA possesses antioxidant properties by suppressing iron-catalyzed oxidative reactions to protect against oxidative damage associated with heart disease (Shi et al., 2018). Furthermore, Champ (2002) mentioned that PA has shown significant potential in inhibiting the development of various experimental tumors such as mammary tumors, human prostate

carcinoma cells, and colon carcinogenesis. For instance, a study by Okda et al. (2021) revealed that PA, known for its anti-tumor properties, efficiently inhibited 1,2 -dimethylhydrazine-induced colorectal cancer. This inhibition was mediated through pathways involving apoptosis, cell proliferation, and antioxidant activity, demonstrating efficacy both in the presence and absence of oxaliplatin treatment.

As reported by Shi et al. (2018), the PA content in whole yellow peas is approximately 9.93 mg/g sample dry matter. PA is characterized by heat-stable properties in nature (Shi et al., 2018). Despite this, hydrothermal treatments considerably reduce the contents of PA in pulses, owing to their water-soluble nature (Kalpanadevi & Mohan, 2013). This phenomenon could be caused by the combination of heat and water facilitating the breakdown and leaching of PA from the seed matrix into a liquid medium, while heating promotes the degradation of inositol hexaphosphate into lower phosphorylated forms such as inositol pentatetraphosphate and even further down to lower phytate forms (Thakur et al., 2019; Kalpanadevi & Mohan, 2013; Champ, 2002). Kalpanadevi & Mohan (2013) explored that the maximum reductions in PA content, up to 100%, can be achieved by autoclaving after soaking or germination.

The determination of phytate content typically involves estimating the concentration of *myo*-inositol hexakisphosphate by leveraging its chelation properties with metal ions. This process can be accomplished using spectrophotometric methods, as described by Haug & Lantzsch (1983) or chromatographic techniques, as demonstrated by Lestienne et al. (2005) and Knuckles et al. (1982). Haug & Lantzsch (1983) introduced a precise, reliable, rapid indirect method used for the determination of PA at low concentrations in a wide range of samples such as soy-based vegetable protein, cereals, cereal-based products, and faba beans (Haug & Lantzsch, 1983; Luo & Xie, 2013). This method is based on the precipitation of phytate as a ferric salt. Initially, the sample extract was heated with an acidic iron-III solution with a known iron concentration. The resulting iron-

phytate complex precipitates out of the solution, leaving a measurable decrease in the iron content of the supernatant. After that, the pink-colored solution was developed upon reaction with 2,2'-bipyridine and excess iron. The intensity of the color, measured spectrophotometrically at 519 nm, is inversely proportional to the PA content (Haug & Lantzsch, 1983). In contrast to the precipitation method, chromatographic separation by High-Performance Liquid Chromatography (HPLC) offers a highly precise alternative for the determination of PA content. This method leverages the highly ionized nature of phytates, utilizing an anion-exchange column to effectively separate PA from other sample components (Purohit et al., 2023; Lestienne et al., 2005; Knuckles et al., 1982; Talamond et al., 1998). For both the precipitation method and the chromatographic approach, quantification of PA is achieved through the creation of an external calibration curve using a series of standard PA solutions (Haug & Lantzsch, 1983; Talamond et al., 1998). A significant limitation of precipitation-based phytate determination arises from its lack of selectivity, as lower inositol phosphate forms such as IP1 – IP5 and/or inorganic phosphates can also precipitate with iron, resulting in misreading and overestimation of true phytate content. In contrast, HPLC presents a high-precision alternative for phytate determination, but it faces a challenge with the co-elution of inositol phosphates with the solvent front, which can overlap with PA peak, complicating accurate quantification (Purohit et al., 2022).

2.4.2.4 Total polyphenols: Characteristics and measurements

Polyphenols, a diverse class of phytochemicals, are abundantly found in tea, coffee, wine, cocoa, cereal grains, fruits, and legume seeds (Cirkovic Velickovic & Stanic-Vucinic, 2018). In pulse seeds, the predominant polyphenolic classes include phenolic acids, flavonoids, and condensed tannins, which are primarily concentrated in the seed coat (Singh et al., 2017).

Polyphenols are widely known for their health-promoting properties, specifically due to their strong antioxidative performance, which has attracted increasing scientific interest. As a result, researchers have focused on establishing optimal strategies, such as selecting appropriate processing methods and plant-based protein sources, to preserve and augment the concentration of polyphenol compounds in pulses (Kumitch et al., 2019; Samtiya et al., 2020, Agboola et al., 2010; Khan et al., 2018; De Camargo & Da Silva Lima, 2019; Han & Baik, 2008). For example, boiling or soaking chickpea, pea, and soybean seeds has been shown to increase polyphenolic contents compared to unprocessed seeds, with higher polyphenol content correlating positively with greater antioxidant activity (Han & Baik, 2008). Moreover, De Camargo et al. (2019) summarized that polyphenols exert their beneficial effects across various health domains, including the prevention of cancers, cardiovascular diseases, diabetes, obesity, and inflammation.

However, despite these positive impacts, polyphenolic compounds, including condensed tannins (CT), are also regarded as anti-nutritional chemicals with a lower absorption capability in the gastrointestinal system (Drulyte & Orlien, 2019; Bessada et al., 2019; Samtiya et al., 2020). Structurally, polyphenolic compounds consist of multiple aromatic rings with one or more hydroxyl substituents, ranging from simple structures to highly complex polymers (Zhou et al., 2024; Nikmaram et al., 2017; Hollman et al., 2011). These compounds exhibit a strong tendency to cross-link with proteins, forming insoluble complexes that hinder the action of hydrolytic enzymes. This interaction further adversely affects protein digestion and reduces amino acid bioavailability (Bessada et al., 2019; Konieczny et al., 2020; Bai et al., 2018). Furthermore, polyphenols suppress the digestion of carbohydrates and lipids by binding to these macronutrients, thereby obstructing their breakdown and absorption by enzymes (Konieczny et al., 2020; Cirkovic Velickovic & Stanic-Vucinic, 2018). Additionally, polyphenolic substances have the tendency to

reduce the bioavailability of dietary minerals, adding to their classification as ANFs (Samtiya et al., 2020).

Unlike other ANFs found in pulses, polyphenolic compounds are particularly abundant in the seed coats of these plants (Agboola et al., 2010). Pulses with highly pigmented or dark-colored seed coats present significantly higher contents of polyphenolic compounds as compared with their light-colored counterparts. For instance, total phenolics, flavonoids, and condensed tannins in colored peas were measured at 31.46, 24.65, and 61.17 mg/g seed coat, respectively, whereas white-colored peas contained only 1.42 mg/g seed coat in total phenolics, with flavonoids and condensed tannins being undetectable (Troszyńska et al., 1997). A similar trend was observed in soybeans, with black soybeans containing more polyphenols than yellow soybeans (Xu & Chang, 2007). The dual nature of polyphenols, with both health benefits and anti-nutritional effects, highlights the complexity of their role in nutrition and health, necessitating careful consideration of their potential adverse effects when evaluating their overall benefits.

In general, yellow peas (light color) were found to contain 1.12 mg catechin equivalent/g of the sample (Shi et al., 2022). Nevertheless, various approaches such as mechanical, thermal, chemical, and biological approaches can be employed on the pulse seeds to lower the levels of polyphenolic compounds, as reported by Xu & Chang (2008), Zhang & Chang (2022), Konieczny et al. (2020), Bai et al. (2018), Agboola et al. (2010), Pedrosa et al. (2020), and Patterson et al. (2017), where each method varies greatly in its efficacy depending on the specific polyphenol composition and seed characteristics. However, a number of studies have found that microwave cooking and roasting enhanced the total phenolic content (TPC) in sorghums and chickpeas, which might be related to the release of bound phenolics (Almaiman et al., 2021; Jogihalli et al., 2017; Khan et al., 2018). Additionally, polyphenols are known to be relatively heat-stable compounds and are not readily degraded by mild heat treatments. For instance, a slight reduction of

approximately 16% in polyphenol content in chickpeas subjected to tempering and heating at 135°C, as demonstrated by previous research by Bai et al. (2018). Therefore, effectively reducing polyphenolic content through thermal treatment alone presents a challenge, necessitating consideration of alternative processing conditions or synergistic applications to achieve desired outcomes in food applications.

The Folin-Ciocalteu (Folin-C) method stands as one of the most fundamental and extensively employed approaches for quantifying the TPC of foods (Makkar et al., 2007). This method relies on the reaction of polyphenolic compounds within samples with the Folin-C reagent, followed by neutralization with sodium carbonate, resulting in the formation of a blue-colored complex that can be detected spectrophotometrically. The intensity of this blue-colored complex correlates directly with the concentration of the polyphenolic compounds present in the samples (Kupina et al., 2018). As described by Kupina et al. (2018), Bai et al. (2018), Siah et al. (2014), Agboola et al. (2010), and Konieczny et al. (2020), the concentration of polyphenolic compounds is then calculated according to the absorbance of the sample relative to a calibration curve established using standard compounds such as gallic acid or catechin, which are commonly used for pulse seeds. These standards are widely chosen for their stabilizing properties and well-established reactivity with the Folin-C reagent, allowing for accurate quantification across different samples. Gallic acid, a phenolic compound, is frequently used due to its cost-effectiveness, high water solubility, and stability (Singleton et al., 1999). Catechin, on the other hand, is specifically utilized to quantify CT within the polyphenolic profile. These standards have been adopted by extensive literature, highlighting their efficacy and relevance in polyphenolic quantification (Bai et al., 2018; Siah et al., 2014; Agboola et al., 2010; Konieczny et al., 2020; Amoussa et al., 2021). Therefore, the quantification is expressed as mg of gallic acid equivalent (GAE)/g sample or as mg (+) catechin equivalent (CE)/g of the sample, providing a standardized

unit for comparison across different samples and studies. A Folin-Denis reagent can be also employed as an alternative to the Folin-C reagent. However, these two reagents essentially are not specific and may not react with all phenolic compounds present in the sample extracts, which can lead to variations in the measured TPC depending on the polyphenolic composition of the sample (Naczk & Shahidi, 2004). The Association of Official Agricultural Chemists (AOAC) has authorized a technique (Method 2017.13) specifically designed for the determination of TPC in extracts from grape seeds, grape skins, cocoa, coffee, and black tea. This method is particularly suitable for samples with a TPC of exceeding 5% (Kupina et al., 2018). However, the procedures for the analysis of pulse seeds are slightly different from that of the official method in terms of the volume of reagents added, different apparatuses, and different calibration standards solutions (Agboola et al., 2010; Konieczny et al., 2020; Bai et al., 2018; Çabuk et al., 2018).

2.4.2.5 Condensed tannins: Characteristics and measurements

Tannins are a group of polyphenolic compounds recognized as anti-nutritional chemicals, characterized by MW typically ranging from 500 to 3000 Da (Drulyte & Orlien, 2019; Bessada et al., 2019; Smeriglio et al., 2017; Thakur et al., 2019). As these compounds are distinguished by their high number of hydroxyl groups (approximately 20), tannins exhibit a strong ability to bind to proteins and other compounds, and also directly sequester and precipitate nutrients including proteins, carbohydrates, enzymes, and other organic compounds, forming insoluble complexes (Samtiya et al., 2020; Smeriglio et al., 2017; Adamczyk et al., 2017, Purohit et al., 2023; Thakur et al., 2019). Consequently, once tannin-protein complexes are generated, the complexes become highly tolerant to the proteolytic activity of enzymes, thereby resulting in a significant decrease in

protein solubility and indispensable amino acid levels, ultimately impairing protein digestibility and quality (Konieczny et al., 2020; Bessada et al., 2019; Thakur et al., 2019).

Nevertheless, tannins play a beneficial role in human health. As a major class of polyphenolic compounds, tannins exhibit strong antioxidant properties, which contribute to the prevention of certain chronic diseases. Tannins derived from medicinal plants provide pharmacological benefits, including antioxidant, anticarcinogenic, antimicrobial, anti-inflammatory, cardioprotective, and antidiabetic actions (Smeriglio et al., 2017). Despite these promising attributes, the beneficial effects of tannins specifically derived from pulses have yet to be conclusively demonstrated in scientific studies.

Tannins are structurally divided into two main categories: hydrolyzable tannins (HTs) and condensed tannins (CTs, also known as proanthocyanidins) (Thakur et al., 2019). Hydrolyzable tannins are further subdivided into gallotannins and ellagitannins, commonly found in certain woods, cereals, nuts, and a few fruits. Gallotannins can be hydrolyzed to particularly yield gallic acids, while ellagitannins mainly yield ellagic acids upon hydrolysis (Samtiya et al., 2020; Smeriglio et al., 2017). However, these hydrolyzable forms are less likely to be found in pulse seeds. On the other hand, condensed tannins are prevalent in colored soybeans, peanuts, and pulse seeds such as common beans, cowpeas, lentils, and peas (Smeriglio et al., 2017; Xu & Chang, 2007). The condensed tannins are polymeric flavonoid compounds composed of flavan-3-ol units linked through carbon-carbon bonds (Smeriglio et al., 2017; Adamczyk et al., 2017). Given their prevalence in pulse seeds, this study will focus on the detection of condensed tannins for pea samples. Peas have been reported to contain CT contents ranging from 0.02 - 10.5 mg/g (Shi et al., 2022; Gilani et al., 2005). Importantly, CTs are likely to be substantially removed by hydrothermal treatment due to their water-soluble nature, which allows them to leach readily into the liquid medium (Ma et al., 2017). Despite being generally heat-stable, it was significantly reduced and

even completely eliminated through specific processing treatments, such as the combination of germination for more than 48 hr + autoclaving, as reported by Kalpanadevi & Mohan (2013).

Prior to measurement, it is preferable to use samples with a larger particle size (greater than 820 µm) as CT content has been observed to decrease with reducing particle size (Deshpande & Cheryan, 1985). For CT determination, two chemical approaches are commonly applied: vanillin-HCl assay and butanol-HCl methods (Makkar et al., 2007). Many studies typically employed the vanillin-HCl method with or without modifications based on the original procedure described by Burns (1971), for the determination of CT contents, which is favored for its reliability and adaptability across different food matrices including chickpeas, peas, sorghum grains, and beans (Goertzen et al., 2020; Konieczny et al., 2020; Burns, 1971; Makkar et al., 2007). Price et al. (1978), using a revision of Burns (1971), presented a sensitive, time-efficient, convenient, accurate and reproducible method entitled modified vanillin-HCl (MV-HCl) assay. Comparatively, this modified vanillin assay involves a 20-minute extraction using 1% HCl in methanol and has been shown to detect higher contents of CT in various sorghum grain varieties compared to the original method (Price et al., 1978). Subsequently, a vanillin-based reagent composed of 1% vanillin in methanol and 8% HCl (i.e., acidified vanillin) reacts with the sample extract to produce a colored final product (Broadhurst & Jones, 1978). Finally, a spectrophotometer technique is used to determine the absorbances of CT in the sample at 500 nm. The measurements of CT commonly utilize catechin as a standard for the construction of a standard curve (Goertzen et al. 2020). Catechin, which belongs to the flavonoid family, serves as a suitable reference because it is structurally similar to the building blocks of CT (Smeriglio et al., 2017). As reported by Broadhurst & Jones (1978), the absorbance readings of the modified vanillin assay are highly sensitive to light, temperature, and time. To ensure accuracy, it is important to conduct the assay in a dark environment, such as by covering the tubes with aluminum foil, as no significant variation in

absorbance was found under these conditions. However, exposing the CT-vanillin mixture to diffuse light and sunlight for 25 min resulted in a 2.5% and 4.5% decline in absorbance, respectively (Broadhurst & Jones, 1978). Additionally, the temperature and time of the reaction must be precisely regulated at 30°C in a water bath and exactly 20 min (Price et al., 1978). These parameters are essential for achieving reproducible and accurate measurements of CT in various foods, ensuring the reliability of the modified vanillin assay for quantitative analysis.

Overall, despite the fact that pulses play a major role in the development of protein sources, their utilization can be restricted due to the presence of these ANFs. A number of food processing techniques have been studied extensively to inactivate and destroy the ANFs to varying degrees. As a result, the pulses can be improved in terms of their nutritional values, protein digestibility, and protein bioavailability (Nosworthy et al., 2018a; Nosworthy et al., 2017a; Ma et al., 2017).

2.4.3 Effects of Food Processing on ANFs

The synergistic application of pre-processing and processing techniques is particularly important because it has been shown to effectively reduce the concentrations of ANFs, such as TIA, PA, tannins, and lectins (Ma et al., 2017; Ma et al., 2011; EL-Suhaibani et al., 2020; Luo & Xie, 2013). Specifically, soaking, which serves as a key pre-processing step, can promote the penetration of water-soluble compounds, such as phenolic antioxidant compounds, into the soaking water. For example, EL-Suhaibani et al. (2020) investigated the ANF contents of goat pea seeds when they were subjected to a variety of processing methods. It found that a combination of germination (48 hr at room temperature) and boiling (20 min) achieved the highest reduction in CT content, with a reduction of 96.30%. Similarly, when goat pea seeds were soaked for six hr and then boiled for 20 min, the CT content was reduced by 89.30%. In contrast, employing soaking

or boiling alone only led to a CT reduction of approximately 37%, demonstrating the limitations of using these methods individually. These findings are consistent with those of Mubarak (2005) and Alonso et al. (2000), who also observed that using pre-treatments alone had a lesser impact on reducing various ANFs. Therefore, by incorporating pre-treatment methods, the nutritional value of foods and the cooking process can be substantially improved.

Thermal treatments are effective in reducing the concentrations of ANFs to varying degrees. Numerous studies have investigated the effects of thermal processing on the reduction of ANFs including trypsin inhibitors, PA, tannins, lectins, total phenolic compounds, and oligosaccharides in pulses such as lentils, peas, faba beans, chickpeas, cowpeas, and common beans (Hefnawy, 2011; Khattab & Arntfield, 2009; Ma et al., 2017; Kalpanadevi & Mohan, 2013; Sánchez-Velázquez et al., 2021; Umoren et al., 1997). Among different food processing, boiling is considered to have a strongly destructive effect on ANFs. Studies have demonstrated that boiling almost completely eliminates TIA and lectins in various presoaked legumes such as peas, cowpeas, kidney beans, fava beans, common beans, and soybeans (Khattab & Arntfield, 2009; Shi et al., 2018). Autoclave has also been shown to result in total inactivation of TIA, PA, lectins, and oligosaccharides. In some cases, the application of pretreatments such as soaking and germination is needed to achieve these effects (Umoren et al., 1997; Kalpanadevi & Mohan, 2013; Khattab & Arntfield, 2009). Boiling and autoclaving have demonstrated the most effectiveness in inactivating ANFs because these compounds are either heat-labile, water-soluble, or both in nature, making them remarkably susceptible to hydrothermal processing (Khattab & Arntfield, 2009; Patterson et al., 2017). Micronization, a process that is typically preceded by tempering, involves preparing seeds to a pre-determined moisture content rather than the use of soaking (Wray, 1999). Micronization has been reported to significantly reduce TIA and polyphenols. For example, Bai et al. (2018) reported that the TIA reductions in un-tempered micronized desi chickpeas reached up to 40%, while desi

chickpeas tempered to 20% moisture content exhibited TIA reductions up to 83%. Notably, the tempering step did not affect the concentrations of polyphenols and CT in their study. However, Khattab & Arntfield (2009) demonstrated that, with the exception of TIA, which achieved a high reduction of 88.80 – 94.35%, micronization after tempering to 24% moisture content also significantly reduced tannins, PA, and oligosaccharides in Canadian and Egyptian cowpeas, kidney beans, and peas. Specifically, tannin contents were reduced by 6.38 – 88.63% (varied between samples), PA by 31.86 – 37.62%, and total oligosaccharides by 14.92 – 20.53% in these pulse varieties. Nevertheless, micronization proved less effective in reducing ANFs than other hydrothermal treatments, such as boiling, pre-germinated boiling, microwaving, autoclaving, and roasting (Khattab & Arntfield, 2009; Ma et al., 2017). With respect to baking, it is classified as a dry-heat process that operates without the direct involvement of water or steam (Drulyte & Orlien, 2019). The effectiveness of baking in inactivating ANFs varies compared to hydrothermal treatments. Sánchez-Velázquez et al. (2021) reported that baking led to a significant decrease in PA, achieving at least a 34% reduction in lentils, split peas, chickpeas, and five common beans. However, there were no significant decreases in saponin contents across any tested samples and in the polyphenols of most of the samples tested. In fact, baking led to a significant increase in saponin contents in red lentils, green lentils, green split peas, red kidney beans and navy beans. Similarly, polyphenol contents increased after baking in red lentils, green split peas, yellow split peas, chickpeas, and navy beans (Sánchez-Velázquez et al., 2021). These results demonstrate that thermal treatments have proven efficacy in reducing ANFs in soybeans and pulse samples, thereby enhancing their suitability for incorporation into a variety of food products. Nevertheless, there remains limited information on the effects of thermal processing on the ANFs in protein-enriched derivatives such as protein concentrates and protein isolates.

While numerous studies have investigated the effects of various processing methods, particularly thermal processing, on the nutritional value, ANFs, and protein quality of pulses, research on the impact of HHP processing on these factors, as well as on pulse protein concentrates, and protein isolates, remains limited. Studies have shown promising results with HHP treatment at 600 MPa, providing reductions in ANFs, and improvements in nutritional values and protein digestibility across various pulses. For example, Lee et al. (2018) reported that HHP at 600 MPa for 5 min significantly reduced TIA and PA in red bean powder by 80.5% and 13.5%, respectively, which were remarkably comparable to the reductions achieved by boiling at 90°C for 15 min), with TIA and PA reduced by 83.3% and 12.0%, respectively. Additionally, the *in vitro* protein digestibility of red bean powder improved significantly from 79.3% to 83.8% following HHP treatment, while it remained unchanged with boiling treatment. Similar trends were observed by Linsberger-Martin et al. (2013), with significant reductions of up to 68.41% in oligosaccharides, 35.65% in PA, 28.17% in total phenolic acid content, and a complete reduction (100%) in TIA, along with improvements in protein digestibility, which increased from 82.3% in unprocessed sample to 85.8% after HHP treatment, particularly observed when split peas were treated at 600 MPa in conjunction with different holding time (30 and 60 min) and temperature (20 and 60°C). Deng et al. (2015) also found that HHP treatment at 600 MPa at 60°C for 30 min resulted in decreases in PA, TIA, tannin, and saponin in buckwheat by 45.49%, 12.63%, 19.88%, and 14.55%, respectively. HHP has been continuously explored for its potential to reduce ANFs and enhance protein digestibility, where these phenomena could be achieved through the structural denaturation of proteins, thereby increasing the accessibility of proteolytic enzymes under high-pressure conditions (Wu et al., 2023). Therefore, according to previous studies, HHP treatment at 600 MPa is astonishingly efficient at reducing ANFs and enhancing the nutritional quality in diverse grains. Overall, HHP treatment, as a novel non-thermal treatment technique, demonstrates significant

potential by maximally preserving desirable organoleptic properties and nutrients, greatly reducing ANFs, and enhancing protein digestibility. Consequently, HHP is an alternative technique to thermal treatment in food processing. However, there is a lack of studies on ANFs, nutritional value, and protein quality in yellow field peas and their derivatives, as well as other pulses by HHP treatment.

In summary, these thermal and non-thermal processing methods could play a critical role in improving the nutritional quality and digestibility of pulses, particularly yellow field peas, by reducing the presence of ANFs and altering seed structure. However, there are trade-offs between each method in terms of nutrient retention and efficiency for maximizing protein quality and minimizing ANF contents.

2.5 IN VITRO PROTEIN QUALITY

2.5.1 Overview of Protein

Proteins, as one of the macronutrients, play a vital role in human growth, development, and maintenance of various physiological functions (Hertzler et al., 2020; Wu, 2016; Sá et al., 2020). Protein content in food is typically estimated by measuring the nitrogen (N) content of a protein source and then multiplying this value by a nitrogen-to-protein conversion factor. This approach is based on the general assumption that the nitrogen content in proteins is approximately 16%, meaning every 100 g of protein contains about 16 g of nitrogen. Consequently, the conversion factor for most foods is given as 6.25, which is derived from the calculation of 100/16 = 6.25. Therefore, the formula used to estimate the protein content is N × 6.25, which is also referred to as the “crude protein” content, as it assumes that all nitrogen present in the food sources originates from proteins, although some nitrogen may come from non-protein sources (Mariotti et al., 2008). However, the appropriate conversion factor varies significantly depending on the type of food

products, in particular the differentiation between animal and plant proteins, due to variations in amino acid profiles, nitrogen content, non-protein nitrogen content, and species. Additionally, processing and the purification conditions used (e.g., the degree of protein purification) influence nitrogen content in different protein sources (Mariotti et al., 2008). Thus, while the average conversion factor of 6.25 is commonly applied, it may not accurately reflect the true protein content for certain cereals, pulses, oilseeds, nuts, and milk (Sosulski & Imafidon, 1990; Mariotti et al., 2008). As a result, to ensure a more accurate assessment of nutritional value, specific conversion factors ranging from 5.24 to 5.64 have been suggested for a number of pulse samples, in particular for pea samples, with specific conversion factors of 5.24, 5.40, 5.44, and 5.45 being recommended, as mentioned in Mariotti et al. (2008) and Pedrosa et al. (2020). Nevertheless, the absence of standardized methods for identifying specific conversion factors has led to inconsistent development and application of new factors (Krul, 2019). Consequently, a conversion factor of 6.25 is always recommended and required for the calculation of crude protein content across most animal and plant protein sources, including pulses, as endorsed by the FAO/WHO (2013) (Sosulski & Imafidon, 1990; FAO/WHO, 2013). Therefore, the continued use of 6.25 ensures consistency in nutritional assessments across different studies and food products.

2.5.2 Overview of Amino Acid Profile and Measurements

The fundamental structure of a protein is primarily composed of 20 standard amino acids that are arranged in many different sequences and linked together by peptide bonds. These 20 amino acids can be categorized based on their unique side chains and their net charge at physiological pH into hydrophobic, hydrophilic uncharged, and hydrophilic charged amino acids (Bessada et al., 2019). Among these amino acids, there are nine amino acids to be considered indispensable or essential, meaning that the human or other mammalian bodies cannot synthesize

them endogenously and they must be acquired through dietary intake. These indispensable amino acids (IAAs) include histidine (HIS), isoleucine (ILE), leucine (LEU), lysine (LYS), methionine (MET), phenylalanine (PHE), threonine (THR), tryptophan (TRP), and valine (VAL) (Bessada et al., 2019). Additionally, certain amino acids are considered conditionally IAAs, as they can be normally synthesized by the body but become indispensable under specific conditions such as illness, stress, rapid growth, pregnancy, or recovery when the body's demand surpasses its synthetic capacity (Bender, 2012; Oni et al., 2021; Lopez & Mohiuddin, 2020; Bessada et al., 2019). Specifically, cysteine (CYS) and tyrosine (TYR) are considered conditionally essential amino acids as they are typically synthesized from their essential precursors: MET and PHE, respectively (Shi, 2021; Bender, 2012). On the other hand, dispensable amino acids (also known as non-essential amino acids) that can be endogenously synthesized by the body, include alanine (ALA), arginine (ARG), aspartic acid (ASP), glutamic acid (GLU), glycine (GLY), proline (PRO), and serine (SER) (Bender, 2012; Frias et al., 2011). Proteins derived from pulses, seeds, and other vegetables are often deficient in certain IAAs, specifically TRP or sulfur-containing amino acids (i.e., MET and CYS) (Bessada et al., 2019). This limitation can lead to lower overall protein quality in plant-derived proteins compared to animal-derived proteins, which are referred to as complete proteins as they generally possess all nine IAAs in adequate proportions relative to human dietary requirements for growth, development, and maintenance of bodily functions (Bessada et al., 2019; Nosworthy & House, 2017; Nosworthy et al., 2017a; Sá & House, 2024; Herreman et al., 2020). Despite the fact that pea proteins contain all nine IAAs, they are deficient in either TRP or sulfur-containing amino acids when compared to human nutritional requirements, which means those are typically considered first limiting amino acids (LAA) (Stone et al., 2019; Nosworthy et al., 2017a). This deficiency underscores the need to complement pea proteins with other protein sources to

achieve a balanced and complete amino acid profile for optimal health benefits (Bessada et al., 2019).

It is important to note that specific processing treatments can lead to the loss of sulfur-containing amino acids which serve as the first or second limiting amino acids in pulse-based samples, thereby impacting the overall nutritional profile of the food product (Rutherfurd & Moughan, 2008; Marshall et al., 1982). Sulfur-containing amino acids including MET and CYS, are relatively unstable and particularly susceptible to food processing treatments such as heating, alkaline conditions, oxidative environments, and isolation processes, leading to chemical changes to form methionine sulfoxide and methionine sulfone from methionine, as well as cysteic acid from cysteine (Rutherfurd & Moughan, 2008; Marshall et al., 1982; Chang et al., 1982; Todd et al., 1984). The oxidized derivatives of sulfur-containing amino acids, specifically methionine sulfone and cysteic acid, are generally considered biologically unavailable to animals and humans. Methionine sulfoxide, however, remains partial bioavailability in limited quantities (Marshall et al., 1982; Chang et al., 1985; Rutherfurd & Gilani, 2009; Xiong & Guo, 2021). For instance, Anderson et al. (1976) discovered that roughly 60% of L-methionine sulfoxide was deemed to be in a bioavailable form and utilized for growth in experimental rats. It is important to consider that when conducting performic acid oxidation followed by acid hydrolysis, which is the official procedure commonly used for the quantification of sulfur-containing amino acids in food samples, the resulting amino acid analysis may still detect oxidized forms of MET and CYS that are not biologically available in processed samples. This can lead to an overestimation of the levels of sulfur-containing amino acids, and consequently an inflated assessment of protein quality in processed products (Rutherfurd & Gilani, 2009).

To quantify the amino acid composition in foods, the protein source typically undergoes three fundamental analytical procedures including protein hydrolysis, separation of amino acids,

and the subsequent detection and quantification of the separated amino acids (Otter, 2012). Protein molecules are initially hydrolyzed to convert them into their constituent amino acids by breaking the peptide bonds that connect the amino acids in the protein chain (Otter, 2012). According to FAO/WHO (2013), three standardized approaches are currently employed to determine amino acid content in proteins:

1. Regular amino acid hydrolysis. This standard method is used to determine all amino acids with the exception of MET, CYS, and TRP. It involves acid hydrolysis through incubating the sample with a strong acid, i.e., 6 N hydrochloric acid (HCl) at 110°C for 24 hr, which effectively cleaves the peptide bonds and releases individual amino acids for subsequent analysis (Rutherfurd & Gilani, 2009; AOAC, 1995; FAO/WHO, 2013). Alternative hydrolysis methods have been explored such as automatic protein hydrolysis with strong cation-exchange resins at 110°C for 20 hr, which yields 70 – 75% of amino acid recovery compared to the standard method while reducing protein contamination through the direct integration of columns with an HPLC system. Other efficient methods include a rapid hydrolysis with 6 N HCl, at 160°C for 1 hr, achieving a mean recovery value of 98.4% for individual amino acids, and microwave-assisted acid hydrolysis using 6 N HCl at 150°C for 3 hr, resulting in a recovery of at least 85.1% of the total protein content (Otter, 2012). Despite the potential advantages of these alternative methods, comprehensive studies on the effectiveness and comparability of these alternatives with the standard hydrolysis method are limited.
2. Oxidized amino acid hydrolysis. This method is specifically used for the determination of MET and CYS. During this process, performic acid oxidation converts CYS into cysteic acid and MET into methionine sulfone, thus stabilizing these amino acids before subsequent acid hydrolysis (FAO/WHO, 2013; Rutherfurd & Gilani, 2009). Notably,

except for TRP, TYR and PHE, which showed significant decreases after oxidation, most amino acids typically measured through acid hydrolysis can also be accurately quantified in samples treated with performic acid, with HIS, in particular, showing an increase of more than 20%, which demonstrates the broad compatibility of the method (Rutherfurd & Gilani, 2009; Thera et al., 2018).

3. Alkaline amino acid hydrolysis. This approach is specifically used to determine TRP because it is susceptible to degradation under acidic conditions, requiring hydrolysis with barium hydroxide, sodium hydroxide, or lithium hydroxide (FAO/WHO, 2013; Rutherfurd & Gilani, 2009). While other amino acids require derivatization to improve their detectability, TRP can be detected without derivatization due to its ability to absorb natural ultraviolet absorption and fluorescence properties (Rutherfurd & Gilani, 2009).

The use of specific hydrolysis procedures for MET, CYS, and TRP contributes to accurate assessment, as these amino acids can be partially or completely degraded under standard acid hydrolysis conditions (Otter, 2012; Rutherfurd & Gilani, 2009). When dealing with processed food protein sources, particularly those that have undergone thermal treatment, specialized measurements for the determination of reactive lysine and reactive sulfur-containing amino acids should be also considered and standardized, which is recommended by FAO/WHO (2013) and Rutherfurd & Gilani (2009).

Following hydrolysis, the separation and quantification of individual amino acids are effectively accomplished through various chromatographic techniques such as Ultra-Performance Liquid Chromatography (UPLC), High-Performance Liquid Chromatography (HPLC), and Gas Chromatography (GC). These techniques are commonly coupled with a range of detectors such as fluorescence (FLR), Ultraviolet-visible (UV-vis), or photodiode array (PDA), and mass

spectrometry (MS) (Otter, 2012; Kaur et al., 2023). UPLC and HPLC are instrumental in amino acid analysis due to their high resolution, sensitivity, and precision. GC technique, while effective, is less commonly utilized for amino acid analysis, as it is intended for specific applications where amino acids are derivatized into volatile forms (Otter, 2012). The amino acid profiles obtained from these chromatographic techniques can be further used to calculate the amino acid score (AAS), an important metric for assessing protein quality by comparing the content of IAAs in the test protein against a reference pattern. This score helps determine the nutritional value of protein sources in human diets, ensuring that they meet the body's requirements for growth, development, and overall health (Nosworthy & House, 2017).

2.5.3 Overview of Protein Digestibility and Measurements

While the amino acid profile offers valuable insights into the potential nutritional value of proteins for protein quality assessment, assessing protein digestibility (PD) is another critical element in determining overall protein quality (Bessada et al., 2019). PD refers to the percentage of dietary proteins that are effectively broken down (digested) into smaller peptides and amino acids and subsequently absorbed in the digestive tract, making them available for synthesis and utilization by the human body. PD is also essential for assessing the bioavailability of amino acids (Bessada et al., 2019; Nosworthy & House, 2017; Sá & House, 2024). Most food proteins derived from plant sources generally exhibit lower digestibility compared to animal-based proteins (Nosworthy et al., 2023; Sá et al., 2019; Gilani et al., 2005; Berrazaga et al., 2019). The lower digestibility of plant-based proteins can be attributed to their inherent structural characteristics, including a compact and rigid protein matrix. This structure arises from a high content of β-sheet conformation and a low proportion of α-helix secondary structure, as well as the presence of non-

starch polysaccharides or fibers, which restrict the accessibility of digestive enzymes (Berrazaga et al., 2019; Bessada et al., 2019). Beyond structural barriers, ANFs such as enzyme inhibitors, phytic acid, and phenolic compounds, can further diminish digestibility. These compounds, which are prevalent in plant-based sources but typically absent in animal-based sources, can bind to digestive enzymes or essential nutrients, inhibiting enzymatic activity and reducing amino acid bioavailability, ultimately limiting the overall nutritional value and quality of plant-based proteins (Berrazaga et al., 2019; Nosworthy et al., 2023; Sá & House, 2024).

PD can be assessed using two primary methods: ileal digestibility and fecal digestibility, which differ considerably in terms of the location within the digestive tract where measurements are taken and the accuracy of estimating amino acid absorption (Bessada et al., 2019). Ileal digestibility (also referred to as apparent, standardized, or true digestibility) involves the collection of digesta samples from the terminal ileum, the final section of the small intestine (Bessada et al., 2019). This method provides a more accurate reflection of nitrogen absorption as it focuses on the site where most nutrient absorption occurs (FAO/WHO, 2013; Berrazaga et al., 2019). By measuring the disappearance of nitrogen at the end of the terminal ileum, ileal digestibility avoids the interfering effects of nitrogen excretion and microbial fermentation by the microflora in the large intestine. However, it is important to note that ileal digestibility is considered invasive due to the need for surgical interventions or specialized techniques to access the ileal contents (Nosworthy & House, 2017; Reynaud et al., 2021; FAO/WHO, 2013). Additionally, a potential limitation of ileal digestibility is the challenge of distinguishing between dietary proteins and endogenous proteins that are secreted into the digestive system and reabsorbed at the terminal ileum. Nonetheless, methods have been developed to estimate and correct for the contribution of endogenous protein secretions, thereby improving the accuracy of ileal digestibility measurements (Bessada et al., 2019; Nosworthy & House, 2017; FAO/WHO, 2013; Rutherfurd et al., 2015).

While ileal digestibility measurements are more commonly used in animal studies, fecal digestibility measurements (i.e., apparent or true protein digestibility) reflect the overall digestibility of a protein source throughout the entire digestive tract. This method estimates digestibility by measuring the difference between the amount of nitrogen ingested and the amount of nitrogen excreted in feces, which represents the proportion of dietary nitrogen that is absorbed by the body and not lost through fecal excretion (Nosworthy et al., 2023; Sá & House, 2024; Nosworthy & House, 2017). To obtain a more accurate assessment, known as true fecal digestibility, it is essential to correct for endogenous nitrogen losses, i.e., proteins secreted into the digestive tract that are not derived from the diet. This correction can be made by feeding control animals a protein-free, casein, or hydrolyzed casein diet (Nosworthy et al., 2023; Sá & House, 2024; Nosworthy & House, 2017; Moughan & Wolfe, 2019). Fecal digestibility assessments can be performed with or without the use of animals (Sá & House, 2024). Fecal digestibility measurements are less invasive and simpler to perform than ileal digestibility, however, their accuracy can be compromised by the net excretion of amino acids by the microflora (leading to an underestimation of the protein quality) and by microbial fermentation (resulting in an overestimation of the protein quality) in the large intestine. These microbial activities can alter the nitrogen composition of feces (Nosworthy & House, 2017; FAO/WHO, 2013). In summary, ileal digestibility provides a more reliable measurement of protein absorption. However, its application is limited by its invasive nature, which raises ethical considerations and procedural complexities. Fecal digestibility, on the other hand, offers a more practical and less invasive alternative.

Overall, both amino acid profile and protein digestibility can contribute to further comprehensive assessment of protein quality.

2.5.4 Overview of Protein Quality and Measurements

Protein quality is a critical parameter that reflects the capability of a protein source to support the physiological and nutritional needs of the human body. It also is integral in establishing protein content claims for food products (Mansilla et al., 2020; Nosworthy & House, 2017). To achieve this, accurate methods for assessing the quality of dietary proteins are necessary to comply with regulatory standards and guarantee consumer health (Mansilla et al., 2020; Marinangeli & House, 2017).

Among the available methods, the Protein-Digestibility-Corrected Amino Acid Score (PDCAAS) method is the most widely used and endorsed in human nutrition. This method was recommended in 1989 by the Joint Food and Agriculture Organization (FAO)/World Health Organization (WHO) Expert Consultation on Protein Quality Evaluation for foods and infant formulas (FAO/WHO, 2013). The PDCAAS method evaluates protein quality by incorporating two essential elements: the lowest AAS of the IAA and protein digestibility (FAO/WHO, 2013). The AAS is calculated by comparing the amount of each IAA in the test protein to a reference requirement pattern, enabling further identification of the IAA with the lowest score relative to this reference pattern (Marinangeli & House, 2017; Nosworthy & House, 2017; FAO/WHO, 2013). This IAA, known as the first limiting amino acid (LAA), is critical in determining the overall score as it represents the "bottleneck" for protein synthesis in the body. The presence of an LAA restricts the body's ability to effectively utilize the other amino acids, regardless of their abundance (Berrazaga et al., 2019). FAO/WHO (1991) recommended using the IAA requirement of preschool children aged 2 to 5 years as the reference requirement pattern for calculating the AAS. This recommendation is based on the fact that the age group of young children have high IAA requirements to support their rapid growth and development. By setting the IAA needs of preschool children as the benchmark, the scoring pattern ensures that a protein source adequate for

this demographic will also meet the requirements of older children and adult populations, whose IAA requirements are typically lower on a mg/kg body weight basis. For infants, however, the amino acid profile of breast milk is used as the reference scoring pattern (**Table 2.3**).

The PDCAAS method can be applied using both *in vivo* and *in vitro* assays to evaluate protein quality (Nosworthy & House, 2017). The *in vivo* PDCAAS assessments typically rely on measuring true fecal nitrogen digestibility (TPD) through the fecal collection in rodent bioassays (Mansilla et al., 2020). Nonetheless, there has been a growing preference for *in vitro* assays due to several advantages over *in vivo* assays since *in vitro* assays are not only less costly and time-consuming but also eliminate the need for animals and reduce the variability that arises from biological differences between test subjects (Prachansuwan et al., 2019; Mansilla et al., 2020; Sá & House, 2024). The *in vitro* protein digestibility (IVPD) can be assessed using both dynamic and static digestion methods (Mansilla et al., 2020). The dynamic digestion method, such as the TNO Gastro-Intestinal Model (TIM system), simulates the complex processes of the human digestive tract with high accuracy and reproducibility. This system employs computer-controlled mechanisms to mimic physiological conditions of the human body, such as digestive enzyme activity, digestion time, pH, temperature, enzymatic secretions, and peristaltic movement (Minekus et al., 1995; Minekus et al., 2014; Dupont et al., 2018; Mansilla et al., 2020). In contrast, static digestion methods, such as pH-drop, pH-stat, two-step, and European INFOGEST models, offer simpler, cost-effective, and more straightforward approaches for evaluating protein digestibility, utilizing specific protocols and enzyme systems to simulate digestion (Sá & House, 2024; Mansilla et al., 2020; Hsu et al., 1977; Mendes et al., 2016; Pedersen & Eggum, 1981; Franczyk, 2018; Bui, 2024). Of which the pH-drop method developed by Hsu et al. (1977), is widely used due to its ease of use and accessibility, accomplished by monitoring the change in pH over a 10-minute period, during which the amino acids and peptides increase in response to

enzymatic digestion, resulting in the release of protons. The enzyme system utilized typically consists of multiple enzymes, including trypsin, chymotrypsin, and protease, which collectively hydrolyze proteins in food samples (Franczyk, 2018; Hsu et al., 1977; Pedersen & Eggum, 1981). Furthermore, different regression equations that account for pH changes observed during digestion have been established in studies by Hsu et al. (1977), Pedersen & Eggum (1981), and Mendes et al. (2016), providing a reliable method for predicting both apparent and true fecal nitrogen digestibility across different protein types, including animal, plant, or both. Summarily, numerous studies have consistently demonstrated a strong correlation between *in vivo* and *in vitro* measurements in terms of protein digestibility and PDCAAS values for pulses, underscoring the reliability of *in vitro* assays for evaluating overall protein quality (Hsu et al., 1977; Pedersen & Eggum, 1981; Nosworthy et al., 2017a; Rutherfurd et al., 2015; House et al., 2019). For instance, a high R^2 value of up to 0.97 between *in vivo* and *in vitro* PDCAAS was observed in the study by Nosworthy et al. (2017a). The strong correlation highlights the effectiveness of *in vitro* methods as a viable and practical alternative to *in vivo* assessments.

In the context of the PDCAAS method, a controversial issue arises from the practice of truncating PDCAAS values that theoretically exceed 100% are truncated to 100% to ensure that all values fall within the 0% to 100% range (Nosworthy & House, 2017; Leser, 2013). This truncation caps the maximum score a protein can achieve, meaning that no protein can be scored higher than that of the reference protein. While this approach standardizes protein quality assessments, it may inadvertently underestimate the true value of exceptionally high-quality proteins that surpass the 100% threshold, such as those found in eggs and cow's milk (Nosworthy & House, 2017; Mansilla et al., 2020; Leser, 2013). In the United States, protein content claims are calculated based on the corrected protein content per Reference Amount Customarily Consumed (RACC) (Marinangeli & House, 2017). This process involves multiplying the crude

protein (%) by the PDCAAS value, and then by the RACC. The resulting PDCAAS-corrected protein value is used to calculate the percent Daily Value (DV), with the daily reference value set at 50 g of protein. Foods meet the criteria for a "good source of protein" if the PDCAAS-corrected protein value ranges from 5 to 9.9 g per RACC, corresponding to 10 – 19.9% of the DV, and an "excellent source of protein" if it equal or greater than 10 g per RACC, which is equivalent to 20% or more of the DV (Marinangeli & House, 2017; Sá & House, 2024; Nosworthy et al., 2023).

The PDCAAS method, recognized as the official method for evaluating protein quality in the United States, has recently been accepted and adopted in Canada by Health Canada (HC). In addition to PDCAAS, the other two primary methods currently available are the Protein Efficiency Ratio (PER) and the Digestible Indispensable Amino Acid Score (DIAAS) (Mansilla et al., 2020; Nosworthy et al., 2023), as summarized in **Table 2.4**.

The PER method, initially established in 1919, is a necessary method officially accepted in both Canada and the United States for *in vivo* protein quality assessment (Mansilla et al., 2020). This method measures body weight gain over a 28-day period in rapidly growing experimental animals, typically weanling rats, on a diet in which the test protein serves as the only source of dietary nitrogen and constitutes approximately 10% of the diet, while a control group is fed a diet containing 10% casein (a standard reference protein) (Marinangeli & House, 2017). The PER is obtained by dividing the total body weight gain (in grams) by the total amount of protein consumed (in grams) during the test period. To ensure consistency and comparability across different studies, the actual PER of the sample (i.e., adjusted PER) is further corrected by multiplying the observed PER_{sample} by the ratio of a standardized reference value of 2.5 to the $PER_{control}$ (Mansilla et al., 2020; Marinangeli & House, 2017; Nosworthy et al., 2023). In Canada, the PER method remains the regulatory standard for determining protein quality claims in food products (Nosworthy & House, 2017). The regulatory protein claims are established based on the protein rating, which is

derived by multiplying the grams of protein in a Reasonable Daily Intake (RDI) by the adjusted PER. A food product qualifies for the claim of "a source of protein" if the calculated protein rating falls between 20 and 39.9. If the protein rating exceeds 40, the product can be labelled as "an excellent source of protein" (Nosworthy & House, 2017; Marinangeli & House, 2017). Notably, Health Canada permits a conversion between PER and PDCAAS to facilitate the determination of protein content claims on food labels in Canada, wherein the estimated PER can be derived by multiplying the established PDCAAS value by a conversion factor of 2.5 (Health Canada, 2023; Nosworthy et al., 2023; Marinangeli & House, 2017; Sá & House, 2024). However, the PER method has several inherent limitations due to its reliance on animal models. Firstly, it is based on the growth response of weanling rats, which have higher amino acid requirements than humans, particularly for certain amino acids such as sulfur-containing amino acids. Consequently, the PER may not accurately reflect human protein requirements, especially for adults or individuals who are not in the growth phases. In contrast, the PDCAAS method is favored because it is based on human amino acid requirements compared to animal bioassays, making it a reliable indicator of how well a given protein source meets human dietary needs (FAO/WHO, 2013; Tome et al., 2024). Secondly, the PER method does not take into account the fact that body weight gain in rats may not directly correlate with an increase in the protein of the body, as weight gain may also include the accumulation of other non-protein components (Bessada et al., 2019). Moreover, there are ethical concerns associated with the use of animal-based assays, given the increasing interest in alternatives without the need for animal involvement (Nosworthy & House, 2017; Bessada et al., 2019).

The DIAAS is the most recently established method advocated by the FAO to replace the PER and PDCAAS approaches (FAO/WHO, 2013; Marinangeli & House, 2017). Unlike PDCAAS, which calculates protein quality based on fecal nitrogen digestibility, DIAAS evaluates

the true ileal digestibility of individual IAAs, providing a more accurate reflection of amino acid absorption and bioavailability at the end of the terminal ileum. This method can utilize different models, including humans, growing pigs, and growing rats. In human studies, the use of a naso-ileal tube allows for sampling of the digesta directly from the small intestine following the ingestion of N-labeled amino acids (FAO/WHO, 2013; Marinangeli & House, 2017; Havenaar et al., 2016). However, due to the invasive nature of direct sampling methods, a study by Havenaar et al. (2016) explored the use of an *in vitro* dynamic gastrointestinal model – tiny TIM system, which correlated strongly with human data ($R^2 = 0.96$) and could serve as a potential alternative to the DIAAS method for accurately predicting protein quality while minimizing the need for animal use. In contrast to the PDCAAS method, the DIAAS approach is untruncated and based on the amino acid requirement pattern for young children (6 months to 3 years) as its reference (**Table 2.3**) (FAO/WHO, 2013; Asen et al., 2023; Havenaar et al., 2016). The DIAAS has been proposed by the FAO for establishing protein content claims, similar to the PDCAAS method utilized in the United States. Under the DIAAS framework, only foods exceeding a threshold of 75% are eligible to make protein content claims, while the rationale for establishing this specific threshold has not been clearly justified. Foods can be labelled as a "good source of protein" if their DIAAS value falls between 75% and 99% and its corrected protein value is between 5 and 10 g per RACC, and an "excellent source of protein" if their DIAAS value is 100% or higher and a corrected protein value is at least 10 g per RACC (Marinangeli & House, 2017; Sá & House, 2024; Nosworthy et al., 2023).

In this study, the protein quality of yellow field peas and their derivatives will be assessed using the *in vitro* PDCAAS method, which focuses on the determinations of the AAS of the first LAA and the *in vitro* protein digestibility. This method offers a reliable and efficient alternative to other methods for evaluating protein quality without the ethical and methodologically complex

challenges associated with *in vivo* methods. This research will contribute to a better understanding of how different food processing treatments can affect the protein quality of yellow field pea samples.

Table 2.3. Recommended IAA scoring patterns according to FAO/WHO (1991) for the PDCAAS method and the most recent FAO/WHO (2013) for the DIAAS method (mg/g protein/day).

Age group	THR	VAL	M+C	ILE	LEU	P+T	HIS	LYS	TRP
Scoring pattern mg/g protein requirements for PDCAAS (FAO/WHO, 1991)									
Pre-school child (2-5 years)	34.0	35.0	25.0	28.0	66.0	63.0	19.0	58.0	11.0
Infant (0-0.5 years)[a]	43.0	55.0	42.0	46.0	93.0	72.0	26.0	66.0	17.0
School child (10-12 years)	28.0	25.0	22.0	28.0	44.0	22.0	19.0	44.0	9.0
Adult	9.0	13.0	17.0	13.0	19.0	19.0	16.0	16.0	5.0
Scoring pattern mg/g protein requirements for DIAAS (FAO/WHO, 2013)									
Pre-school child (0.5-3 years)	31.0	43.0	27.0	32.0	66.0	52.0	20.0	57.0	8.5
Infant (0-0.5 years)[a]	44.0	55.0	33.0	55.0	96.0	94.0	21.0	69.0	17.0
Older child, adolescent, adult (3-10 years)	25.0	40.0	23.0	30.0	61.0	41.0	16.0	48.0	6.6

[a] Amino acid composition of human milk.

Abbreviations: THR = Threonine; VAL = Valine; M+C = Methionine + Cysteine; ILE = Isoleucine; LEU = Leucine; P+T = Phenylalanine + Tyrosine; HIS = Histidine; LYS = Lysine; TRP = Tryptophan.

Table 2.4. Overview of the mechanisms, advantages, and disadvantages of PER, PDCAAS, and DIAAS for the determination of protein quality.

Methods	Mechanisms	Advantages	Disadvantages
PER	• Weight gain measurement of growing rats per unit of protein consumed	• Historically used • Straightforward	• Growth-specific, limited to growing animals • Not directly applicable to humans • Ethical concerns
PDCAAS	• Amino acid score • Apparent or true fecal protein digestibility • Both *in vivo* and *in vitro* assays	• Human-relevant • Reduced time/cost, and no ethical concerns with *in vitro* methods	• Invasiveness in *in vivo* methods • Overestimated the amount of amino acid absorbed due to the fecal collection in nature • Truncated at 100%, which can underestimate high-quality proteins
DIAAS	• Amino acid score • Ileal or true, or standardized digestibility of individual amino acids • Currently focusing on *in vivo* DIAAS	• More accurate reflection of protein quality • Measures digestibility at the ileal level, avoiding microbial effects in large intestinal.	• More complex and resource-intensive • Invasive sampling required in *in vivo* studies • Limited data on human digestibility values.

2.5.5 Effects of Food Processing on Protein Quality

Based on the processing methods in use, the amino acid composition and protein digestibility of food products can be altered to different extents, thereby influencing the overall protein quality of pulses including yellow field peas, as mentioned in numerous studies by Sá & House (2024), Bai et al. (2018), Nosworthy et al. (2017a), Gilani et al. (2005), Lalitha & Singh (2020), Çabuk et al. (2018), Khattab et al. (2009), Wang et al. (2019), Sánchez-Velázquez et al. (2021), and Nosworthy et al. (2018b). A study by Nosworthy et al. (2017a) investigated the AAS, IVPD, and *in vitro* PDCAAS of split pea seeds that were processed by extrusion, baking and boiling. Their findings showed that the IVPD values for the baked yellow and green split peas were approximately 82%, whereas the boiled yellow and green split peas exhibited higher IVPD values of approximately 87%. Correspondingly, lower *in vitro* PDCAAS values were observed in baked yellow and green split peas, which were 65.49% and 69.88%, respectively. The *in vitro* PDCAAS values of boiled yellow and green split peas were reported to be 67.44% and 71.17%, respectively. However, Sánchez-Velázquez et al. (2021) discovered inconsistent findings with the preceding study. In their study, boiled yellow and green split peas had IVPD values of 88.77% and 88.71%, respectively, which was highly similar to those of 87.68% and 88.10% for baked yellow and green split peas. Moreover, with respect to the *in vitro* PDCAAS of both split peas, the baking treatment had a more pronounced effect on enhancing the *in vitro* PDCAAS of both yellow and green split peas as compared to boiling. Especially in comparison to the boiled yellow split pea (67.87%), the baked one had up to 84.93% of *in vitro* PDCAAS. The existing research indicates gaps in studies on the amino acid profile, protein digestibility, as well as overall *in vitro* protein quality of many pulses under specific processing methods such as baking, infrared heating, and autoclaving processing. Furthermore, although the impacts of most food processing techniques on the *in vitro* protein quality in diverse pulse flours have been extensively explored, there is a scarcity

of research examining the effects on protein quality in protein concentrates, and isolates derived from corresponding flours.

It is important to note that the presence of ANFs in pulse-based protein sources, such as trypsin and chymotrypsin inhibitors, polyphenols, tannins, lectins, and phytic acid, can potentially impede amino acid bioavailability and protein digestibility (Leser, 2013). Many attempts have been made to prove that inactivation of ANFs can be accomplished through the use of different processing methods such as infrared heating, dehulling, fermentation, extrusion, boiling, and baking (Bai et al., 2018; Lalitha & Singh, 2020; Çabuk et al., 2018; Nosworthy et al., 2018c; Devi et al., 2020; Sánchez-Velázquez et al., 2021). For instance, Bai et al. (2018) reported that chickpeas subjected to tempering-heat conditions (20% moisture content and 135°C) exhibited substantial reductions in TPC, CT, TIA, and chymotrypsin inhibitors, while the IVPD was raised from 76.46% to 79.28% and *in vitro* PDCAAS significantly improved from 64.99% to 70.94%. Despite these findings, there remains a lack of comprehensive studies on the correlations between various ANFs and protein quality parameters (e.g., protein digestibility and amino acid bioavailability). Specifically, the extent to which the presence and concentration of various ANFs influence protein quality is yet to be understood. Moreover, the overestimation of protein quality by PDCAAS can also be attributed to the limited restricted bioavailability of specific amino acids that are damaged by food processing, such as unavailable lysine (Leser, 2013).

2.6 REACTIVE LYSINE ANALYSIS

2.6.1 Overview of Lysine and Reactive Lysine

Lysine is one of the indispensable amino acids, implying that it cannot be synthesized in the human and animal body and therefore needs to be offered from food protein sources in the

daily diet (Rutherfurd, 2015). In general, lysine is most abundant in legumes and is present in smaller quantities in cereals. As such, lysine content serves as a critical indicator for evaluating the nutritional quality of foods or feedstuffs (Rutherfurd & Moughan, 2007). Lysine is a basic amino acid characterized by a positively charged ε – amino group [ε – NH_2] at the end of its side chain (Moughan & Rutherfurd, 2008; Rutherfurd & Moughan, 2007). During heat processing or prolonged storage, the ε – amino group of lysine has a strong affinity to react with a variety of compounds present in foods, such as reducing sugars, fats, polyphenols, vitamins, food additives, and other amino acids, which can significantly reduce the availability and ileal digestibility of lysine, as well as diminishing its nutritional value (Rutherfurd & Moughan, 2007; Stein et al., 2006; Rutherfurd, 2015; Brestenský et al., 2014). These reactions generate modified derivatives that are not nutritionally available, which are referred to as modified, unreactive, or unavailable lysine. In contrast, lysine with a free or unmodified ε – amino group is defined as unmodified, reactive, or available lysine (Rutherfurd, 2015).

2.6.2 The Fate of Lysine in Processing

The most significant and well-known chemical reaction involving lysine occurs when it reacts with reducing sugars under thermal treatments, a process known as the Maillard reaction (Rutherfurd, 2015). During this reaction, the ε – NH_2 group of lysine initially reacts with carbonyl groups of reducing sugars such as glucose under heat, forming an initial lysine - reducing sugar complex through a reversible condensation reaction. This leads to the production of Schiff's base, as illustrated in **Figure 2.1 (**Rutherfurd & Moughan, 2007; Rutherfurd, 2015**)**. Notably, the study by Finot et al. (1977) observed that these modified forms (Schiff's base) formed during the initial stages of the Maillard reaction were almost completely utilized and nearly as effective as free

lysine in supporting the growth of rats. However, as the Maillard reaction progresses, the Schiff's base subsequently undergoes an irreversible rearrangement, leading to the formation of ε – N-deoxyketosyllysine (also known as the Amadori product or early Maillard product). Further reactions lead to the formation of brown-colored compounds referred to as melanoidins, which are considered late Maillard products (Rutherfurd & Moughan, 2007; Rutherfurd, 2015). Both the early (Amadori product) and late (melanoidins) Maillard products are considered nutritionally unavailable and less digestible within the body. The irreversible nature of these reactions, particularly the formation of Amadori products, permanently alters the lysine residues such that they cannot be utilized or retained as a source of lysine in the body (Rutherfurd & Moughan, 2007; Finot et al., 1977; Faist & Erbersdobler, 2001).

In general, lysine content is typically assessed through traditional (regular) amino acid analysis, a process that involves hydrolyzing the peptide bonds that connect amino acids by treating proteins with concentrated acid and heat (6 M HCl at 110°C for 24 hr). This hydrolysis releases individual amino acids, including lysine, from the protein matrix (Rutherfurd, 2015). However, when analyzing processed samples containing early Maillard products, an important complication arises. Early Maillard products (Amadori products) are unstable and tend to revert back to lysine during acid hydrolysis. This reverted lysine, however, shares the same structure as unmodified lysine, it is chemically altered and nutritionally unavailable when absorbed to contribute to protein synthesis or further metabolism (Rutherfurd, 2015; Rutherfurd & Moughan, 2007). Despite this, it is still detected as part of the total lysine content when regular amino acid analysis is employed, as both reverted and unmodified lysine (i.e., reactive lysine) are indistinguishable by this method. This presents a significant challenge in accurately assessing the reactive lysine in processed foods. Since reverted lysine is detected along with unmodified lysine, traditional amino acid analysis (measuring the sum of reactive lysine and reverted lysine), can lead

to an overestimation of the nutritionally available lysine in processed samples (Rutherfurd, 2015; Bestenský et al., 2014; Torbatinejad et al., 2005).

It is important to note that the reverted lysine produced from early Maillard products remains nutritionally unavailable for the body. These early Maillard products do not revert to lysine under physiological conditions, such as those found in the mammalian or avian digestive tract. Furthermore, late Millard products, which possess a chemical structure distinctly different from lysine, cannot revert back to lysine under any conditions. Consequently, both early and late Maillard products cannot be broken down into lysine or utilized by the body under any conditions (Brestenský et al., 2014; Rutherfurd & Moughan, 2007; Rutherfurd, 2015). Therefore, it is necessary to use specific analytical methods to distinguish between total lysine and reactive lysine.

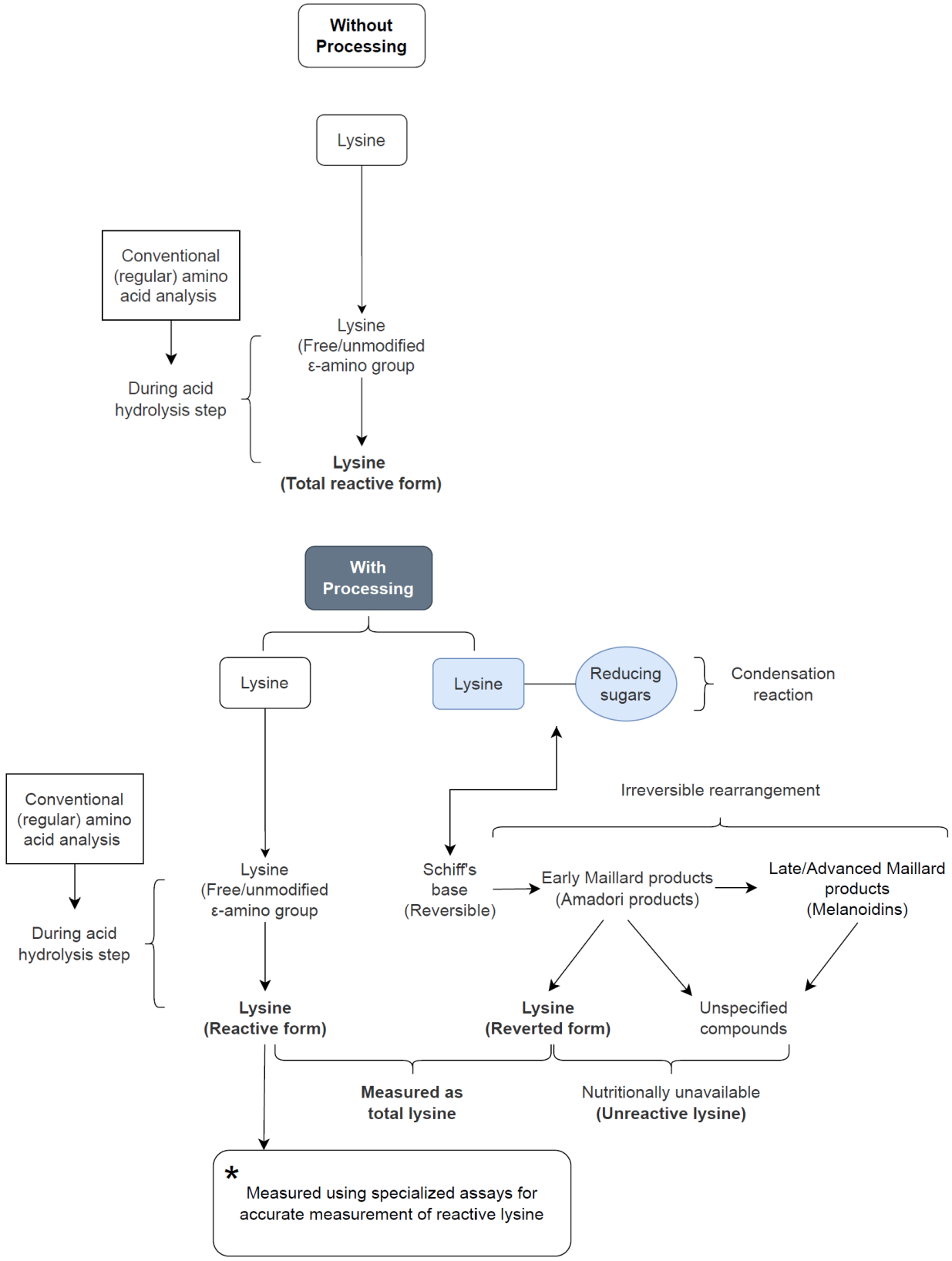

Figure 2.1. *The fate of lysine under food processing and analysis. Adapted from Rutherfurd (2015) and Rutherfurd & Moughan (2007).*

2.6.3 Specialized Methods for Measuring Reactive Lysine

Several analytical methods have been developed and employed to accurately quantify the amounts of reactive lysine in food and feed samples, including the Fluoro-di-nitro-benzene (FDNB) method (Malomo et al., 2013), the guanidination method (Rutherfurd et al., 2012), the *ortho*-phthaldialdehyde (OPA) (Barba et al., 2013), the furosine method (Desrosiers et al., 1989), and the dye-binding method (Hurrell et al., 1979). Each of these methods has a specific approach to targeting lysine with free-reacting side chain amino groups, except for the furosine method, which specifically quantifies the amount of furosine (= the amounts of reverted lysine) derived from early Maillard products during acid hydrolysis (Rutherfurd, 2015). Each method offers unique advantages, contributing to a comprehensive assessment of lysine availability and nutritional quality in processed foods.

The guanidination method is widely recognized for its specificity and effectiveness in targeting ε- amino group of lysine, where the free or peptide-bound ε-amino group of lysine can react with an O-methylisourea (OMIU) reagent, resulting in the formation of homoarginine as the end product. Interestingly, the specificity of the OMIU reagent is one of its key advantages, as it selectively interacts with the ε-amino group of lysine without reacting with other functional groups, including the N-terminal amino group of lysine (Rutherfurd & Moughan, 2007, Rutherfurd, 2015). The homoarginine product converted from reactive lysine is known for its exceptional resistance to acidic conditions. This property is particularly advantageous during the subsequent steps of amino acid analysis. The homoarginine product formed from reactive lysine undergoes the harsh acid hydrolysis process without degradation, thereby allowing for accurate chromatographic detection. This stability under acidic conditions ensures the integrity of the homoarginine product throughout the analytical procedure. Given sufficient incubation time, the amount of reactive lysine, which is equivalent to the amounts of homoarginine, ultimately can be calculated by

multiplying a molar weight ratio is 0.7767 (Rutherfurd & Moughan, 2007; Fontaine et al., 2007). However, the guanidination method is considerably time-consuming, primarily due to the prolonged incubation periods required for optimal accuracy. The incubation time after adding the OMIU reagent is critical for achieving maximum conversion of reactive lysine to homoarginine. This incubation period is particularly extended for heat-treated foods, which typically necessitate a reaction time of 3 to 7 days to ensure complete conversion, as reported in Moughan & Rutherfurd (1996), Rutherfurd et al. (1997), Rutherfurd et al. (2012), and Rutherfurd & Moughan (1997). Moreover, numerous studies have compared the guanidination method with alternative techniques for determining reactive lysine (Rutherfurd & Moughan, 2007; Rutherfurd et al., 1997; Torbatinejad et al., 2005). Torbatinejad et al. (2005) found a strong correlation between the guanidination method and the FDNB, with an R^2 of 0.985. The FDNB method, which is also widely used for reactive lysine determination, involves the reaction of FDNB with the free ε-amino group of lysine to form dinitrophenyl derivatives, which are then quantified by spectrophotometer or HPLC. Although the FDNB method is generally faster than the guanidination method, it is less specific for the side-chain amino group of lysine, as FDNB may interact with other amino acids and functional groups (e.g., the N-terminal amino group of free amino acids or peptides) present in the samples. Therefore, while the guanidination method is preferred for its specificity and accuracy, the FDNB method may be chosen for its relative simplicity and shorter processing time, provided the possibility of overestimation is considered (Rutherfurd & Moughan, 2007).

Furthermore, another outstanding method for the determination of reactive lysine is the automated OPA fluorometric assay, which is adapted from the manual fluorometric technique, and is distinguished by its precision, time and reagent efficiency, ability to operate continuously, and ease of operation (Barba et al., 2013). This method has been widely adopted for the quantification of amino acids, as well as reactive lysine in a variety of food products, including liquid foods (e.g.,

soy, oat, and quinoa beverages, and liquid sterilized milk) and powdered samples (e.g., infant formulas) (Barba et al., 2013; EL-Suhaibani et al., 2020). Briefly, this method works by reacting OPA with primary amines in the presence of a thiol compound to form highly fluorescent derivatives (Morales et al., 1995). The fluorescence intensity of these derivatives can be subsequently quantified using a fluorometer, providing an accurate measurement of reactive lysine content (Barba et al., 2013). Calibration curves are constructed using a range of proteins with varying lysine contents, such as lysozyme, ovalbumin, and k-Casein. One of the significant advantages of the OPA method is that it does not require hydrolysis or amino acid analysis, nor does it necessitate heating and solvent extraction. Instead, it uses sodium dodecyl sulphate (SDS) to denature proteins (Barba et al., 2013; Vigo et al., 1992). Despite the advantage of a very short reaction time when reacting with OPA reagent, this method faces a significant challenge due to the instability of the fluorescent compounds formed. These compounds degrade quickly, necessitating that fluorescence measurements be taken precisely within 2 min after the reaction to ensure accurate and comparable results (Aalaei et al., 2018). Considering the less widespread availability of fluorometers, an alternative spectrophotometric technique of the OPA method has been developed. This adaptation is based on the principle of fluorescence quenching by peptide bonds (Vigo et al., 1992). The reactive lysine contents in different dairy samples with or without heating applied, were effectively measured using the spectrophotometric and fluorometric OPA methods, which have been demonstrated by Vigo et al. (1992) and Ferrer et al. (2003), respectively, to be as reliable, reproducible, and accurate as the FDNB method, especially in samples that are free of interfering carbohydrates. Vigo et al. (1992) highlighted that the OPA method is particularly applicable for soluble proteins, with the absorbance contribution from α – amino groups limited to no more than 10% of total absorbance. Overall, the OPA method remains an excellent choice for routine analysis in settings where high throughput and efficiency are required.

However, it is important to carefully consider the potential limitations, such as the instability of fluorescent compounds and the specific conditions required to achieve optimal performance.

2.6.4 Effects of Food Processing on Total and Reactive Lysine

Numerous studies, as described by Rutherfurd et al. (2012), Moughan & Rutherfurd (1996), and Torbatinejad et al. (2005), have consistently highlighted discrepancies between the total lysine contents, determined by standardized amino acid analysis, and the reactive lysine contents measured using more specific assays like the guanidination method in heat-treated protein sources. Specifically, previous studies have found that the contents of reactive lysine tended to be lower than that of total lysine because the guanidination method selectively targets the ε- amino groups of lysine residues, whereas the standardized amino acid analysis accounted for all lysine residue, including those that may be nutritionally unavailable. Rutherfurd & Moughan (1997) clearly demonstrated the effect of thermal processing on both total and reactive lysine contents, as illustrated in **Figure 2.2**. In untreated field peas, the contents of reactive lysine were nearly identical to the total lysine content, indicating that lysine remained in its unmodified, bioavailable form, and no early Maillard products were formed. However, with increasing thermal exposure at 110, 135, 150, and 165°C for 15 min, both total and reactive lysine contents showed significant decreases to varying degrees as a result of the Maillard reaction. Notably, reactive lysine was more susceptible to thermal degradation compared to total lysine (Rutherfurd & Moughan, 1997). There is a noticeable gap in the literature regarding the effects of different thermal (e.g., baking and micronization) and non-thermal processing methods (e.g., HHP) on reactive lysine content. This exploration could provide valuable insights into how different processing techniques influence lysine availability.

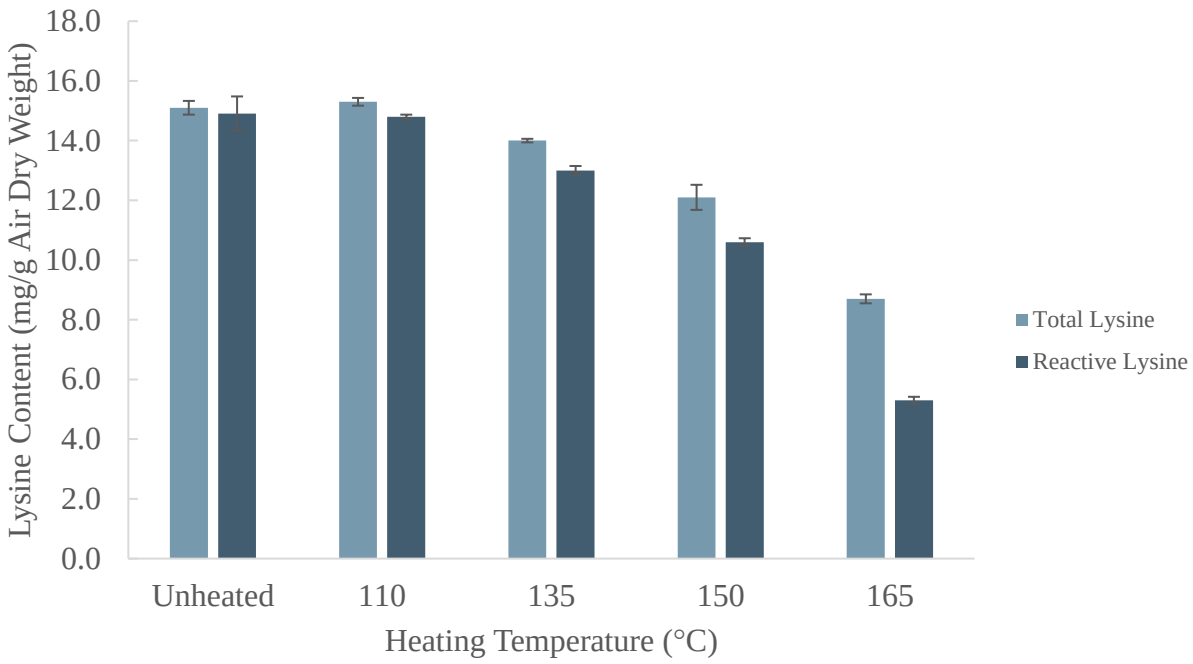

Figure 2.2. *Total lysine content determined by standardized amino acid analysis and reactive lysine content by guanidination method for unheated and heat-treated field peas. Adapted from Rutherfurd & Moughan (1997).*

2.7 RESEARCH GAPS

While there is an extensive body of research on the effects of traditional thermal processing methods, such as boiling and autoclaving, on yellow field pea seeds, limited attention has been paid to less common thermal techniques such as micronization and baking and non-thermal techniques, particularly HHP. The impact of these methods on ANF contents, *in vitro* protein quality (amino acid profile and *in vitro* protein digestibility), and reactive lysine contents in yellow field pea samples, especially their derivatives, remain underexplored. This gap is especially evident in the comprehensive ANF profiling, where several ANFs, including trypsin inhibitors, lectins, phytic acid, polyphenols, and condensed tannins, have yet to be fully examined in both untreated and treated yellow field pea derivatives, despite the extensive focus on their nutritional composition.

Moreover, the preservation of nutrients, including reactive lysine in the processed yellow field pea samples has not been thoroughly explored. A noticeable deficiency in the literature is the comparison between total lysine (measuring through conventional amino acid analysis) and reactive lysine (using the OPA fluorometric assay) contents in untreated and treated peas and their derivatives, particularly in relation to lesser-studied processing methods like baking, micronization, and HHP.

By bridging this knowledge gap, this research could deliver valuable insights into optimizing yellow field pea protein processing for enhanced nutritional quality and functional applications in food systems.

CHAPTER 3: HYPOTHESES & OBJECTIVES

3.1 HYPOTHESES

3.1.1 Hypotheses of Study 1

H_{a1}: The amounts of ANFs in whole yellow field pea flour samples will be significantly reduced by both thermal and non-thermal processing methods (including boiling, autoclaving, micronization, baking and HHP) when compared to the unprocessed form. Additionally, yellow field pea protein concentrate, 80% protein isolate, and 90% protein isolate subjected to baking and HHP treatments will exhibit lower ANF contents than their corresponding unprocessed forms.

- H_{01}: The amounts of ANFs in all yellow field pea samples will not be significantly altered by the applied processing methods as compared to their respective unprocessed forms.

3.1.2 Hypotheses of Study 2

H_{a1}: Amino acid profile, *in vitro* protein digestibility, and *in vitro* protein quality of yellow field pea samples will be significantly improved by different processing methods.

- H_{01}: Amino acid profile, *in vitro* protein digestibility, and *in vitro* protein quality of pea samples will not be altered by different processing methods.

H_{a2}: The reactive lysine contents of processed yellow field pea samples will significantly differ from their respective total lysine contents. Moreover, the concentration of reactive lysine in the yellow field pea samples will be significantly reduced by food processing methods.

- H_{02}: There will be no significant difference between total lysine and reactive lysine contents in any of the tested samples. Moreover, the concentration of reactive lysine in the yellow field pea samples will not be significantly changed by processing methods.

3.2 OBJECTIVES

3.2.1 Objectives of Study 1 (Chapter 4)

1. **Evaluate the Impact of Various Processing Methods on ANFs in Yellow Field Pea Flour**. Assess the effect of thermal processing methods, including boiling, autoclaving, micronization, and baking, as well as non-thermal treatment (i.e., HHP), on the concentrations of key ANFs in yellow field pea flours. The ANFs of interest include trypsin inhibitors, phytic acid, lectins, total polyphenols, and condensed tannins.
2. **Assess the Effects of Baking and HHP on ANFs in Yellow Field Pea Derivatives.** Examine and compare the impacts of baking and HHP on the contents of the five ANFs in yellow field pea protein concentrate, 80% protein isolate, and 90% protein isolate with their corresponding unprocessed forms.

3.2.2 Objectives of Study 2 (Chapter 5)

1. **Analyze the Effects of Different Processing Methods on *in Vitro* Protein Quality in Yellow Field Pea Samples.** Investigate the effect of the aforementioned processing methods on amino acid profile, *in vitro* protein digestibility, and overall *in vitro* protein quality via the *in vitro* PDCAAS method of yellow field pea samples.
2. **Compare Total and Reactive Lysine in Untreated and Treated Yellow Field Pea Samples.** Evaluate and compare total lysine content (measured using conventional amino acid assay) with reactive lysine content (determined using a specific assay) across a range of unprocessed and processed yellow field pea samples.

CHAPTER 4: IMPACT OF DIFFERENT PROCESSING METHODS ON ANTI-NUTRITIONAL FACTORS OF YELLOW FIELD PEAS AND THEIR DERIVATIVES (STUDY 1)

4.1 ABSTRACT

Background: Yellow field peas (*Pisum sativum* L.) are characterized as a highly nutritious, cost-effective, hypoallergenic, environmentally sustainable plant-based protein source, and are underutilized due to the presence of anti-nutritional factors (ANFs). Trypsin inhibitor activity (TIA), lectins, phytic acid (PA), total phenolic content (TPC), and condensed tannins (CT) are examples of ANFs commonly found in most pulses, which can negatively affect the nutritional value, protein digestibility, and overall protein quality of pulse-based food products. Various processing methods could influence the concentrations of ANFs in yellow field pea products.

Objectives: This study investigated the impact of thermal (boiling, autoclaving, micronization, and baking) and non-thermal (high-hydrostatic pressure (HHP)) processing methods on whole yellow field pea flour. Additionally, the effect of baking and HHP treatments were further explored on protein concentrate, 80% protein isolate and 90% protein isolate, all generated from the same lot of whole yellow field pea seeds. A starch-rich byproduct (coarse fraction) along with the protein-enriched fine fraction (protein concentrate) from air classification was also analyzed. The research centered on the concentrations of major ANFs including TIA, lectins, PA, TPC, and CT expressed on both dry matter and protein bases.

Results: In untreated yellow field pea samples, the protein concentrate and flour exhibited higher contents of TIA, PA, and TPC, particularly on a protein basis. The 90% protein isolates showed the highest lectin concentration, while 80% protein isolates had the highest content of CT. Thermal treatments, particularly boiling and baking, significantly reduced TIA, lectin, PA, and TPC. Boiling was the most effective, reducing lectin content by 99.88% and TIA by over 88.60% in the flour. Baking also led to substantial reductions in all samples. HHP also demonstrated a reducing effect on TIA, PA, and TPC, while notably increasing lectin and CT contents in most samples, with flour showing a dramatic rise in CT of up to 521.57%.

Conclusion: Thermal treatments, particularly boiling, were highly effective in reducing ANFs, thereby potentially improving the nutritional value and quality of yellow field pea products. However, HHP showed limited benefits in reducing ANFs and unexpectedly increased the lectin and CT contents.

Significance and Novelty: This study underscores the importance of striving to minimize the limitations posed by ANFs to optimize the utilization of yellow field pea proteins for researchers and food processors. It also provides novel insights into the contrasting effects of HHP, which unexpectedly increased the contents of lectins and CT while retaining some nutrients.

4.2 INTRODUCTION

Peas (*Pisum sativum* L.) are among the most extensively cultivated grain crops globally, playing a dominant role in both the global and Canadian pulse markets (Bessada et al., 2019). Major production regions include Canada, Russia, China, India, and the United States, with Canada leading as the largest producer and exporter of both green and yellow peas (Lu et al., 2019). In 2024 – 2025, Canadian pea production is forecasted to reach 3 million tonnes, and exports will reach 2.4 million tonnes (Government of Canada, 2024). The growing demand for plant-based proteins is driving the expansion of the pea market, positioning peas as a key crop for sustainable food production and innovation (Lu et al., 2019; Nosworthy et al., 2017a).

Yellow field peas, in particular, are a rich source of plant-based protein, with protein content ranging from 21.2% to 32.9% on a dry matter basis. They are also high in carbohydrates (36.9 – 49.0%), dietary fibers (14 – 26%), and essential vitamins and minerals, such as B-complex vitamins, potassium, and phosphorus (Pedrosa et al., 2020; Stone et al., 2019; Frias et al., 2011; Nosworthy et al., 2017a; Nosworthy et al., 2017c; Dahl et al., 2012; Shanthakumar et al., 2022). This nutrient-rich profile makes yellow field peas an important ingredient in many diets and value-added products, including protein concentrates, isolates, and hydrolysates (Hertzler et al., 2020).

However, one of the primary concerns in utilizing plant-based protein sources, including yellow field peas, is the presence of naturally occurring anti-nutritional factors (ANFs). Common ANFs in yellow peas include phytic acid, condensed tannins, polyphenols, trypsin inhibitors, chymotrypsin inhibitors, α – amylase inhibitors, lectins, and oligosaccharides (Alonso et al., 1998; Khattab & Arntfield, 2009). These compounds, while beneficial for plant defense through protection against environmental threats such as pests and pathogens, can reduce amino acid bioavailability and protein digestibility, thereby lowering the overall protein quality of food products (Makkar et al., 2007; Troszyńska & Ciska, 2011; Rahate et al., 2021; Bessada et al., 2019).

Structurally, ANFs are categorized into protein-based (e.g., trypsin inhibitors, lectins) and non-protein-based (e.g., phytic acid, polyphenols, and condensed tannins) groups (Bessada et al., 2019). Trypsin inhibitors inhibit the proteolytic activity of pancreatic enzymes such as trypsin and chymotrypsin, thereby reducing protein digestion, while lectins impair nutrient absorption by binding to carbohydrates on cell surfaces, leading to red blood cell agglutination (Avilés-Gaxiola et al., 2018; Shi et al., 2018; Bessada et al., 2019). Non-protein-based ANFs, such as phytic acid, bind to essential minerals such as calcium, iron, and zinc to form insoluble complexes that hinder mineral absorption and bioavailability (Shi et al., 2018; Bessada et al., 2019); and polyphenols, including condensed tannins, mainly bind to proteins to form insoluble complexes that reduce protein digestion and nutritional value (Konieczny et al., 2020; Smeriglio et al., 2017; Samtiya et al., 2020).

Numerous studies demonstrated that thermal processing methods such as boiling, microwave heating, and autoclaving, drastically reduce the contents of ANFs in pulses. These reductions are mainly attributed to the leaching of ANFs into the soaking and cooking medium, degradation, and protein denaturation (Hefnawy, 2011; Khattab & Arntfield, 2009; Kalpanadevi & Mohan, 2013; Avilés-Gaxiola et al., 2018). High-hydrostatic pressure (HHP) is a non-thermal treatment commonly used for food preservation with better retention of nutritional values and sensory properties, but research activities on the effect on ANFs are still limited (Yamamoto, 2017; Gharibzahedi & Smith, 2021).

This study, therefore, aims to evaluate the effect of various thermal (boiling, autoclaving, micronization, and baking) and non-thermal (HHP) processing methods on the key ANFs in yellow field pea flours, as well as to examine the effect of baking and HHP on those ANFs in yellow field pea protein concentrate and protein isolates. The targeted ANFs include trypsin inhibitors, phytic acid, lectins, total polyphenols, and condensed tannins, with results reported on both a dry matter

(DM) and protein bases. The yellow field pea coarse fraction (a starch-rich byproduct) along with a protein concentrate (fine fraction) separated via air classification of the whole seed flour will be also included in this study.

4.3 MATERIALS AND METHODS

4.3.1 Chemicals

All chemicals utilized in Study 1 were procured from the suppliers listed in **Table 4.1**. Common reagents including sodium hydroxide (NaOH), hydrochloric acid (HCl), and methanol were sourced from Fisher Scientific (Ottawa, ON, Canada). If no particular remarks are provided, reverse osmosis (RO) water was used for most experiments in this study.

Table 4.1. Information of chemicals used for all analyses in Study 1.

Analysis	Chemical	Supply	Catalog NO.
TIA	Trizma® base – Tris(hydroxymethyl) aminomethane	Sigma-Aldrich (Oakville, ON, Canada)	T1503
	Calcium chloride dihydrate	Sigma-Aldrich	223506
	Nα -Benzoyl-DL-arginine ρ-nitroanilide hydrochloride	Sigma-Aldrich	B4875
	Dimethyl sulfoxide	Sigma-Aldrich	472301
	Trypsin from bovine pancreas with at least 10,000 benzoyl-L-arginine ethyl ester (BAEE) units/mg protein	Sigma-Aldrich	T1426
	Glacial acetic acid	Sigma-Aldrich	1.00063
Phytic acid	Phytic acid sodium salt hydrate (with a phosphorus purity of 93.7%)	Sigma-Aldrich	68388
	Ammonium iron (III) sulfate dodecahydrate	Sigma-Aldrich	221260
	2,2′-Bipyridyl	Sigma-Aldrich	D216305
	Thioglycolic acid	Sigma-Aldrich	T3758
Lectin	Gibco™ Phosphate buffered saline (PBS), pH 7.2 (1X)	Fisher Scientific (Ottawa, ONs, Canada)	20-012-027
	Glutaraldehyde stabilized rabbit red blood cells (10%)	Cedarlane (Burlington, ON, Canada)	CLD48
Condensed tannin	(+)-Catechin hydrate (purity of 99%)	Sigma-Aldrich	C1251
	Vanillin	Sigma-Aldrich	V1104
Polyphenol	Folin & Ciocalteu′s phenol reagent	Sigma-Aldrich	F9252
	Sodium carbonate decahydrate	Sigma-Aldrich	71360
	(+)-Catechin hydrate (purity of 99%)	Sigma-Aldrich	C1251
	Gallic acid (purity of 95.3% ± 0.3%	Sigma-Aldrich	91215

4.3.2 Sample Procurement and Preparation

Yellow field pea seeds (*Pisum sativum L.*) and two yellow field pea protein isolates with protein contents of 80% and 90% were sourced from a local food manufacturer (Winnipeg, MB, Canada), all derived from the same lot of seeds. The 80% and 90% protein isolate samples were produced using an aqueous pH-phased method. However, the specific procedural details and conditions of the isolation process were not disclosed by the supplier. Before undergoing the specific food processing methods, all samples received were stored at room temperature, and then individually and thoroughly homogenized to ensure consistency.

As illustrated in **Figure 4.1**., a large portion of the yellow field pea seeds was processed into pea protein concentrates, while the remaining seeds were subjected to a variety of processing methods, including thermal treatments (i.e., boiling, autoclaving, micronization, and baking) and a non-thermal treatment (i.e., HHP). Baking and HHP treatments were applied exclusively to the protein concentrate and both protein isolates. Each processing treatment was completed in triplicate.

4.3.3 Processing Treatments

4.3.3.1 Air-classification

Air-classification of yellow field pea flour was conducted at the Richardson Centre for Food Technology and Research (Winnipeg, MB). A total of 120 kg of whole seeds were conveyed from a hopper to an M-21 Prater-Sterling Impact Mill (Prater, Bolingbrook, USA) at an average feed rate of 113 kg/hr. The miller was equipped with a 1 mm screen and operated at a milling power of 40 Hz to generate coarse flour. Prior to operation, the milling system was flushed with 5 kg of seeds to ensure the cleanliness and consistency of the system. Subsequently, 100 kg of the

coarse pea flour collected from the pre-breaking process was further milled using the same miller but with a 0.3 mm screen. Similarly, approximately 5 kg of the coarse pre-milled flour was utilized to flush the miller and air classifier systems. The resulting flour from the 0.3 mm screen was then passed through a MAC-0 Prater-Sterling air classifier (Prater, Bolingbrook, USA). The miller had a hopper feed rate of approximately 104 kg/hr and a milling power of 40 Hz, while the air classifier's primary and secondary air settings were 75% and 20%, respectively, accompanied by an air classifier power of 25 Hz. The air classification resulted in two distinct fractions: coarse and fine, the latter of which is identified as pea protein concentrate. Moisture content, mass yield, protein content, protein yield, and particle size distribution (PSD) of both fractions were measured and calculated as presented in **Table 4.2**. Although the air classification procedure was performed once only, however, both fractions included in the current study were individually sub-sampled in triplicate for further analysis.

Table 4.2 Properties of the coarse and fine fractions of whole yellow field peas obtained by air classification.

Measurement	**Coarse Fraction**	**Fine Fraction**
Dry matter (%)	97.16	91.76
Mass yield (%, DM)	69.25	20.48
Crude protein (%, DM)	13.83	48.77
Protein yield (%, DM)	40.98	42.74
PSD (μm)[1]	D [4,3]: 78.00	D [4,3]: 66.23
	Dv (90): 205.67	Dv (90): 31.63
	Dv (50): 36.53	Dv (50): 12.00
	Dv (10): 17.33	Dv (10): 4.15

[1]D [4,3] represents the mean particle size of a sample; Dv (90), Dv (50), and Dv (10) represent the particle size below which 90%, 50%, and 10% of the particles in the sample lie, respectively.

4.3.3.2 Boiling

Approximately 0.8 kg of cleaned and thrice washed yellow field pea seeds, were soaked in tap water at room temperature with a ratio of 1:5 (seed:water. w/v). To determine the optimal soaking duration, drained pea seeds were weighed every 2 hr until they achieved a constant weight, to yield maximum seed hydration. Therefore, the optimum soaking time of the pea seeds was approximately 18 hr (Shi et al., 2018; Khattab et al., 2009). The pre-soaked seeds were rinsed once and drained prior to boiling. Subsequently, they were brought to boiling water at a 1:5 (seed:water, w/v) ratio and steadily cooked at atmospheric pressure until most of the seeds were softened when squeezed by fingers, which was after approximately 35 min. Afterwards, the boiled pea seeds were drained and freeze-dried.

4.3.3.3 Autoclaving

Another approximately 0.8 kg of cleaned yellow field pea seeds (rinsed three times) were soaked in tap water at a ratio of 1:5 (seed:water. w/v) for 18 hr at room temperature, rinsed once and drained. They were then packed in the double layers of autoclavable polypropylene bags at a ratio of 1:5 (seed:water, w/v), before being autoclaved at 121°C for 30 min using the Steris Amsco Century SV-120 Scientific Prevacuum Sterilizer (Steris, Ohio, USA) until most of the seeds were soft when squeezed between fingers. After processing, the autoclaved pea seeds were drained and freeze-dried.

4.3.3.4 Micronization

Before infrared heating, approximately 5 kg of yellow field pea seeds were tempered to 20% moisture content by spraying a pre-determined volume of distilled water (0.606 kg) directly

toward the seeds while mixing them thoroughly. The mixing process was carried out in a cement mixer for 18 hr under ambient temperature and atmospheric pressure conditions. The following formula was employed to calculate the amount of water necessary to achieve the desired final moisture content of 20%. However, the actual moisture content of 19.3% was obtained.

According to AACC Official Method 26-95.01 (AACC, 1999):

$$Weight\ of\ water\ required\ = \frac{W_S \times (M_T - M_O)}{100 - M_T} \quad \text{(Eq. 4.1)}$$

Where

W_S = Sample weight,

M_T = Target moisture content (e.g., 20%), and

M_O = Original moisture content of the seeds (i.e., of un-tempered seeds).

All the tempered pea seeds were fed onto a vibratory conveyor of the infrared heating system using a pilot-scale micronizer (Micronizing Company, Suffolk, UK) with a slope of 1° and a feed rate of 45%. Approximately 2 kg of the tempered seeds were introduced during the pre-heating of the micronizer. During this period, the maximum surface temperature of the seeds was recorded at least three times by holding an infrared thermometer near the collection hopper for 20 sec. Once the seeds achieved a steady mass flow rate and a constant seed surface temperature (115 – 120°C), approximately 1 kg of micronized seeds were collected. After cooling to room temperature, the micronized seeds were freeze-dried.

4.3.3.5 Baking

The baking procedures were conducted as stated in a previous paper (Nosworthy et al., 2018). The yellow field pea seeds underwent the same milling procedure as described in **Section 4.3.4**, resulting in fine flour that passed through a 200 µm screen. Optimal moisture level, baking

temperature, baking time, and dough size were evaluated in preliminary trials. Then 0.8 kg of pre-milled flour, protein concentrate, as well as 80% and 90% protein isolates, were individually blended at pre-determined flour-to-water ratios (1:0.5, 1:0.5, 1:1.7, 1:2.0, respectively) using a spatula. Each mixture was further mixed in a stand mixer with a dough hook attachment at speed #1 for 3 min. Each dough was rolled into a hydrated non-sticky dough before being divided and rolled by hand into cylindrical doughs (15 cm in length and 1 cm in diameter). Subsequently, the rolled doughs were allowed to rest on baking trays coated with parchment paper for 30 min. The dough was then baked in a pre-heated double-racked oven with a convection baking mode set at 380F for 30 min and the trays were rotated at the 15 min mark for even baking. After cooling to room temperature, the baked samples were cut into smaller pieces with scissors before freeze-drying.

4.3.3.6 High-hydrostatic pressure (HHP)

Yellow field pea seeds were processed in accordance with the previous methods of Lee et al. (2018) and Deng et al. (2015). Each type of sample, including pea flour (after grinding to a particle size of 200 µm), protein concentrate, 80% isolate, and 90% isolate, was mixed with the Milli-Q (MQ) water at a ratio of 1:10 (w/v, i.e., 180 g of flour sample/1.8 L of MQ water). Each sample type was replicated three times but processed in the same HHP batch. The solution was solubilized while ensuring that its temperature did not exceed 26°C as well as preventing the formation of a thick, gel-like solution. To achieve this, the sample powder was divided into four, and each quarter was added to MQ water in a 2 L beaker and mixed for approximately 10 sec with a hand blender at high speed until all the powder had been incorporated. After that, the solution was gently agitated overnight on a stir plate in a walk-in refrigerator at around 4°C.

Each mixture was packed in a plastic bag and exposed to high-pressure processing at 600 MPa for a 10-min holding time at room temperature using a commercial Hiperbaric 135 L HHP unit (Hiperbaric, Burgos, Spain) with water serving as the pressure transmitting fluid. The pressurization rate reached approximately 240 MPa/min, followed by immediate decompression after treatment. The temperature was maintained consistently at around 17°C throughout the process. The pressurized samples were subsequently frozen before undergoing freeze-drying. The aforementioned procedures were carried out at the Laval University (Quebec, Canada). The processed samples were ultimately milled to a fine flour through a 200 µm screen at the University of Manitoba.

4.3.4 Post-Processing Procedures

All unprocessed and processed samples were subjected to a specific milling procedure in which the processed samples were stored in a freezer at approximately -20°C prior to milling. The milling process began with all samples, both unprocessed and previously frozen processed samples, being ground through a Retsch SR 200 miller (Retsch GmbH, Haan, Germany), equipped with a 4 mm screen size to produce coarse flour. Subsequently, these samples were cooled in a -20°C freezer and further milled through a 200 µm mesh. The primary purpose of the freezing step was to prevent excessive heat generation due to the relatively small sieve holes. The resulting fine flours were sub-sampled using a coning and quartering method (CQM) to ensure homogeneity and representativeness. However, due to the insufficient weight of the HHP-treated samples, they were exempted from the CQM technique. Finally, all milled samples were preserved in the -20°C freezer until further analysis.

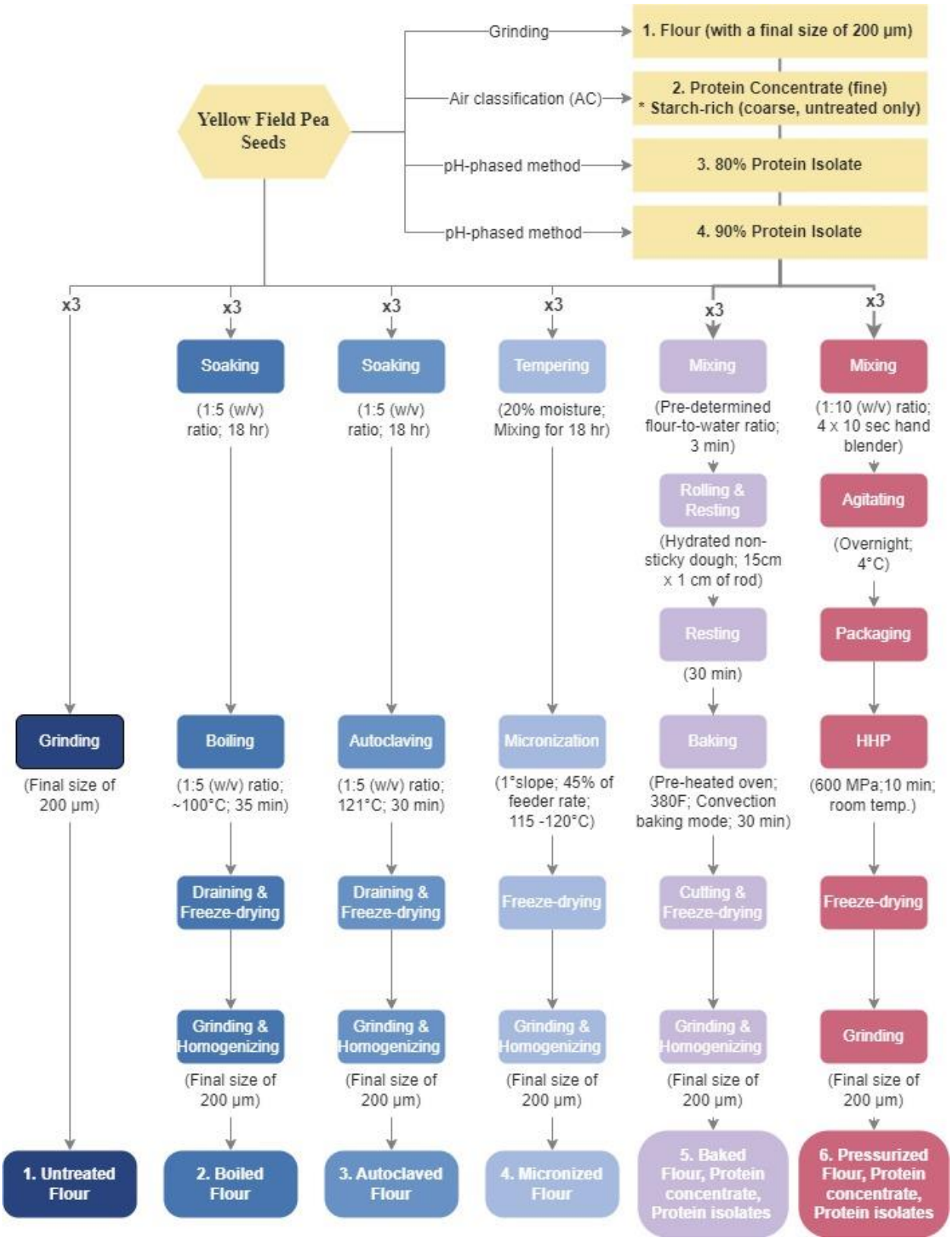

Figure 4.1. *A flow chart of processing yellow field peas by various food processing methods.*

4.3.5 Proximate Analysis and Particle Size Distribution

The following proximate and particle size distribution analyses were conducted in duplicate.

All untreated and treated ground samples underwent the measurements of dry matter (DM%) in accordance with a standardized method of 925.10 (AOAC, 1995). Approximately 1.000 ± 0.005 g of the well-mixed sample was weighed in a pre-weighed metal dish with a lid, after which it was dried overnight in an air oven at 105°C, with the lid partially opened. The next day, the dish containing the dried sample was closed and weighed again after cooling to room temperature in a desiccator.

$$Moisture\ content\ (\%) = \frac{Weight\ of\ initial\ sample - Weight\ of\ dried\ sample}{Weight\ of\ initial\ sample} \times 100 \quad \text{(Eq. 4.2)}$$

$$Dry\ matter\ (\%) = 1 - moisture\ content\ (\%) \quad \text{(Eq. 4.3)}$$

The content of crude protein (CP%) was determined by the Central Testing Laboratory (Winnipeg, MB, Canada) utilizing the Dumas combustion method. The determined nitrogen content was then converted to crude protein content through a standard nitrogen conversion factor of 6.25 (Mariotti et al., 2008).

In this study, the results of the chemical analyses were expressed on both dry matter and protein bases. The conversions from an as is basis were calculated using the following formulas.

$$Dry\ matter\ basis\ (\%) = \frac{Result\ on\ an\ as\ is\ basis\ (\%)}{Dry\ matter\ (\%)} \times 100 \quad \text{(Eq. 4.4)}$$

$$Protein\ basis\ (\%) = \frac{Result\ on\ an\ as\ is\ basis\ (\%)}{Protein\ content\ (\%)} \times 100 \quad \text{(Eq. 4.5)}$$

Particle size distribution (PSD) was done using a laser diffraction particle size analyzer, Mastersizer 3000 (Malvern Instruments Ltd., Worcestershire, UK). Two scoops (approximately 4 g) of each sample were transferred into the instrument's feed hopper along with a standard

operating procedure outlined in the user manual. The PSD results, which included the volume-weighed mean size (D [4,3]) and three standard percentiles, were created by the computer software based on the average of two measurements. The three percentiles, Dv (10), Dv (50), and Dv (90) represent the size values below which 10%, 50% and 90% of all particles were found, respectively.

4.3.6 Analyses of Anti-nutritional Factors

4.3.6.1 Trypsin inhibitor activity

Trypsin inhibitor activity (TIA) was evaluated according to Liu et al. (2021). Initially, a preliminary test must be carried out, in singlet, involving the dilution of sample extract with RO water at an appropriate dilution factor, such that trypsin inhibition (TI%) in 1.0 mL of the diluted sample extract falls within the range of 30% and 70%. To achieve this, 1.000 ± 0.001 g of each sample, was weighed and extracted with 50 mL of 10 mM NaOH in a 100 mL beaker. The extraction process involved stirring at 500 rpm at room temperature for 3 hr, as described by Chen et al. (2020) and Liu et al. (2021). A series of different concentrations of the sample extract was prepared by transferring varying amounts of the sample extract while stirring and then diluting each with RO water to achieve a final volume of 10 mL. The subsequent steps followed the procedures of TIA assay as outlined below. To enhance the accuracy and efficiency of the measurement, Liu et al. (2021) suggested that TIA values can be corrected by converting the TI% to 50% based on the TI% values obtained in the preliminary trial. This adjustment helps to consistently achieve approximately 50% inhibition level in subsequent runs, thereby improving the reliability and reproducibility of the assay. The TI% can be confirmed by a calculation using the following equation:

$$TI\% = \frac{(Abs_R - Abs_{RB}) - (Abs_S - Abs_{SB})}{(Abs_R - Abs_{RB})} \times 100 \quad \text{(Eq. 4.6)}$$

Where

Abs_R is the reference reading, Abs_{RB} is the reference blank, Abs_S is the sample reading, and Abs_{SB} is the sample blank.

Once an appropriate dilution factor had been determined, the TIA assay was repeated in the same manner, but with the quantity of each sample reduced to 0.5 g. The extraction was performed in the singlet, using a 50 mL beaker with 25 mL of 10 mM NaOH, following the same extraction conditions as described above.

For the analysis of the TIA analysis, a series of reagents were prepared, including 1) 0.05 M of Tris buffer (pH 8.2): 6.05 g tris (hydroxymethyl) aminomethane and 2.94 g calcium chloride dihydrate ($CaCl_2 \cdot 2H_2O$) were dissolved in 900 mL RO water. The solution was adjusted to a pH of 8.2 using 2 M HCl before being filled to a final volume of 1 L with RO water; 2) 40 mg/mL of Nα-benzoyl-DL-arginine-ρ-nitroanilide hydrochloride (DL-BAPA) stock solution: 0.2 g of DL-BAPA was dissolved in 5 mL of dimethyl sulfoxide. This solution was stored in an amber bottle at room temperature; 3) 0.4 mg/mL of freshly prepared DL-BAPA working solution: 1.0 mL of the DL-BAPA stock solution was diluted to 100 mL with the Tris buffer that has been pre-warmed in a 37°C water bath. The BAPA working solution was kept in the water bath at 37°C during use; 4) 1 mM of HCl solution: 0.736 g $CaCl_2 \cdot 2H_2O$ was dissolved in 900 mL RO water, followed by the addition of 0.5 mL of 2 M HCl. The final volume of solution was brought to 1 L by using RO water; 5) 0.2 mg/mL of trypsin stock solution: 0.01 g of bovine trypsin was dissolved by 50 mL of the HCl solution; 6) 20 µg/mL of trypsin working solution: 5 mL of the trypsin stock solution was diluted to a level of 50 mL with the HCl solution; 7) 30% acetic acid solution: 30 mL of glacial acetic acid was diluted with 70 mL of RO water.

The solutions applied for the TIA assay include 1) diluted sample extract or RO water; 2) DL-BAPA working solution; 3) trypsin working solution; and 4) 30% acetic acid solution. To

initiate the TIA assay, the solutions were added into a set of 11 mL Pyrex glass tubes following the order and volume described in **Table 4.3.** The test tubes for sample readings and reference readings were prepared in duplicate. Instead of using a diluted sample extract, RO water was utilized as a reference. During the assay, each tube containing the first reagent (diluted sample extract or RO water) was pre-warmed in a water bath at 37°C and then vortexed for 6 sec with the second reagent. The timing was started immediately after the third reagent was introduced, with a 6-second vortexing period. After exactly 10 min, the enzymatic reaction was halted by immediately adding and mixing the fourth reagent for 6 sec. The sample and reference blanks were prepared in a similar manner, except that the 30% acetic acid solution was added before the trypsin working solution was applied. The resulting yellowish mixture in each tube was centrifuged at 1500×g for 5 min and then measured with an Evolution One Spectrophotometer with Insight™ Pro Software (Thermo Fisher Scientific Inc., Waltham, USA) at 410 nm, following the same time intervals as the previous addition of the third reagent.

The TIA results were expressed as trypsin inhibitory units (TIU) per g sample and converted to both DM and protein bases, where one TIU was defined as a 0.02 absorbance unit increase. The value of TIA was calculated using the following formula:

$$TIA\ (TIU\ per\ g\ sample) = (Abs_R - Abs_{RB}) - (Abs_S - Abs_{SB}) \div 0.02$$
$$\div [(concentration\ of\ a\ diluted\ extract\ in\ mg\ sample\ per\ mL)$$
$$\times (mL\ of\ a\ diluted\ extract\ in\ the\ assay)]$$
$$= (Abs_R - Abs_{RB}) - (Abs_S - Abs_{SB}) \div 0.02 \div g\ sample\ in\ the\ assay \quad \text{(Eq. 4.7)}$$

Where

Abs_R, Abs_{RB}, Abs_S, and Abs_{SB} were the absorbances of the reference reading, reference blank, sample reading, and sample blank, respectively.

Table 4.3. Volumes and sequence of adding solutions for measuring TIA for a single sample.

Reagent solution	**Volume (mL)**	**Sample reading 1 (Abs_S 1)**	**Sample reading 2 (Abs_S 2)**	**Sample blank (Abs_{SB})**	**Reference reading 1 (Abs_R 1)**	**Reference reading 2 (Abs_R 2)**	**Reference blank (Abs_{RB})**
Diluted sample extract	1.0	First	First	First			
RO water	1.0				First	First	First
BAPA working solution	2.5	Second	Second	Second	Second	Second	Second
Trypsin working solution	1.0	Third	Third	Fourth	Third	Third	Fourth
Acetic acid solution	0.5	Fourth	Fourth	Third	Fourth	Fourth	Third

4.3.6.2 Lectin activity

The quantification of lectin content or hemagglutinating activity (HA) was determined by a hemagglutination assay as described previously by Shi et al. (2018) and Makkar et al. (1997), with some modifications. Approximately 0.5 g of ground sample was extracted, in singlet, in a 15 mL Falcon centrifuge tube with 5 mL of phosphate buffered saline (PBS) by agitating for 1 min at 3000 rpm to prevent the sample from sticking to the bottom of the tube. The mixture was then rotated on a rotator at a speed setting of 40 in a walk-in fridge at approximately 4°C overnight (~18 hr). The sample extract was centrifuged at 3,000×g for 10 min at 4°C. Subsequently, 1.5 mL of the resulting supernatant was transferred in duplicate to microcentrifuge tubes and further centrifuged at 14,200×g for 30 min at 4°C. The supernatants were stored in the -20°C freezer for further analysis. In a 96-well V-shaped-bottom microplate, the supernatant underwent a two-fold serial dilution, starting from no dilution in Well 1 to well 22 (1:2,097,152 (extract:PBS, v/v). To fulfill this requirement, a volume of 0.05 mL PBS was added to each well except for the first well, while 0.1 mL of the supernatant was pipetted into the first well, followed by a transfer of 0.05 mL of the mixture from the well 1 (dilution factor 1) to the well 2 (dilution factor 2) after mixing. Again, 0.05 mL of the mixture was transferred from well 2 to well 3 (dilution factor 4). The procedure was followed in the same manner until the final well 22 with a dilution factor of 2,097,152. To attain the same volume of solution in each well tube, 0.05 mL of the volume in the last well was discarded. Finally, 0.05 mL of 1% rabbit red blood cells (RBC, 0.5 mL of 10% RBC + 4.5 mL of PBS) was added to each well, obtaining a total volume of 0.1 mL. The blank was composed of 0.05 mL of PBS and 0.05 mL of 1% RBC. The microplates were then left at room temperature without agitation for 2 hr, as the hemagglutinin activity of the samples was stable after 2 hr.

The HA was evaluated visually following the method described by Makkar et al. (1997), Labba et al. (2021), and Liu et al. (2013). A positive result of HA was characterized by a homogeneous suspension with minimal or no RBC clumping at the bottom, while a negative result indicated no agglutination, presenting as a circular clump of RBC with a clear margin identical to that of the blank. The highest dilution giving positive HA was defined as one hemagglutinating unit (HU). The HA results were calculated and expressed as HU per g sample and converted to both DM and protein bases.

The HA was calculated as follows:

$$HA\ (HU/g\ sample)\ =\ \frac{D_a \times D_b \times S}{V} \quad \text{(Eq. 4.8)}$$

Where

V = Volume of extract in well 1 (= 0.05 mL),

S = mL sample extract/g flour (e.g., 5 mL/500 g = 0.01 mL/g),

D_a = Dilution factor of extract in well 1 (= 1, in the condition of a sample extract that has not been diluted), and

D_b = Dilution factor of the tube in which the agglutination result occurred at the highest dilution.

4.3.6.3 Phytic acid

PA assay was performed following the procedures of Haug & Lantzsch (1983), using phytic acid sodium salt hydrate (with a phosphorus purity of 93.7%) as a standard. A phytate stock used for building a standard curve was prepared by dissolving 0.15 g of phytic acid sodium salt hydrate in 50 mL of RO water. Subsequently, a diluted phytate stock was prepared daily, by combining 2.7 mL of phytate stock with 5 mL of 1 M HCl and then adjusting the volume to 25

mL with RO water to achieve a final HCl concentration of 0.2 M. The standard curve was constructed based on a serial dilution of the diluted phytate stock that was further prepared in 2 mL microcentrifuge tubes in a range from 0.03 – 0.3 mg/mL with 0.2 M HCl.

To initiate the analysis, 0.025 – 0.200 g of yellow field pea samples (e.g., 0.1 g of raw pea flour and 0.025 g of 90% protein isolate) were weighed in an 11 mL Pyrex glass tubes and extracted with 5.0 mL of 0.2 M HCl on a rotator at a speed 40 for 3 hr at room temperature. The sample weight was determined by ensuring that the absorbance of the sample fell within the absorbance range of the standard concentrations. The sample extract was vortexed for 6 sec at 3000 rpm and allowed to rest for 10 min. A 0.5 mL aliquot of the sample extract from the interface between the bubble layer and the turbid bottom layer was pipetted in duplicate into 2 mL of microcentrifuge tubes. Each standard concentration and sample were mixed with 1 mL of ferric solution (0.1 g of ammonium iron (III) sulphate dodecahydrate in 50 mL of 2 M HCl, with a final volume of 500 mL with RO water). The microcentrifuge tubes for the standards and samples were then heated on a heat block at 100°C for 30 min and centrifuged for 30 min at 3000×g at room temperature after cooling to room temperature. Subsequently, 0.5 mL of each supernatant was transferred to a disposable cuvette and absorbance was measured in a spectrophotometer at 519 nm after reacting with 0.75 mL of bipyridine solution (5 g of 2,2'-bipyridyl with 5 mL of thioglycolic acid and made up to a final volume of 500 mL with RO water) for 50 – 55 sec. A blank used for zeroing the spectrophotometer, was prepared by using 0.5 mL of 0.2 M HCl instead of 0.5 mL of supernatant and following the same procedure above.

The phytate content was calculated from the standard curve and blank and reported as mg phytic acid equivalent/g of sample (DM basis) or g of protein, as follows:

$$PA\ (mg\ PA\ equivalent/g\ sample) = \frac{(Abs_S - b)}{a} \times S \times D \quad \text{(Eq. 4.9)}$$

Where

Abs_s = Absorbance of the sample,

b and a = Intercept and slope of the standard curve, respectively,

S = Volume of sample extract (mL)/sample weight (g) (e.g., 5 mL/0.025 g = 200 mL/g), and D = Dilution factor = 1.5 mL/0.5 mL × 1.25 mL/0.5 mL = 7.5 mL.

4.3.6.4 Total phenolic content

The total phenolic content (TPC) was measured using the Folin-Ciocalteu assay according to Konieczny et al. (2020) and Amoussa et al. (2021). Briefly, 1.0 g of each ground flour was extracted, in singlet, in a 50 mL Oak Ridge centrifuge tube with 5 ml of methanol solution (1% (w/v) concentrated HCl in absolute methanol) by vigorous vortexing for 15 sec at 3000 rpm and shaking it on a rotator at a speed of 40 for 2 hr at room temperature in a dark environment. The resulting sample extract was then centrifuged at 1050×g for 10 min. The supernatant was then decanted into a new test tube, while the pellet in the original tube was re-dissolved in 5 mL of methanol solution. The extraction procedures were repeated an additional two times, but the extraction time was reduced to 20 min. The three supernatants were combined after each extraction cycle. Additional centrifugation was needed because the supernatants were turbid. To achieve this, approximately 1.5 mL of the pooled supernatants were transferred to microcentrifuge tubes in duplicate and then centrifuged at the highest speed (i.e., 12700 rpm) for 10 min at room temperature using a microcentrifuge.

To prepare the standard curve for the TPC assay, a 1 mg/mL catechin and/or gallic acid stock solution was prepared and subsequently diluted with 1% HCl in methanol to achieve catechin and/or gallic acid concentrations ranging from 0.1 to 0.5 mg/mL. To initiate the TPC assay, a set

of test tubes was set up, including 1) blank: 0.125 mL of 1% HCl in methanol (used to zero the spectrophotometer); 2) 0.125 mL of each standard concentration (0.1 – 0.5 mg/mL of catechin and/or gallic acid); 3) 0.125 mL of the pooled sample extract. Each tube was then combined with 1.5 mL of RO water. Subsequently, the mixture in the test tube was mixed with 0.125 mL of Folin-Ciocalteu reagent. After 5 min, the mixture was immediately reacted with 1.25 mL of 7% (w/v) Na_2CO_3 (by gradually dissolving 94.50 g of sodium carbonate decahydrate in RO water and then bringing the volume to 500 mL). The tubes were incubated at room temperature in a dark environment for 1 hr and 40 min. The intensity of the blue-colored mixture was then detected spectrophotometrically as absorbance at 550 nm, 750 nm, and/or 760nm against the blank. The results were reported in mg of catechin equivalent (CE) or gallic acid equivalent (GAE) per g of sample on a DM basis and per g of protein, calculated based on the standard curve generated with catechin and/or gallic acid standard solution.

$$TPC\ (mg\ CE\ or\ GAE/g\ sample) = \frac{(Abs_S - b)}{a} \times \frac{15\ mL\ of\ extract}{S} \quad \text{(Eq. 4.10)}$$

Where

Abs_S = Absorbances of the sample,

b and a = Intercept and slope of the standard curve, respectively, and

S = Sample weight (g).

4.3.6.5 Condensed tannins

The procedure for estimating CT in this study differed slightly from the method of Shi (2022). To construct a standard curve, a 0.3 mg/mL catechin stock solution (0.003 g catechin in 10 mL absolute methanol) was prepared daily. Then, catechin concentrations of 0 – 0.15 mg/mL were prepared by dispensing the catechin stock of 0, 0.4, 0.8, 1.2, 1.6, and 2 mL into test tubes,

and filled with absolute methanol to a volume of 4.0 mL. A 1 mL Aliquot of each standard solution was individually transferred to two sets of six test tubes to generate the standard curve in duplicate, before being maintained in a water bath at 30°C for 10 min. To initiate the reaction, 5 mL of 4% concentrated HCl in absolute methanol (for blank) was transferred to one set of tubes at 1-min intervals. Then 5 mL of a freshly prepared vanillin working solution (1% vanillin in absolute methanol in 8% concentrated HCl in absolute methanol at a ratio of 1:1) was added to another set of tubes at 1-min intervals. Additionally, 0.5 g of each ground sample was weighed in triplicate and extracted in a 50 mL Falcon centrifuge tube, where threads were wrapped four times with Teflon tape, and with the tubes containing 10 mL of absolute methanol. Samples were then rotated at 40 rpm at room temperature for 20 min. The mixture was centrifuged at 3000×g for 5 min. After transferring 1.5 mL of the supernatant to a set of four microcentrifuge tubes individually, the supernatant was further centrifuged at the highest speed (i.e., 12700 rpm) for 5 min using a microcentrifuge. Then 1 mL of supernatant was added to each of the four test tubes. Similarly, 5 mL of 4% concentrated HCl in absolute methanol was transferred into the first two tubes as a sample blank at 1-min intervals. Another two of the test tubes that served as sample readings received 5 mL of the vanillin working solution at 1-min intervals. The tubes containing standard solutions and samples were incubated at 30°C for exactly 20 min and then the absorbance was measured in a spectrophotometer at 500 nm following the 1-min intervals using quartz cuvettes.

The actual content of condensed tannins was determined by correcting the sample blanks and comparing them to a standard curve generated on the same day. The spectrophotometer was zeroed using 4% HCl in methanol. The results were reported as mg CE/g of sample (DM basis) and mg CE/g of protein and calculated using the following calculation:

$$CT\ (mg\ CE/g\ sample) = \frac{(Ab_{s1} - Ab_{s0} - b)}{a} \times 10\ mL\ of\ extract \div 0.5\ g\ of\ sample \quad \text{(Eq. 4.11)}$$

Where

Abs_1 and Abs_0 = the absorbances of the sample treated with vanillin working reagent and with 4% HCl solution, respectively, and

b and a = the standard curve's intercept and slope, respectively.

4.3.7 Statistical Analysis

Each treatment type including both untreated and treated samples, used in this study was conducted in triplicate. All analyses were carried out at least twice. Data were represented as the mean of three replicates and statistically evaluated using one-way analysis of variance (ANOVA) followed by a Tukey's multiple comparison test to identify significant differences among treatments for each pea sample type (pea flour, protein concentrate, 80% protein isolate, and 90% protein isolate). A pooled standard error (SE) and significance level of $p < 0.05$ for each sample type were determined. Additionally, linear regression was applied to assess the relationships between two standards used in the polyphenol assay. Statistical analyses were performed through Microsoft Excel 2021, GraphPad Prism, and R.

4.4 RESULTS AND DISCUSSION

4.4.1 Proximate Analysis and Particle Size Distribution

Variations in moisture content, crude protein (CP) content, which was expressed on a dry matter (DM) basis, and particle size distribution (PSD) of yellow field pea flour, protein concentrate, and both the 80% and 90% protein isolates before and after different processing methods, including data for the untreated coarse fraction, are detailed in **Table 4.4.** The untreated pea flour and protein concentrate had comparable moisture contents of 8.33% and 8.24%, respectively. These values were notably higher than that of untreated 80% isolate and 90% isolate, which had moisture contents of 4.41% and 4.99%, respectively. The reduced moisture contents observed in both protein isolates could be attributed to the extensive purification processes to remove non-protein components and excess water in order to achieve a higher protein concentration. This finding aligns with the observations reported by Shi et al. (2022), although different isolation methods were used. The coarse fraction had a lower moisture content of 2.84%, compared to the protein concentrate, which measured at 8.24%.

After exposure to the processing methods, among the flour samples, the moisture contents of thermally processed flour showed a significant loss ($p < 0.05$) ranging from 40.22 to 70.47% (baking > boiling > autoclaving > micronization). Similarly, baking significantly decreased ($p < 0.05$) the moisture content of the 80% and 90% protein isolates by 29.48% and 41.08%, respectively. In contrast, the moisture content of the baked protein concentrate sample surprisingly increased ($p < 0.05$) by 15.90%. In the present study, the moisture contents of the boiled and baked flours did not show significant differences ($p > 0.05$), which is identical to the observation of Nosworthy et al. (2017a), where the boiled and baked yellow split peas had similar moisture contents, at 2.56% and 4.19%, respectively. However, the baked flour showed a lower moisture

content of 2.46% in the current study, likely due to the consistent high-intensity temperature applied throughout the baking period and the subsequent involvement of the freeze-drying step, compared to the tunnel conveyor oven with three distinct temperature zones employed by Nosworthy et al. (2017a), which resulted in varied moisture losses. On the other hand, the application of HHP resulted in significant decreases ($p < 0.05$) in the moisture content of flour and protein concentrate, reducing by 39.98% and 33.13%, respectively. However, no significant decreases ($p > 0.05$) were observed in the moisture contents of HHP-treated 80% and 90% isolates, which were reduced by 9.07% and 22.24%, respectively. These non-significant reductions may be due to the fact that the initial purification processes had already lowered their moisture content, resulting in minimal further reduction through HHP treatment.

In terms of CP, the untreated pea flour, protein concentrate, 80% isolate, and 90% isolate had protein contents of 23.37%, 48.77%, 82.15%, and 88.90% on a DM basis, respectively. This observation is consistent with previous studies, where Nosworthy et al. (2017a), Frias et al. (2011), Carmo et al. (2020), Konieczny et al. (2020), and Shi (2022) reported protein contents ranging from 23.10% to 23.78% in yellow pea flours, 44.0 % to 49.31% in air-classified protein concentrates, and up to 91.79% in protein isolates. Additionally, the coarse fraction obtained from air classification, primarily consisting of starch, had a lower protein content of 13.83% on a DM basis. As a result, the protein contents of both protein concentrate and coarse fraction obtained in this study were higher than those reported by Carmo et al. (2020), who found protein contents of 44.0% DM and 8.9% DM for the protein concentrate and coarse fraction of whole yellow peas, respectively. These differences may be caused by variations in air classification processing conditions and yellow pea variety.

After thermal processing (boiling, autoclaving, micronization, and baking) was applied to the pea seeds, it was observed that boiling and autoclaving gave statistically significant increases

($p < 0.05$) in protein contents by 3.21% and 3.81%, respectively. Conversely, except for the baked protein concentrate, which exhibited a significant increase ($p < 0.05$) in protein content of 5.74%, both baking and HHP processing led to minor reductions in protein contents across the tested samples, with a maximum reduction of 1.20%. Nosworthy et al. (2017a) observed that extrusion of yellow split pea increased its protein content, whereas boiling and baking led to a decrease. These findings highlight that processing methods can induce slight changes in protein content, either increasing or decreasing to varying extents, depending on the different processing conditions, sample types, and sample cultivars (Nosworthy et al., 2017a; Frias et al., 2011; Zhong et al., 2015).

PSD results typically include several parameters D [4,3], Dv (10), Dv (50), and Dv (90), where D [4,3] is known as the mean diameter over a volume of a sample, and Dv (90), Dv (50), and Dv (10) represent the particle size below which 90%, 50%, and 10% of the particles in the sample lie, respectively. These parameters offer insights into the range of size and uniformity of distribution of a sample. In untreated samples, the pea flour, which underwent milling by passing through a 200 µm mesh exhibited the highest D [4,3] and Dv (90) values of 102.63 µm and 189.00 µm, respectively. The protein concentrate and protein isolates were derived from whole seeds following dry fractionation and the aqueous pH-phased method, respectively, resulting in reduced particle sizes. Specifically, D [4,3] and Dv (90) values for protein concentrate were measured at 66.23 µm and 31.63 µm, respectively. In particular, the Dv (90) value for protein concentrate represented the smallest particle size among all tested samples. This can be attributed to the removal of the coarse fraction through air classification. In comparison, the coarse fraction exhibited larger particle size, with D [4,3] and Dv (90) values of 78.00 µm and 205.67 µm, respectively. As expected, De Angelis et al. (2021) demonstrated in their scanning electron micrographs that the coarse fraction mainly contained large, spherical starch granules with a small number of starch macro-complexes embedded in the protein matrix. In contrast, the fine fraction

(protein concentrate) predominantly consisted of protein bodies and exhibited smaller particle sizes. Both 80% and 90% isolates had comparable PSD: 56.70 µm and 63.00 µm of D [4,3], 21.63 µm and 21.13 µm of Dv (10), 50.53 µm and 52.10 µm of Dv (50), and 101.00 µm and 112.33 µm of Dv (90), respectively.

After subjecting pea seeds and their derivative samples to various processing treatments, all thermally and non-thermally treated samples were freeze-dried and ultimately milled through a 200 µm mesh sieve. Among the flour samples, the particle sizes corresponding to Dv (90) ranged insignificantly ($p > 0.05$) from 105.67 to 144.67 µm, with the order from smallest to largest particle size: baking < HHP < boiling < autoclaving < micronization. However, all of these processing methods resulted in a significant reduction ($p < 0.05$) in Dv (90) when compared to the untreated flour. The observed lower particle sizes based on D [4,3] and Dv (90) values in the baked and HHP-treated flours could be attributed to the initial pre-milling step carried out prior to these treatments in the current study, representing the decrease in particle size with increasing number of the grinding (Tinus et al., 2012). In contrast, both baking and HHP treatments induced significantly increased ($p < 0.05$) [Dv (90)] variations in protein concentrates and 80% protein isolate (except for HHP-treated 80% isolate). Dehnad et al. (2024) explained that HHP treatment, at higher pressure levels (e.g., 600 MPa) can result in a reduction of aggregate size by breaking down larger clusters into smaller, more homogeneous units, while simultaneously inducing both protein association and aggregation. Additionally, the differences in PSD values among the tested samples may also be associated with their different protein types, structural properties, and conformational stability (Dehnad et al., 2024).

Overall, the moisture content generally decreased with treatments across all categories, with the exception of baked protein concentrate, where it increased. Baking and boiling had the greatest impact on the moisture content in pea flour. CP content on a DM basis was minimally

affected by most treatments of the pea flour and the derivatives, except for boiled flour, autoclaved flour, and baked protein concentrate, which showed significant increases in CP. PSD varied significantly across treatments, with baking and HHP notably reducing [Dv (90)] values in the pea flour, while increasing them in derivatives, except for 90% protein isolates. The observed changes in moisture content, CP, and PSD highlight the differential effects of thermal and non-thermal treatments on both the composition and physical properties of yellow field pea products. Furthermore, moisture content and CP results will serve as a basis for expressing the outcomes of other analyses, allowing for accurate and comprehensive comparisons across samples with differing moisture or protein contents.

Table 4.4. Moisture content, crude protein content and particle size distribution of untreated and treated whole yellow field pea flours, protein concentrates, coarse fraction, 80% protein isolates, and 90% protein isolates.

Sample Description	Moisture Content (%)		Crude Protein (%, DM)		Particle Size Distribution (µm)			
	Result (%)	Relative Change (%)	Result (%)	Relative Change (%)	D [4,3]	Dv (10)	Dv (50)	Dv (90)
Whole Pea Flour								
Untreated	8.33^{a}	0.00	23.37^{b}	0.00	102.63^{a}	3.72^{c}	21.30^{e}	189.00^{a}
Boiled	3.52^{bc}	-57.74	24.12^{a}	+3.21	79.23^{ab}	12.07^{a}	61.60^{a}	133.00^{b}
Autoclaved	4.49^{b}	-46.10	24.26^{a}	+3.81	76.60^{bc}	11.40^{a}	50.03^{b}	139.00^{b}
Micronized	4.98^{b}	-40.22	23.59^{ab}	+0.94	62.37^{bcd}	7.83^{b}	27.93^{d}	144.67^{b}
Baked	2.46^{c}	-70.47	23.22^{b}	-0.64	50.67^{d}	6.01^{b}	29.07^{d}	105.67^{b}
HHP-treated	5.00^{b}	-39.98	23.09^{b}	-1.20	54.47^{cd}	12.63^{a}	37.33^{c}	118.33^{b}
Pooled SE	**0.4910**	**/**	**0.0636**	**/**	**77.50**	**0.6130**	**0.9622**	**239.0**
***P*-value**	**<0.0001**	**/**	**0.0004**	**/**	**0.0001**	**<0.0001**	**<0.0001**	**0.0005**
Protein Concentrate								
Untreated	8.24^{b}	0.00	48.77^{b}	0.00	66.23^{a}	4.15^{c}	12.00^{d}	31.63^{d}
Baked	9.55^{a}	+15.90	51.57^{a}	+5.74	65.30^{a}	4.46^{c}	26.80^{c}	104.33^{c}
HHP-treated	5.51^{c}	-33.13	48.55^{b}	-0.45	58.80^{a}	14.40^{b}	48.67^{a}	115.67^{b}
Coarse fraction	2.84^{d}	-65.53	13.83^{c}	-71.64	78.00^{a}	17.33^{a}	36.53^{b}	205.67^{a}
Pooled SE	**0.1160**	**/**	**0.0566**	**/**	**57.10**	**0.0678**	**1.167**	**6.006**
***P*-value**	**<0.0001**	**/**	**<0.0001**	**/**	**0.0758**	**<0.0001**	**<0.0001**	**<0.0001**

Data are expressed as mean of three replicates (two measurements per replicate). Mean values followed by different superscript letters within the column of each yellow field pea type indicate statistically significant differences between treatments ($p < 0.05$) using one-way ANOVA along with Tukey's multiple comparison test. *Abbreviations:* SE = Standard error; DM = Dry matter.

Table 4.4. Continued.

Sample Description	Moisture Content (%)		Crude Protein (%, DM)		Particle Size Distribution (µm)			
	Result (%)	Relative Change (%)	Result (%)	Relative change (%)	D [4,3]	Dv (10)	Dv (50)	Dv (90)
80% Protein Isolate								
Untreated	4.41^{a}	0.00	82.15^{a}	0.00	56.70^{b}	21.63^{a}	50.53^{a}	101.00^{b}
Baked	3.11^{b}	-29.48	81.88^{ab}	-0.33	72.67^{a}	3.89^{c}	39.03^{a}	121.33^{a}
HHP-treated	4.01^{a}	-9.07	81.60^{b}	-0.67	55.93^{b}	17.90^{b}	48.90^{a}	103.57^{ab}
Pooled SE	**0.0286**	**/**	**0.0403**	**/**	**24.30**	**0.2110**	**32.89**	**61.57**
***P*-value**	**0.0002**	**/**	**0.0442**	**/**	**0.0098**	**<0.0001**	**0.0969**	**0.0373**
90% Protein Isolate								
Untreated	4.99^{a}	0.00	88.90^{a}	0.00	63.00^{a}	21.13^{a}	52.10^{a}	112.33^{a}
Baked	2.94^{b}	-41.08	88.24^{b}	-0.74	55.70^{a}	4.81^{c}	37.37^{b}	107.00^{a}
HHP-treated	3.88^{ab}	-22.24	88.11^{b}	-0.89	61.57^{a}	17.67^{b}	53.20^{a}	115.67^{a}
Pooled SE	**0.5890**	**/**	**0.0535**	**/**	**42.00**	**0.1110**	**16.90**	**112.9**
***P*-value**	**0.0462**	**/**	**0.0117**	**/**	**0.4007**	**<0.0001**	**0.0056**	**0.6255**

Data are expressed as mean of three replicates (two measurements per replicate). Mean values followed by different superscript letters within the column of each yellow field pea type indicate statistically significant differences between treatments ($p < 0.05$) using one-way ANOVA along with Tukey's multiple comparison test. *Abbreviations:* SE = Standard error; DM = Dry matter.

4.4.2 Analyses of Anti-Nutritional Factors

4.4.2.1 Trypsin inhibitor activity

Changes in trypsin inhibitor activity (TIA), expressed as both trypsin inhibitor units (TIU)/g sample on a DM basis and TIU/g protein, for whole yellow field pea flour, protein concentrate, 80% protein isolate, and 90% protein isolate before and after different processing methods applied, as well as for the untreated coarse fraction, are given in **Table 4.5.** During the TIA assay, it is essential to dilute the sample extract appropriately to achieve a 30% – 70% trypsin inhibition (TI) range in 1 mL of diluted extract (Liu et al., 2021). The volume of the sample extract for the dilution was estimated based on pre-knowledge of the extent of TIA inactivation in thermally processed and non-thermally processed samples, combined with some guesswork (Hamerstrand et al., 1981). It was observed that diluting 1.2 mL of the extract from untreated yellow field pea flour to 10 mL met this requirement, which can be considered as a baseline. Based on this, the extract volumes for treated samples were adjusted accordingly: the extract volume was increased for thermally processed samples, while a similar volume to the untreated flour extract was used for non-thermally processed samples. Specifically, for thermally processed samples, it was expected that TIA would be significantly reduced, necessitating a larger volume of sample extract to fall within the target inhibition range. Conversely, for the samples subjected to HHP, the TIA reduction may not be as significant as that with thermal treatments, therefore, a similar volume of sample extract as for the untreated flour may be sufficient to achieve the desired range (Liu et al., 2021). Additionally, TI% values among all the samples ranged from 43.86% to 62.6%. To facilitate a comparison of their TIA results, the TIA data were corrected by converting these TI% values to a consistent value of 50%.

In the case of untreated samples, the protein concentrate exhibited the highest TIA (14906.34 TIU/g sample DM and 30562.11 TIU/g protein). This was followed by the flour sample (6639.23 TIU/g sample DM and 28406.88 TIU/g protein) and 90% protein isolate (5166.58 TIU/g sample DM and 5811.50 TIU/g protein). The lowest TIA (4004.25 TIU/g sample DM and 4874.50 TIU/g protein) was observed in 80% protein isolate. Compared to the protein concentrate, the coarse fraction obtained through air classification of the whole pea seed flour showed a TIA value of 23623.54 TIU/g protein. Compared to the TIA result for the untreated protein concentrate in the present study, the air-classified pea protein-enriched flour (PPEF) reported by Konieczny et al. (2020) had a significantly higher TIA value of 38.35 TIU/mg (i.e., 38350 TIU/g) of sample on a DM basis, despite both samples having similar protein contents. Furthermore, in the present study, the protein content in the untreated protein concentrate was approximately double that of untreated whole pea seed flour, with a proportionally higher TIA, nearly twice as much. This enrichment is likely due to the removal of starch and other large granular compounds during the dry air classification process (Konieczny et al., 2020). Similarly, Liu et al. (2021) reported that the TIA result in pea protein concentrate was twice as high as that in pea flour, with values of 5.86 TIU/mg sample (as is) and 2.93 TIU/mg sample (as is), respectively. However, a contrasting trend was observed in soybean-derived products, where raw soybean flour exhibited the highest TIA at 45.65 TIU/mg sample (as is), followed by soy protein concentrate at 10.49 TIU/mg sample (as is), and soy protein isolate at 7.39 TIU/mg sample (as is) (Liu et al., 2021). In the present study, both protein isolate samples, despite their protein contents being as high as 88.90%, did not exhibit a correspondingly high TIA compared to the flour and protein concentrate. This finding aligns with those of Fernández-Quintela et al. (1997), who reported that pea protein isolate showed nearly twice the reduction in TIA when compared to the corresponding pea seeds. The significant reduction in TIA is likely due to the effects of the protein isolation process. As a result, protein

isolates are characterized by a higher protein purity and a lower presence of TIA, making them particularly suitable as value-added ingredients for enhancing the nutritional quality and digestibility of food products.

Thermal treatments had significant reductions ($p < 0.05$) in the TIA of yellow field pea samples. The effectiveness of these treatments in the case of flour samples varied, with TIA reductions ranging from 70.96% to 88.95% on both unit bases across different methods (boiling > baking > autoclaving > micronization). Boiling was identified as the most effective method in reducing TIA, reaching a reduction exceeding 88.60% on both units. This finding is consistent with the results reported by Ma et al. (2017), where boiling yellow field peas for 30 min resulted in the maximum decrease in TIA (87.2%) among various processing methods. Additionally, Khattab and Arntfield (2009) documented that TIA results were even completely inactivated through boiling (100°C for 35 min after presoaking for 4 hr), roasting (180°C for 15 min), microwaving (on high mode for 15 min), and autoclaving (121°C for 20 min) in both Canadian and Egyptian peas. These methods were highly effective, rendering the TIA almost undetectable. However, in the present study, autoclaving showed a relatively lower efficacy in reducing TIA, achieving a reduction of up to 81.24%, despite the pea seeds being pre-soaked and autoclaved at 121°C for a prolonged period of 30 min. Among the thermal processing methods tested in this study, micronization had the least impact on TIA reduction in the flour sample, resulting in approximately a 71% reduction on both bases. In contrast, Khattab and Arntfield (2009) reported that micronization (pre-tempering to a 24% moisture content and heating up to 90°C for 2.5 min) led to significant TIA reductions of up to 94% across different varieties of peas. These discrepancies in TIA reduction could be attributed to variations in the initial contents of Kunitz inhibitors/Bowman-Birk inhibitors in peas, slight differences in process parameters (e.g., milling temperature, storage conditions, and processing conditions), as well as differences in pea cultivars

(Zhang & Chang, 2022). In this study, baking was also found to be significantly effective in reducing TIA in yellow field peas and their derivative products. The effectiveness of TIA reduction through baking may be attributed to a higher temperature employed in this thermal treatment. However, it is important to note that TIA is particularly susceptible to moist heating (Ma et al., 2017; Avilés-Gaxiola et al., 2018). Consequently, despite the high temperature involved in baking, its classification as a dry heating method indicates that it has a comparatively lesser effect on TIA inactivation in the flour sample than boiling in the current study (Drulyte & Orlien, 2019). Among the four types of samples in the current study, baked protein concentrates exhibited the highest TIA reduction of up to 89.82%. This was followed by flour with up to 83.68% reduction, 80% protein isolate with up to 52.69% reduction, and 90% protein isolate with up to 43.73% reduction on both unit bases. In an attempt to elucidate the reasons for the reduced TIA, Kumitch et al. (2019), Lee et al. (2018), and Avilés-Gaxiola et al. (2018) emphasized that TIA is a heat-labile compound primarily composed of low-molecular-weight proteins. Thermal treatments expose these proteins to elevated temperatures for a specific period of time, leading to the denaturation of proteins (including anti-nutritional proteins), and disruption of intermolecular bonds that are crucial for maintaining the tertiary structure of these inhibitors, including the active binding sites of trypsin inhibitors. As a result, their ability to bind and inhibit trypsin is diminished, effectively reducing their activity (Kumitch et al., 2019; Avilés-Gaxiola et al., 2018). In summary, the significant reduction in TIA highlighted in this study underscores the importance of thermal processing in mitigating heat-labile ANFs in yellow field peas, thereby enhancing their nutritional value.

Following non-thermal treatment using HHP at 600 MPa for 10 min in this study, TIA reductions were also significant ($p < 0.05$) but less pronounced compared to thermal treatments. On both unit bases, HHP-treated flour and protein concentrate samples showed effective reductions in TIA ranging from 21.32 to 24.83%. In contrast, both protein isolates treated with

HHP experienced significantly lower decreases in TIA, with reductions ranging from 4.00 to 5.62%. The inactivation in TIA achieved through HHP treatment could be attributed to the induced denaturation and subsequent aggregation of proteins, including anti-nutritional protein compounds (Avilés-Gaxiola et al., 2018; Gharibzahedi & Smith, 2021). Under extreme pressure, HHP can disrupt the native conformation of proteins by breaking intramolecular interactions, such as hydrophobic and electrostatic bonds that maintain the stability of the protein. This disruption exposes hydrophobic and sulfhydryl groups that are typically sequestered within the core of the protein structure, promoting protein-protein interactions and the formation of soluble aggregates (Avilés-Gaxiola et al., 2018; Gharibzahedi & Smith, 2021; Ohanenye et al., 2022; Dehnad et al., 2024). The lower TIA reductions observed in protein isolates may be attributed to the concentration and composition of proteins altered during the isolation process, which may further restrict the extent of pressure-induced structural changes (Gharibzahedi & Smith, 2021). There is compelling evidence that TIA can be significantly reduced by HHP treatment, as reported by Linsberger-Martin et al. (2013), Lee et al. (2018), and Deng et al. (2015). For instance, Linsberger-Martin et al. (2013) investigated the effect of various HHP conditions, including pressure levels (100 or 600 MPa), holding times (30 or 60 min), and temperatures (20°C or 60°C) on TIA reduction in split peas and whole white beans. The study revealed that TIA results were significantly reduced ($p < 0.05$) in both peas and beans under all tested combinations of HHP conditions. Notably, the most substantial reductions were observed at the higher temperature (60°C), regardless of pressure levels and holding times, with reductions ranging from 71.3% – 100% in peas and 37.9% – 83.8% in beans. The combination of the experimental parameters (600 MPa, 60°C, and 30 min) yielded the maximum reduction, achieving 100% in split peas. Appropriate thermal processing conditions can assist in enhancing the catalytic activity of enzymes and improve reaction rates (Khan et al., 2018). This suggests that synergizing HHP with mild

heating could further improve the efficiency of reducing TIA and even other ANFs. Nevertheless, even at 20°C, HHP significantly reduced ($p < 0.05$) TIA contents, by 22.1% – 41.9% and 22.4% – 33.4% for peas and beans, respectively. Specifically, HHP-treated peas (600 MPa, 30 min, 20°C) showed a TIA value of 0.96 TIU/mg DM, representing a 29.4% reduction compared to the untreated form. This reduction is comparable to the 24.83% reduction observed in the present study, where whole pea flour was processed under similar conditions (600 MPa, 10 min, room temperature). Lee et al. (2018) explored the HHP treatment (wet-grinding + HHP at 600 MPa with a holding time of 5 min + freeze-drying) and compared it to a thermal treatment (boiling at 90°C for 15 min + wet-grinding + freeze drying) for the removal of TIA of red bean powder (RBP). The study found a significant reduction of 80.5% in the TIA of RBP following the HHP treatment. Interestingly, the TIA in HHP-treated RBP (0.07 mg of pure trypsin/g fresh weight) was remarkably comparable to that in the boiled RBP (0.06 mg of pure trypsin/g fresh weight), whereas the untreated RBP possessed 0.36 mg of pure trypsin/g fresh weight. Similarly, Deng et al. (2015) reported that both HHP-treated (600 MPa, 30 min, 60°C) and microwave-treated (2450 MHz, 850 W, 30 min) buckwheat displayed comparable TIA results, at 5.19 g/100 g DM and 5.18 g/100 g DM, respectively. Therefore, as a non-thermal treatment, HHP proved to be effective in the inactivation of TIA, with the added advantage of preserving the nutritional and sensory qualities of food that might be damaged by thermal treatments (Lee et al., 2018; Gharibzahedi & Smith, 2021). Further optimization of HHP parameters, such as combining pressure with mild heating, could synergistically improve the reduction of TIA and other ANFs, making it a versatile and efficient processing technique for a wide range of food applications.

Overall, both thermal and non-thermal processing methods employed in this study demonstrated significant feasibility for the inactivation of TIA.

Table 4.5. Effect of different processing methods on the **trypsin inhibitor activity (TIA)** of whole yellow field pea flour, protein concentrate, coarse fraction, 80% protein isolate, and 90% protein isolate.

Sample Description	Sample Extract Diluted to 10 ml (mL)	TI%	DM Basis		Protein Basis	
			Corrected TIA (TIU/g sample, DM)[1]	Relative Change (%)[2]	Corrected TIA (TIU/g protein)[1]	Relative Change (%)[2]
Whole Pea Flour						
Untreated	1.20	46.45^{b}	6639.23^{a}	0.00	28406.88^{a}	0.00
Boiled	N/A	47.55^{b}	757.05^{f}	-88.60	3138.38^{f}	-88.95
Autoclaved	5.91	46.62^{b}	1292.61^{d}	-80.53	5328.88^{d}	-81.24
Micronized	3.86	47.61^{b}	1927.95^{c}	-70.96	8171.20^{c}	-71.24
Baked	6.91	44.65^{b}	1083.54^{e}	-83.68	4667.73^{e}	-83.57
HHP-treated	1.40	59.28^{a}	4990.90^{b}	-24.83	21615.06^{b}	-23.91
Pooled SE	**/**	**12.59**	**1765**	**/**	**56661**	**/**
***P*-value**	**/**	**0.0034**	**<0.0001**	**/**	**<0.0001**	**/**
Protein Concentrate						
Untreated	0.53	46.41^{c}	14906.34^{a}	0.00	30562.11^{a}	0.00
Baked	5.09	46.80^{bc}	1604.25^{d}	-89.24	3110.76^{c}	-89.82
HHP-treated	0.60	56.28^{a}	11673.92^{b}	-21.68	24046.19^{b}	-21.32
Coarse fraction	2.33	52.65^{ab}	3266.85^{c}	-78.08	23623.54^{b}	-22.70
Pooled SE	**/**	**5.586**	**2167**	**/**	**37920**	**/**
***P*-value**	**/**	**0.0023**	**<0.0001**	**/**	**<0.0001**	**/**

Data are expressed as mean of three replicates (two measurements per replicate) and presented on the DM and protein bases. Mean values followed by different superscript letters within the column of each yellow field pea type indicate statistically significant differences between treatments ($p < 0.05$) using one-way ANOVA along with Tukey's multiple comparison test.

[1] Values were corrected by setting TI% to 50%.

[2] Untreated sample of each category is considered as a baseline. The relative change of each treated sample was calculated based on the baseline.

Abbreviations: TI% = % of trypsin inhibition; TIU = Trypsin inhibitor units; DM = Dry matter; N/A = Not applicable; SE = Standard error.

Table 4.5. Continued.

Sample Description	Sample Extract Diluted to 10 ml (mL)	TI%	DM Basis		Protein Basis	
			Corrected TIA (TIU/g Sample, DM)[1]	Relative Change (%)[2]	Corrected TIA (TIU/g Protein)[1]	Relative Change (%)[2]
80% Protein Isolate						
Untreated	1.93	47.78[a]	4004.25[a]	0.00	4874.50[a]	0.00
Baked	4.03	49.79[a]	1894.39[c]	-52.69	2313.52[c]	-52.54
HHP-treated	1.83	54.39[a]	3779.31[b]	-5.62	4631.28[b]	-4.99
Pooled SE	**/**	**20.77**	**31.24**	**/**	**127.1**	**/**
***P*-value**	**/**	**0.2674**	**<0.0001**	**/**	**<0.0001**	**/**
90% Protein Isolate						
Untreated	1.51	43.86[b]	5166.58[a]	0.00	5811.50[a]	0.00
Baked	2.62	45.13[b]	2906.99[c]	-43.73	3294.58[c]	-43.31
HHP-treated	1.41	62.60[a]	4915.49[b]	-4.86	5578.90[b]	-4.00
Pooled SE	**/**	**5.087**	**1667**	**/**	**1519**	**/**
***P*-value**	**/**	**<0.0001**	**<0.0001**	**/**	**<0.0001**	**/**

Data are expressed as mean of three replicates (two measurements per replicate) and presented on the DM and protein bases. Mean values followed by different superscript letters within the column of each yellow field pea type indicate statistically significant differences between treatments ($p < 0.05$) using one-way ANOVA along with Tukey's multiple comparison test.

[1] Values were corrected by setting TI% to 50%.

[2] Untreated sample of each category is considered as a baseline. The relative change of each treated sample was calculated based on the baseline.

Abbreviations: TI% = % of trypsin inhibition; TIU = Trypsin inhibitor units; DM = Dry matter; N/A = Not applicable; SE = Standard error.

4.4.2.2 Lectin activity

The summaries of lectin content, expressed in hemagglutinating units (HU)/g of sample on a DM basis and HU/g of protein, for untreated and treated whole yellow field pea flour, protein concentrate, and both 80% and 90% protein isolates, as well as for the untreated coarse fraction, are shown in **Table 4.6**. The assay used is based on the ability of lectins/hemagglutinins to bind to specific carbohydrates on the surface of red blood cells (RBCs), Positive hemagglutinating activity (HA) is indicated by the formation of stable lectin - RBC complexes, preventing RBCs from settling on the bottom of the microplate well and maintaining a homogenous suspension (Makkar et al., 2007; Liu et al., 2013; Makkar et al., 1997; Tang et al., 2024). The endpoint of hemagglutination is identified by the presence of agglutination in the last well of a two-fold serial dilution that shows a positive response, corresponding to one HU (Labba et al., 2021; Liu et al., 2013). For example, in the current study, the HA of untreated yellow field pea flour was observed on average in the 11th dilution well (= 11 HU). Boiling resulted in the most pronounced reduction in HA, with activity being detected in an average of only 1.33 wells (= 1.33 HU).

The lectin content in the untreated yellow field pea samples varied significantly. The 90% isolate had the highest content of lectin (1.72E+06 HU/g of sample, DM and 1.94E+06 HU/g of protein). Conversely, the 80% isolate had the lowest lectin content (2.68E+04 HU/g of sample, DM and 3.26E+04 HU/g of protein). On the other hand, the lectin content of the protein concentrate (4.46E+05 HU/g of sample) was twice as high as that of the flour (2.23E+05 HU/g of sample) on the DM basis. However, when expressed on a protein basis, the lectin contents of the protein concentrate and the flour were nearly identical, at 9.15E+05 HU/g of protein and 9.56E+05 HU/g of protein, respectively. Additionally, the coarse fraction showed a substantial lectin content of 7.62E+05 HU/g of protein. However, the results for the untreated pea flour in the present study

show significantly higher lectin contents compared to the observations reported by Shi et al. (2018), Alonso et al. (1998), and El-Adawy et al. (2003). In those studies, the lectin contents in pea samples were reported as 5.1 – 6.2 HU/mg DM (equivalent to 5100 – 6200 HU/g DM) by Shi et al. (2018) and Alonso et al. (1998), and 6400 HU/g flour (El-Adawy et al., 2003). The differences in lectin content could be attributed to several factors, including variations in pea varieties, crop years, growing conditions, and the molecular properties of lectin. Additionally, variability may arise from differences in measurement methods, the definition of HA unit, and the types and metabolic states of RBCs used in the hemagglutination assay (Shi et al., 2018; Pedrosa et al., 2012; Alonso et al., 1998; Marquardt et al., 1974). Furthermore, the lectin content of the untreated 90% protein isolate did not decrease significantly and remained the highest lectin content among the four pea sample types, despite the higher degree of purification during the isolation process. However, the lack of comparable data on lectin content in protein fraction samples, such as protein concentrates and isolates, in the available literature complicates direct comparisons.

Thermal treatments played an outstanding role in reducing lectin content in the pea samples in the current study. All tested thermal methods significantly diminished ($p < 0.05$) the lectin content in the flour samples, with reductions ranging from 74.07% to 99.88% on both unit bases (boiling > baking > micronization > autoclaving). Boiling was the most effective method for the nearly complete elimination of lectins with a 99.88% reduction. Baking followed with a reduction of over 88.17%, while micronization and autoclaving were less effective, showing comparable reductions in lectin content of up to -76.10% and -74.88%, respectively, on both bases. Additionally, baking demonstrated a highly effective impact on reducing lectin content in different protein fractions. Reductions reached up to 99.68% in the 90% isolate, 95.00% in the protein concentrate, 93.83% in the 80% isolate, and 88.25% in the flour on both unit bases. However, for the 90% isolate and protein concentrate, the reductions in lectin content were not statistically

significant ($p > 0.05$) when compared to their respective untreated forms, despite achieving the highest reductions among the baked samples. Sánchez-Velázquez et al. (2021) reported changes in the protein profiles of thermally treated yellow split peas, particularly in the intensity of lectin bands at 35–37 kDa, as the lectins are a type of anti-nutritional proteins. In that study, boiled and baked peas exhibited lower lectin band intensity compared to untreated peas, indicating significant lectin denaturation caused by thermal treatments. However, while baking resulted in lower band intensity than boiling, this does not necessarily reflect a higher degree of lectin denaturation or degradation. Instead, baking may promote greater protein aggregation, making lectins less detectable on electrophoresis (Sánchez-Velázquez et al., 2021). Numerous studies, including those conducted by Shi et al. (2018), Mubarak (2005), and Kalpanadevi & Mohan (2013), have consistently demonstrated the remarkable reduction of lectins in a variety of pulses and oilseeds following boiling. Shi et al. (2018) found that boiling (95°C, 1 hr) after 4 hr of presoaking, nearly eliminated lectins from peas, lentils, fava beans, common beans, and soybeans, achieving reductions ranging from 96.98% to 99.81%. Similarly, Mubarak (2005) observed that presoaking mung beans for 12 hr followed by 90 min of boiling resulted in complete lectin elimination. There is a lack of data regarding the efficacy of baking in reducing lectin content in pulses for comparison. Baking involves exposure to higher temperatures in the current study, demonstrating a highly effective reduction in lectin content. Furthermore, in the studies of Umoren et al. (1997), Mubarak (2005), and Luo & Xie (2013), lectin contents in mung beans, cowpeas, and faba beans were completely destroyed after autoclaving at 121°C for 35 min, 105°C for 30 min, and 121°C for 20 min, respectively. However, despite applying similar or lower temperatures and processing times in these studies, the reduction in lectin content observed in the autoclaved flour in the current study was notably less effective. The significant reduction in lectin content during thermal processing is primarily due to the heat-labile nature of lectins, which undergo degradation into subunits or

conformational changes when exposed to elevated temperatures (Luo & Xie, 2013; Shi et al., 2018). Tang et al. (2024) reported that when the temperature reached 80°C, the tetrameric structure of lectins was almost completely denatured. This denaturation process disrupts the quaternary structure of the proteins, leading to the breakdown into smaller components (e.g., dimers, monomers, or degraded fragments) or the formation of soluble aggregates. These aggregates, formed at temperatures exceeding 70°C, are facilitated by intermolecular interactions and lack the ability to bind carbohydrates effectively. Such thermally induced structural changes can effectively reduce lectin content and mitigate their anti-nutritional and HA effects. However, Luo & Xie (2013), Alonso et al. (2000), and Alonso et al. (1998) reported that pulses such as faba beans, kidney beans, and peas showed no significant reduction in lectin concentrations after dehulling, soaking, or germination.

In this study, the inactivation of lectins varied significantly between thermal and non-thermal processing methods. HHP, as a non-thermal method, significantly increased ($p < 0.05$) lectin content by up to 328.10% in the 90% protein isolate, 290.71% in the flour, and 159.30% in the protein concentrate. However, no difference in lectin content was observed between untreated and HHP-treated 80% protein isolates. These findings contrast with the observations reported by Liu et al. (2013), where the lectin content of red kidney beans was considerably inactivated by HHP processing at pressures exceeding 250 MPa. It explained that HHP can significantly interfere with the quaternary structure of proteins (i.e., lectins) through mechanisms such as conformational changes and induce the aggregation of lectins. This disruption may lead to denaturation and loss of lectin content (Liu et al., 2013). However, there are limited studies investigating the effects of HHP on lectin content in pulse samples, and no studies have reported a direct increase in lectin content as a result of HHP treatment. According to Liu et al. (2013), HHP can enhance the crude extraction yields of lectins primarily by disrupting cellular structures. The application of increased

pressure mechanically breaks down the cell walls and membranes, facilitating the release of intracellular components. Consequently, an apparent increase in lectin content in the extract may be observed, which could be attributed to improved extraction yields by extreme pressure. This phenomenon is comparable to the observed increase in condensed tannin content, where enhanced extraction efficiency often results in increased yields of target compounds, as stated by Das et al. (2020). Furthermore, Siah et al. (2014) reported that the phenolic compound extraction yields increased following autoclaving, with higher yields in autoclaved broth when compared to boiled broth. The high-pressure conditions of autoclaving were suggested to disrupt cellular structures and enhance the release of intracellular components, including phenolic compounds and potentially lectins. Therefore, HHP could be a potential method for improving the extraction of such compounds. However, while Liu et al. (2013) reported improved overall extraction yields of lectins from red kidney beans under HHP, they noted that this potentially induced an increase in non-lectin compounds but was not directly correlated with increased HA. Future studies are needed to explore how lectin behavior changes with increasing extraction yields under HHP processing conditions for various pulses, as well as to explore the effect of HHP on the conformation of pea lectins.

The hemagglutination assay, while relatively safe, straightforward, time-saving, and cost-effective, is primarily utilized to assess the effectiveness of treatments in mitigating seed toxicity (Luo & Xie, 2013; Kong et al., 2022). In the present study, this assay is recognized as having limited precision for accurately estimating lectin content. Various factors can influence the agglutination of lectins, including the molecular properties of lectins, the surface characteristics and metabolic state of cells, and the conditions of the assay in terms of temperature, cell concentration, blood type, and mixing (Shi et al., 2018; Luo & Xie, 2013). In this study, a 1% rabbit red blood cell solution without trypsin treatment was used to evaluate HA in pea samples,

acknowledging the assay's inherent limitations in precision (Hirabayashi, 2014; Alonso et al., 2000; Makkar et al., 1997; Makkar et al., 2007). Furthermore, the hemagglutination assay provides insights into the anti-nutritional effects of lectins by measuring their activity based on their carbohydrate-binding ability. However, its limitations lie in its inability to detect lectins that lack HA but may still exert anti-nutritional impacts, such as monomeric or fragmented lectins, whereas structural integrity of the tetrameric form is essential for lectins to exhibit HA (Kong et al., 2022; Tang et al., 2024). Therefore, while the hemagglutination assay is effective in assessing functional HA, it cannot provide a comprehensive assessment of all anti-nutritional effects associated with lectins. Nevertheless, despite these shortcomings, the hemagglutination assay, used in the present study, provided a practical means to evaluate the relative efficacy of various processing methods in reducing lectin content by comparing untreated and treated samples. This facilitated an understanding of the comparative effectiveness of different treatments. To overcome the limitations of the hemagglutination assay, an immunoassay involving the use of monoclonal antibodies targeting lectins presents a potentially effective, specific, and reproducible alternative for the accurate detection and quantification of lectins (Tang et al., 2024; Kong et al., 2022).

In summary, lectins in pea flour, protein concentrate, and both protein isolates were highly sensitive to thermal treatments, with remarkable reductions observed. However, HHP treatment did not effectively degrade lectins and even resulted in a notable increase in lectin content, likely due to enhanced extraction efficiency. Future studies should focus on improving analytical methods to comprehensively determine all lectin forms, including those contributing to anti-nutritional effects, for more accurate and detailed assessments.

Table 4.6. Effect of different processing methods on the **lectin content** of whole yellow field pea flour, protein concentrate, coarse fraction, 80% protein isolate, and 90% protein isolate.

Sample Description	Well # at Highest Dilution		DM Basis		Protein Basis	
	Result	Relative Change (%)[1]	Lectin (HU/g of sample, DM)	Relative Change (%)[1]	Lectin (HU/g of protein)	Relative Change (%)[1]
Whole Pea Flour						
Untreated	11.00[b]	0.00	2.23E+05[b]	0.00	9.56E+05[b]	0.00
Boiled	1.33[d]	-87.88	2.77E+02[d]	-99.88	1.15E+03[d]	-99.88
Autoclaved	8.67[c]	-21.21	5.79E+04[c]	-74.07	2.40E+05[c]	-74.88
Micronized	9.00[c]	-18.18	5.39E+04[c]	-75.88	2.28E+05[c]	-76.10
Baked	8.00[c]	-27.27	2.62E+04[cd]	-88.25	1.13E+05[cd]	-88.17
HHP-treated	13.00[a]	+18.18	8.62E+05[a]	+285.99	3.73E+06[a]	+290.71
Pooled SE	**0.4444**	**/**	**3.68E+08**	**/**	**6.63E+09**	**/**
***P*-value**	**<0.0001**	**/**	**<0.0001**	**/**	**<0.0001**	**/**
Protein Concentrate						
Untreated	12.00[b]	0.00	4.46E+05[b]	0.00	9.15E+05[b]	0.00
Baked	7.67[d]	-36.11	2.36E+04[b]	-94.72	4.57E+04[b]	-95.00
HHP-treated	13.33[a]	+11.11	1.15E+06[a]	+158.72	2.37E+06[a]	+159.30
Coarse fraction	10.00[c]	-16.67	1.05E+05[b]	-76.39	7.62E+05[b]	-16.74
Pooled SE	**0.1667**	**/**	**6.15E+10**	**/**	**2.48E+11**	**/**
***P*-value**	**<0.0001**	**/**	**0.0019**	**/**	**0.0028**	**/**

Data are expressed as mean of three replicates (two measurements per replicate) and presented on the DM and protein bases. Mean values followed by different superscript letters within the column of each yellow field pea type indicate statistically significant differences between treatments ($p < 0.05$) using one-way ANOVA along with Tukey's multiple comparison test.

[1] Untreated sample of each category is considered as a baseline. The relative change of each treated sample was calculated based on the baseline.

Abbreviations: HU = Hemagglutinating unit; DM = Dry matter; SE = Standard error.

Table 4.6. Continued.

Sample Description	Well # at Highest Dilution		DM Basis		Protein Basis	
	Result	**Relative Change (%)[1]**	**Lectin (HU/g of sample, DM)**	**Relative Change (%)[1]**	**Lectin (HU/g of protein)**	**Relative Change (%)[1]**
80% Protein Isolate						
Untreated	8.00[a]	0.00	2.68E+04[a]	0.00	3.26E+04[a]	0.00
Baked	3.67[b]	-54.17	1.65E+03[b]	-93.83	2.02E+03[b]	-93.81
HHP-treated	8.00[a]	0.00	2.67E+04[a]	-0.42	3.27E+04[a]	+0.24
Pooled SE	**0.4444**	**/**	**6.87E+05**	**/**	**1.03E+06**	**/**
***P*-value**	**0.0003**	**/**	**<0.0001**	**/**	**<0.0001**	**/**
90% Protein Isolate						
Untreated	13.67[b]	0.00	1.72E+06[b]	0.00	1.94E+06[b]	0.00
Baked	5.67[c]	-58.54	5.49E+03[b]	-99.68	6.23E+03[b]	-99.68
HHP-treated	16.00[a]	+17.07	7.38E+06[a]	+328.10	8.38E+06[a]	+332.13
Pooled SE	**0.5556**	**/**	**1.02E+12**	**/**	**1.31E+12**	**/**
***P*-value**	**<0.0001**	**/**	**0.0003**	**/**	**0.0003**	**/**

Data are expressed as mean of three replicates (two measurements per replicate) and presented on the DM and protein bases. Mean values followed by different superscript letters within the column of each yellow field pea type indicate statistically significant differences between treatments ($p < 0.05$) using one-way ANOVA along with Tukey's multiple comparison test.

[1] Untreated sample of each category is considered as a baseline. The relative change of each treated sample was calculated based on the baseline.

Abbreviations: HU = Hemagglutinating unit; DM = Dry matter; SE = Standard error.

4.4.2.3 Phytic acid

Changes in phytic acid (PA) content, quantified as mg PA/g of sample on a DM basis and as mg PA/g of protein, in pea flour, protein concentrate, and both 80% and 90% protein isolates subjected with various thermal and HHP processing methods, along with the PA content of the untreated coarse fraction for comparison, are presented in **Table 4.7**. It is important to note that phytic acid sodium salt hydrate was used as the standard in this study. To ensure greater accuracy, PA results should ideally be expressed as sodium phytate equivalent/g of sample rather than pure phytic acid, as the same expression was used in Osuna-Gallardo et al. (2023). However, according to previous studies that reported the results as mg PA equivalent per gram of sample, it remains unclear whether those values were corrected by sodium content (Shi et al., 2018; Sánchez-Velázquez et al., 2021; Linsberger-Martin et al., 2013; Khattab & Arntfield, 2009). Therefore, to ensure consistency and facilitate direct comparison with the literature, the current study adopts the same format for expressing the PA results, while acknowledging the limitation introduced by potential inaccuracies in PA content expressions.

In terms of untreated samples, PA contents were measured at 8.84 mg/g DM in the flour, 20.54 mg/g DM in the 80% protein isolate, 21.21mg/g DM in the protein concentrate, and 22.66 mg/g DM in the 90% protein isolate. Yellow peas typically exhibit PA contents ranging from 7.29 to 12.40 mg/g, as reported in previous studies (Shi et al., 2018; Shi et al., 2022; Pedrosa et al., 2020; Khattab & Arntfield, 2009; Sánchez-Velázquez et al., 2021). This agrees with the present study's result, where the PA content in the pea flour fell within this established range. Furthermore, Chitra et al. (1995) indicated a strong positive correlation ($R^2 = 0.94$) between PA content and protein content. Shi et al. (2022) further demonstrated that protein isolates with high protein content exhibited higher PA contents (ranging from 19.08 to 35.04 mg/g on a fresh weight

basis) compared to flours (ranging from 7.29 to 17.54 mg/g on a fresh weight basis) across three fava bean varieties, pea, and soy. Consistent with this, the present study found higher PA content in the protein concentrate obtained through dry fractionation compared to the flour, reinforcing the relationship between PA content and protein levels. However, comparable PA contents were observed between the protein concentrate and both protein isolates, suggesting that the isolation process may influence PA retention or removal in the protein isolates. Moreover, the PA contents in the pea flour and 90% protein isolate observed in the present study align with the findings of Shi et al. (2022), who reported PA contents of 7.29 mg/g on a fresh weight basis in pea flour and 23.29 mg/g on a fresh weight basis in pea protein isolate (with a protein content of 86.9%). Conversely, Pedrosa et al. (2020) reported that the PA content in pea flour was higher, measured at 11.07 mg/g DM, compared to pea protein isolate (with a protein content of 68.85%), which had a PA content of 9.59 mg/g DM. Additionally, PA (IP6) constituted the predominant portion of total inositol phosphates, accounting for 80% in pea flour and 83% in pea protein isolate. This was followed by IP5, a lower phosphorylated form, which comprised 13% of the total inositol phosphates in both pea flour and protein isolate. These discrepancies could be related to the variations in sample varieties, processing conditions, and inherent differences in the nutritional composition of the samples. Shi et al. (2022) explained that variations in PA content among different samples could be attributed to differences in protein composition, protein surface properties, and their binding interactions with PA. As a result, the correlation between PA and protein content emphasizes the need for balanced processing methods that can enhance protein content while effectively managing ANFs. When expressed on a protein basis rather than a DM basis, the PA contents in flour and protein concentrate were significantly higher compared to their values expressed on a DM basis, achieving 37.80 mg/g of protein and 43.48 mg/g of protein, respectively. In contrast, the PA contents in both protein isolates were lower compared to those

in the flour and protein concentrate, with 25.00 mg/g of protein in the 80% protein isolate and 25.49 mg/g of protein in the 90% protein isolate. Furthermore, the PA content of the coarse fraction was considerably lower than that of the protein concentrate on a DM basis, at 4.80 mg/g DM. When expressed on a protein basis, the PA content of the coarse fraction increased to 34.73 mg/g of protein, although it remained lower than that of the protein concentrate. De Angelis et al. (2021) observed that the coarse fractions of red lentil, yellow lentil, green pea, and Kabuli chickpea samples exhibited lower PA contents, ranging from 5.06 to 6.86 mg/g DM when compared to their fine fractions, which ranged from 8.72 to 14.06 mg/g DM. This pattern can be attributed to the localization of PA within protein bodies in the cotyledons of seeds. Consequently, the protein concentration following dry fractionation is closely related to the PA content.

Among all thermal treatments, the concentrations of PA in yellow field pea samples significantly decreased ($p < 0.05$). For flour samples, PA contents decreased by 20.48% to 48.20% on both bases (boiling > autoclaving > baking > micronization), with boiling showing the greatest reduction (over -46.61%). This was followed by autoclaving with a decrease of over 29.30%, baking with a decrease of over 27.09%, and micronization with a decrease of over 20.48%. Additionally, baking showed the highest efficacy for the protein concentrate, achieving a PA reduction of above 42.67%. Baking resulted in the reduction of PA by above 21.92% in the 80% protein isolate and above 20.32% in the 90% protein isolate. The higher reduction of PA in the boiled flour in the current study may be attributed to the longer soaking time (18 hr) compared to the study by Shi et al. (2018), which found a PA reduction of 20.68 - 23.00% for pre-soaked (4 hr) boiled yellow peas. Similarly, Sánchez-Velázquez et al. (2021) reported that yellow split peas showed a reduction of more than 70% after 16 hr of soaking followed by boiling. Consistent with these findings, Kalpanadevi & Mohan (2013) reported a 28% PA reduction in boiled cowpeas without soaking, which increased to 68% with 12 hr of soaking. They also reported that

hydrating cowpea seeds for 12, 24, 48, 72, and 96 hr gradually decreased PA contents from 31% to 95%. Therefore, the effective reduction of PA underscores the significance of presoaking combined with thermal treatments, as evidenced by the effects of boiling and autoclaving in the current study. Interestingly, the effects of autoclaving on PA reduction in the present study conflict with the findings reported by Kalpanadevi & Mohan (2013), Hefnawy (2011) and Khattab & Arntfield (2009), where autoclaving was found to be superior to boiling in reducing PA content. In the study of Khattab & Arntfield (2009), autoclaving at 121°C for 20 min showed the most significant reductions of 67.48% and 70.22% in PA content in Canadian and Egyptian peas, respectively, whereas boiling achieved PA reductions of 54.72% and 55.17% for the same varieties, respectively. Micronization, characterized by rapid heating, was observed to have the least, but still significant impact on PA reduction among the thermal treatments tested. In terms of baking (a dry heat process), Sánchez-Velázquez et al. (2021) showed a reduction of 34% in the PA content of yellow split peas after baking at 193°C for 35 min. Notably, the capacity for PA reduction by boiling was twice that of baking, which is consistent with the findings obtained in the present study. The observed reductions in PA content during thermal treatments could be attributed to multiple mechanisms. First, the water-soluble nature of PA facilitates its release into the soaking medium during the extended hydration period, which also activates endogenous phytase enzymes. These enzymes can further catalyze the degradation of PA (IP6) into lower inositol phosphates, thereby reducing its concentration (Kohli & Singha, 2024; Kalpanadevi & Mohan, 2013; Sánchez-Velázquez et al., 2021; Khattab & Arntfield, 2009; Nakitto et al., 2015). Additionally, thermal processing can induce the thermal degradation of PA, breaking it down by heat from the more highly phosphorylated inositol phosphates to lower forms and releasing bound minerals (Kohli & Singha, 2024; Samtiya et al., 2020; Kalpanadevi & Mohan, 2013; Khattab & Arntfield, 2009). Therefore, optimizing hydrothermal treatments or combining

presoaking with appropriate thermal treatments can effectively reduce PA content in pulses, thereby enhancing the nutritional quality and mineral bioavailability in pulse-based food products.

HHP treatment showed a significant ($p < 0.05$) effect on reducing PA content in flour and protein concentrate, with reductions of up to 19.28% in the protein concentrate and 16.06% in the flour on both unit bases. However, PA content in both protein isolates was not significantly lower ($p > 0.05$) when compared to their respective untreated samples. There was even a slight increase (0.31 – 1.22% on DM and protein bases) in the PA content of the 90% protein isolate after HHP. A study by Linsberger-Martin et al. (2013) demonstrated that PA contents were reduced by up to 36% in split peas and 11% in whole white beans by HHP processing under various conditions (pressure levels of 100 or 600 MPa; holding times of 30 or 60 min; and temperatures of 20 or 60°C). Specifically, the HHP processing parameter of 600 MPa for 30 min at 20°C caused a 27% reduction in PA content, decreasing from 1.15 g/100 g DM in untreated peas to 0.84 g/100 g DM in HHP-treated peas. In comparison, the effect of 600 MPa for 10 min at room temperature in the present study led to a 16% reduction in PA content in the pea flour, from 8.84 mg/g DM to 7.42 mg/g DM. Lee et al. (2018) also reported that the PA content in red bean powder (RBP) significantly decreased following HHP treatment. In their study, the PA content of untreated RBP was 9.47 mg/g fresh weight. After applying HHP at 400, 500, and 600 MPa for 5 min, the PA contents decreased to 9.04, 8.33, and 8.19 mg/g fresh weight, respectively. Notably, the PA content of HHP-treated RBP at 600 MPa was even lower than that of boiled RBP, which had a PA content of 8.33 mg/g fresh weight after boiling at 90°C for 15 min. Similarly, Deng et al. (2015) applied a specific set of HHP conditions (600 MPa for 30 min at 60°C) was applied to buckwheat, resulting in a PA content of 9.85 g/100g DM. This was significantly lower than the PA content of microwaved buckwheat with 12.03 g/100 g DM. These studies highlight the variability in PA reduction, influenced by factors such as different sample types, sample cultivars, pre-treatment

conditions (e.g., split vs. whole seeds), and storage conditions. Additionally, the interactions between pressure level, temperature, and holding time contributed to the significant reduction of PA contents, as demonstrated by Linsberger-Martin et al. (2013) and Buta et al. (2019). The substantial reduction in PA content could be attributed to the disruptive effects of HHP on internal cell structures and membranes of seeds. These changes not only allow endogenous enzymes (i.e., phytases) to more easily access and interact with PA but also promote the enzymatic hydrolysis of PA by phytases into lower inositol phosphates such IP4 and IP5 (Lee et al., 2018; Deng et al., 2015; Ohanenye et al., 2022; Buta et al., 2019). In particular, IP4 to IP6 are predominant forms presented after hydrolysis (Linsberger-Martin et al., 2013), which is consistent with the findings of Pedrosa et al. (2020) and Frias et al. (2011). These lower forms of PA exhibit reduced mineral chelating capacity, thereby improving the bioavailability of the minerals (Gupta et al., 2013; Wu et al., 2023).

In summary, thermal treatments significantly reduced PA content in yellow field pea samples. The most effective reduction was observed with optimized thermal treatments, particularly when presoaking was followed by boiling, due to the water solubility of PA. This condition minimized the PA content and potentially improved the bioavailability of the minerals. HHP treatment only effectively reduced the PA content in the protein concentrates and flours.

Table 4.7. Effect of different processing methods on the **phytic acid (PA)** content of whole yellow field pea flour, protein concentrate, coarse fraction, 80% protein isolate, and 90% protein isolate.

Sample Description	DM Basis		Protein Basis	
	Corrected PA (mg PA/g of sample, DM)[1]	**Relative Change (%)[2]**	**Corrected PA (mg PA/g of protein)[1]**	**Relative Change (%)[2]**
Whole Pea Flour				
Untreated	8.84[a]	0.00	37.80[a]	0.00
Boiled	4.72[d]	-46.61	19.58[d]	-48.20
Autoclaved	6.25[c]	-29.30	25.75[c]	-31.88
Micronized	7.03[bc]	-20.48	29.79[bc]	-21.19
Baked	6.39[c]	-27.71	27.56[c]	-27.09
HHP-treated	7.42[b]	-16.06	32.12[b]	-15.03
Pooled SE	**0.1379**	/	**2.571**	/
***P*-value**	**<0.0001**	/	**<0.0001**	/
Protein Concentrate				
Untreated	21.21[a]	0.00	43.48[a]	0.00
Baked	12.16[c]	-42.67	23.57[c]	-45.79
HHP-treated	17.12[b]	-19.28	35.26[b]	-18.91
Coarse fraction	4.80[d]	-77.37	34.73[b]	-20.12
Pooled SE	**0.3743**	/	**1.902**	/
***P*-value**	**<0.0001**	/	**<0.0001**	/

Data are expressed as mean of three replicates (two measurements per replicate) and presented on the DM and protein bases. Mean values followed by different superscript letters within the column of each yellow field pea type indicate statistically significant differences between treatments ($p < 0.05$) using one-way ANOVA along with Tukey's multiple comparison test.

[1]Values were corrected based on a 93% phosphorus content in phytic acid sodium salt hydrate.

[2] Untreated sample of each category is considered as a baseline. The relative change of each treated sample was calculated based on the baseline.

Abbreviations: DM = Dry matter; SE = Standard error.

Table 4.7. Continued.

Sample Description	DM Basis		Protein Basis	
	Corrected PA (mg PA/g of sample, DM)[1]	**Relative Change (%)[2]**	**Corrected PA (mg PA/g of protein)[1]**	**Relative Change (%)[2]**
80% Protein Isolate				
Untreated	20.54[a]	0.00	25.00[a]	0.00
Baked	15.98[b]	-22.20	19.52[b]	-21.92
HHP-treated	20.03[a]	-2.48	24.54[a]	-1.84
Pooled SE	**0.6962**	**/**	**1.061**	**/**
***P*-value**	**0.0010**	**/**	**0.0011**	**/**
90% Protein Isolate				
Untreated	22.66[a]	0.00	25.49[a]	0.00
Baked	17.92[b]	-20.92	20.31[b]	-20.32
HHP-treated	22.73[a]	+0.31	25.80[a]	+1.22
Pooled SE	**0.3951**	**/**	**0.4552**	**/**
***P*-value**	**0.0001**	**/**	**<0.0001**	**/**

Data are expressed as mean of three replicates (two measurements per replicate) and presented on the DM and protein bases. Mean values followed by different superscript letters within the column of each yellow field pea type indicate statistically significant differences between treatments ($p < 0.05$) using one-way ANOVA along with Tukey's multiple comparison test.

[1]Values were corrected based on a 93% phosphorus content in phytic acid sodium salt hydrate.

[2] Untreated sample of each category is considered as a baseline. The relative change of each treated sample was calculated based on the baseline.

Abbreviations: DM = Dry matter; SE = Standard error.

4.4.2.4 Total phenolic content and condensed tannins

The present study utilized two reference standards, catechin and gallic acid, which are typically employed for quantifying polyphenolic compounds, particularly in various pulse varieties. Using both standards in the present study allowed for enhanced comparability across different studies and experiments, thus ensuring consistency and reliability in polyphenolic quantification. Additionally, previous studies have used different combinations of wavelengths and reference standards for polyphenolic quantification, such as catechin at 550 nm in Shi et al (2022), catechin at 760 nm in Bai et al (2018), and gallic acid at 550 nm in Konieczny et al. (2020) and Goertzen et al. (2020). However, the current study identified 750 nm as the optimal wavelength for both standards, achieving the highest absorbance values (in-house data) and the most reliable calibration curves, with R^2 values reaching up to 0.9995. These findings, illustrated in **Figures 4.2 and 4.3**, demonstrate the consistency and accuracy of using 750 nm for both standards on both unit bases.

Changes in polyphenol (measured at 750 nm) and condensed tannin (CT) contents, expressed as mg catechin equivalent (CE)/g of sample on a DM basis and mg CE/g of protein, in pea flour, protein concentrate, and both 80% and 90% protein isolates under thermal and non-thermal processing methods, along with data for the untreated coarse fraction, are summarized in **Tables 4.8**, **4.9**, and **4.12**. For untreated samples, the total phenolic content (TPC) ranged from 1.40 to 3.61 mg CE/g of sample on a DM basis, following the order: protein concentrate > 80% isolate > flour > 90% isolate. When expressed on a protein basis, the TPC ranged between 1.58 and 9.61 mg CE/g of protein, with the order: flour > protein concentrate > 80% isolate > 90% isolate. Flour and protein concentrate, with lower protein contents, contain seed coats and more complex matrices that help preserve polyphenols and other compounds. Conversely, the higher

degrees of isolation processing involved in wet extraction methods for the protein isolates result in the removal of non-protein fractions and greater losses of water-soluble polyphenols, particularly in the 90% isolate (Sánchez-Velázquez et al., 2021). Regarding the CT content of the untreated samples, the ranking was consistent across both unit bases: 80% isolate > protein concentrate > 90% isolate > flour. The 80% isolate had the highest CT content (0.394 mg CE/g of sample, DM and 0.479 mg CE/g of protein), followed by the protein concentrate (0.099 mg CE/g of sample, DM and 0.203 mg CE/g of protein), 90% isolate (0.096 mg CE/g of sample, DM and 0.109 mg CE/g of protein), and flour (0.024 mg CE/g of sample, DM and 0.102 mg CE/g of protein). Additionally, the TPC and CT in the coarse fraction obtained through air classification (8.78 mg CE/g of protein and 0.528 mg CE/g of protein, respectively), which retains more large granules such as seed coats, were notably higher than those found in the protein concentrate (7.39 mg CE/g of protein and 0.203 mg CE/g of protein, respectively). As an important group of polyphenols, CT consistently exhibits lower values compared to the broader TPC measurements. Additionally, polyphenolic compounds including CT are typically concentrated in the seed coats of pulses and their contents are associated with the seed coat color, therefore, yellow peas characterized by light-colored seed coats, always contain small or undetectable amounts of both TPC and CT in the present study (Shi et al., 2022; Konieczny et al., 2020). Compared to the TPC of 2.86 mg GAE/g of sample DM measured at 550 nm in the present study, Han & Baik (2008) reported a similar TPC of 2.5 mg GAE/g of sample, measured at 505 nm, for yellow peas. In comparison to the current study, Shi et al. (2022) reported a lower TPC of 1.12 mg CE/g (as is) in pea flour but a higher TPC of 3.19 mg CE/g (as is) in pea isolate when measured at 550 nm. In the current study, TPC was measured at 3.05 mg CE/g in the pea flour and 2.66 mg CE/g in the 90% pea isolate at the same wavelength. Interestingly, Shi et al. (2022) also reported CT contents of 0.02 mg/g (as is) in pea flour and 0.17 mg/g (as is) in pea isolate, which are highly similar to those

observed in our study. However, in that study, higher TPC and CT results were observed in pea isolates compared to flours, which appears contradictory to the TPC trends observed in the current study. Pedrosa et al. (2020) discovered that pea isolate (with a protein content of 68.85%) extracted from pea flour exhibited a 35% reduction in TPC, decreasing from 5.89 mg CE/g dry weight (d.w.) in the flour to 3.82 mg CE/g (d.w.) in the isolate. In contrast, the TPC of bean protein isolate (BPI) was higher at 5.35 mg CE/g (d.w.) compared to that of its corresponding raw bean flour, which contained only 2.27 mg CE/g (d.w.). Similar outcomes in TPC for the pea seed sample and its protein isolate were observed by Agboola et al. (2010). Furthermore, Goertzen et al. (2020) reported the trend of CT shift contrary to the current study, where the CT value was not detectable in chickpea protein isolate derived from chickpea flour, which initially contained 1.76 mg CE/100g. Therefore, the differences in TPC and CT may be due to variations in sample varieties used, isolation processes, extraction conditions, and processing conditions (e.g., milling). In view of their anti-nutritional effects, understanding the polyphenol and CT contents in different pea-derived products is pivotal for advancing their utilization in food applications.

The effects of different thermal processes on the TPC of whole yellow field pea samples (expressed on both DM and protein bases) were investigated, with a particular focus on measurements at the previously determined optimal wavelength of 750 nm, and with catechin used as the reference standard, as illustrated in **Tables 4.8** and **4.9**. For the flour type, thermal processing resulted in significant decreases ($p < 0.05$) in the TPC, with the reductions following the order: boiling > autoclaving > baking > micronization. Specifically, TPC decreased by at least 55.11% in the boiled flour, 36.89% in the autoclaved flour, 34.13% in the baked flour, and 23.11% in the micronized flour on both unit bases. Similarly, **Tables 4.10** and **4.11** showed comparable trends in TPC reduction in pea flour samples before and after thermal processing, quantified using gallic acid as the reference standard on both DM and protein bases, with the reductions in TPC of at least

54.11% in the boiled flour, 36.23% in the autoclaved flour, 33.71% in the baked flour, and 22.22% in the micronized flour. Furthermore, baking showed moderate efficacy in reducing TPC, with reductions ranging from 36.09 to 41.27% in the protein concentrate, 35.59 to 37.62% in the 80% protein isolate, 33.71 to 34.67% in the flour, and 22.73 to 22.86% in the 90% protein isolate, regardless of whether catechin or gallic acid equivalents were used. As illustrated in **Figures 4.2 and 4.3**, there were strong correlations (at least $R^2 > 0.9977$) between TPC results expressed in catechin and gallic acid equivalents, which indicated that TPC reported using catechin equivalent is comparable to those reported using gallic acid equivalent. Therefore, these findings suggest that either standard provides consistent and reliable measurements of TPC, facilitating comparability across studies employing different reference standards.

Changes in CT content (expressed as mg CE/g of sample on the DM basis and as mg CE/g protein) in the pea flour samples before and after different processing methods, along with the data for the untreated coarse fraction, are summarized in **Table 4.12**. Among the flour samples, boiling resulted in a complete elimination of CT from yellow field pea seeds. Conversely, other thermal processing methods led to notable increases in CT content, with baking causing a significant rise ($p < 0.05$) of up to 516.67%, while micronization (up to 59.80%) and autoclaving (8.33%) resulted in non-significant increases, on both unit bases. Additionally, significant increments ($p < 0.05$) in CT content were observed after baking in treated derivative samples: up to 223.96% in the 90% isolate and 50.10% in the 80% isolate on both bases, as compared to their respective untreated forms. In contrast, the protein concentrate showed no significant difference ($p > 0.05$), with a maximum rise of 9.09%.

In the current study, PA and TPC had similar reduction patterns in flour samples following the application of thermal treatments, with the order of effectiveness being boiling > autoclaving > baking > micronization. This implied that PA and TPC have comparable sensitivities to heating,

particularly hydrothermal treatments. Similar to the mechanisms underlying PA reduction, the significant reduction in TPC is likely due to the leaching of water-soluble phenolic compounds into the soaking and cooking media, and the activation of polyphenol oxidase that can break down the complex phenolic structures into smaller compounds. Heat-induced denaturation further contributes to the reduction of phenolic compounds (Sánchez-Velázquez et al., 2021; Khattab & Arntfield, 2009; Xu & Chang, 2008). The findings of Xu & Chang (2008) are in agreement with the present study, demonstrating a 45.6% decrease in TPC (measured at 765 nm, and expressed as GAE) in yellow peas after boiling for 90 min. Similar reductions in TPC were observed in other pulses, including green peas (48.4 – 50.8%), chickpeas (33.3 – 37.5%), and lentils (50.1 – 55.8%), regardless of boiling time. Conversely, the study of Sánchez-Velázquez et al. (2021) explored the different effects of extrusion, boiling, and baking on the TPC of various pulses, noting that the impacts varied depending on the type of pulses. For instance, they observed significant decreases in TPC for extruded and boiled green split peas, whereas baking significantly increased TPC in both green and yellow split peas. This is contrary to the phenomenon of decreasing TPC observed in the current study. Our study indicated that autoclaving, baking, and micronization also reduced TPC, but to a lesser extent than boiling, likely due to variations in processing conditions such as temperature and processing time, and the nature of thermal treatments. In particular, baking and micronization, by utilizing dry heating, might preserve more phenolic compounds compared to hydrothermal treatments like boiling and autoclaving. Similarly, the mechanism regarding the effects of leaching and enzymatic degradation helps explain the observed decrease in CT after boiling (Sánchez-Velázquez et al., 2021; Khattab & Arntfield, 2009). Khattab & Arntfield (2009) identified boiling as the most effective method for reducing CT content in Canadian and Egyptian cowpeas, kidney beans, and peas, with reductions ranging from 68.49% to 99.29%.

In contrast to our findings, previous studies have generally reported decreases in CT content following other thermal treatments, similar to boiling. Khattab & Arntfield (2009) observed significant reductions across all tested samples (cowpeas, kidney beans, and peas) subjected to various processing methods, including soaking (35.00 – 86.62%), autoclaving (62.79 – 95.05%), microwaving (42.82 – 95.43%), roasting (8.69% – 95.45%), and micronization (7.29 – 88.63%). Similarly, Bai et al. (2018) investigated the effect of micronization on TPC and CT in desi chickpeas and hull-less barley. Their findings showed significant reductions in TPC under different combinations of conditions (with/without tempering plus infrared heating to 115°C or 135°C), with maximum reductions of approximately 16% in desi chickpeas and 23% in hull-less barley. In terms of CT content, there was a reduction of at least 43.75% in desi chickpeas, though this reduction was not significant, whereas hull-less barley showed a significant reduction of 46.23%. The reductions in CT content observed with autoclaving, baking, and micronization in previous studies are inconsistent with the findings of the current study, which reported increases in CT content in yellow field pea samples under similar treatments. Given the limited research reporting increases in CT content, it is important to relate the mechanisms by which thermal treatments (except for boiling) can enhance phenolic compounds including CT. The observed increase in CT, particularly during baking, could be due to the breakdown of cellular structures, leading to the release of bound phenolic compounds into the surrounding liquid, including CTs, that were previously trapped within the plant matrix. Thermal treatments, with the appropriate combination of temperature and processing time, could enhance the extractability of CT. Additionally, Maillard reactions and thermally induced transformations may lead to the formation of new phenolic structures, including new condensed tannins (Sánchez-Velázquez et al., 2021; Bai et al., 2018; Das et al., 2020; Rehman et al. 2002; Ciuperca et al., 2023). Furthermore, Xu & Chang (2008) and Siah et al. (2014) found that boiling and autoclaving significantly reduced the phenolic

compounds in green peas, chickpeas, lentils, yellow peas, and faba beans. Siah et al. (2014) specifically examined the retention and leaching of phenolic compounds after processing. Their findings indicated that while boiling and autoclaving partially destroyed total phenolic, flavonoid and CT contents, a significant portion of the remaining phenolic and flavonoid compounds was primarily retained in the seeds and cooking broths. Autoclaving caused greater leaching of these compounds into the liquid medium than boiling. However, the effect of autoclaving on CT content was found to vary among different faba bean varieties (Siah et al., 2014). Therefore, the effectiveness of thermal treatments in retaining or reducing specific phenolic compounds depends on the processing conditions, the pulse varieties, and the phenolic compound profiles in pulses.

As shown in **Tables 4.8 – 4.11**, HHP treatment resulted in a significant decrease ($p < 0.05$) in TPC, quantified using both catechin and gallic acid standards on both DM and protein bases, across all yellow field pea samples, except for the 90% protein isolate. Specifically, at the previously determined wavelength of 750 nm using both standards, reductions in TPC ranged from 14.61 to 16.62% in the protein concentrate, 11.30 to 13.17% in the 80% isolate, and 7.67 to 9.78% in the flour. However, TPC reduction in the 90% isolate was not significant, with only a slight decrease of 4.43% to 5.30%. Conversely, HHP treatment significantly increased ($p < 0.05$) CT contents, with dramatic increases of up to 521.57% in the pea flour, followed by up to 149.54% in the 90% isolate, and 50.94% in the 80% isolate. Although the protein concentrate showed a notable increase of up to 109.85% on the protein basis, this change was not statistically significant ($p > 0.05$). The study by Linsberger-Martin et al. (2013) explored the effects of HHP on TPC and found variable outcomes, with either increasing or decreasing depending on the specific HHP conditions. Significant reductions in TPC of up to 28% in peas and 30% in beans were observed under certain combinations of parameters. Notably, TPC tended to be lower when HHP was applied alongside mild heating (60°C), regardless of the pressure level and processing time. Similarly, Lee et al.

(2018) reported TPC reductions of 21 – 33% in red bean powder (RBP) treated with HHP at 400 – 600 MPa for 5 min, though these decreases were not significant. Additionally, the CT content under the same conditions was significantly reduced by 25 – 36%. Deng et al. (2015) also found that HHP has been shown to affect tannin content in buckwheat, with reductions of up to 20% observed under the conditions of 600 MPa, 30 min, and 60°C. Moreover, studies by Alsalman & Ramaswamy (2020) and Belmiro et al. (2020) have also highlighted the capacity of HHP to diminish CT content in common beans and chickpeas. These studies collectively support the argument that HHP can mitigate the levels of polyphenol and CT, potentially enhancing the overall nutritional quality of food products. However, the findings from these studies are inconsistent with the notable increase in CT content observed in the current study. For the specific purposes for which TPC and CT are valued for their bioactive properties, such as antioxidant benefits, HHP treatment could be a preferable method to improve the extractability and preservation of these beneficial compounds as compared to thermal treatments, depending on the specific processing parameters (Lee et al., 2018). The reduction effect of HHP on TPC could be attributed to the disruption of cellular integrity, which facilitates the release of intracellular enzymes such as polyphenol oxidase. This enhances enzymatic interactions and activity, leading to the degradation of phenolic compounds and a subsequent decrease in overall TPC. Interestingly, while HHP generally resulted in a decrease in TPC, an increase in particular phenolic compounds such as CT was observed. It is noteworthy that HHP can also enhance the extractability of phenolic compounds that were previously bound within cellular structures, liberating these specific phenolic compounds and making them more accessible for extraction and measurement (Khan et al., 2018).

In summary, the significant reductions in TPC and CT highlight the substantial impact of thermal treatments, particularly boiling, on these compounds in pea flour. The water-soluble nature of phenolic compounds including CT and their breakdown into smaller molecules, could explain

these phenomena. Interestingly, CT significantly increased following all thermal treatments, except for boiling, where it was totally eliminated in pea flour. HHP also reduced TPC while simultaneously increasing CT concentrations, as observed for the pea samples. Furthermore, TPC and CT, while delivering their anti-nutritional effects, present their antioxidant properties and other health benefits. Beyond addressing the constraints posed by ANFs, the processing methods examined in the current study provide unexpected results, offering valuable insights for researchers and food processors aiming to optimize the extraction and retention of these bioactive compounds.

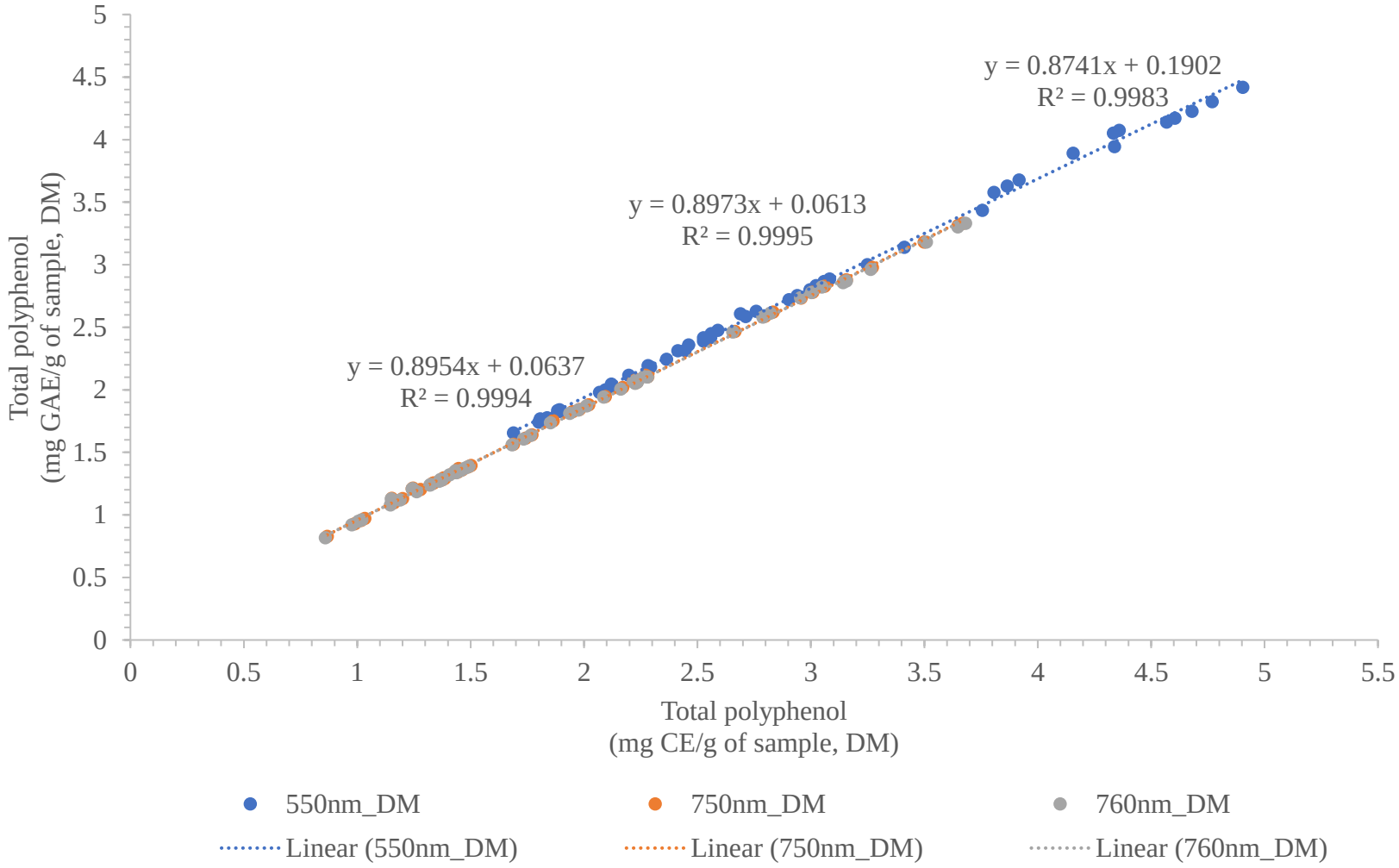

Figure 4.2. *Comparison of* ***total polyphenol content*** *reported as between mg CE/g of sample and mg GAE/g of sample for untreated and treated whole yellow field pea flour, protein concentrate, 80% protein isolate, and 90% protein isolate* ***(DM basis).***

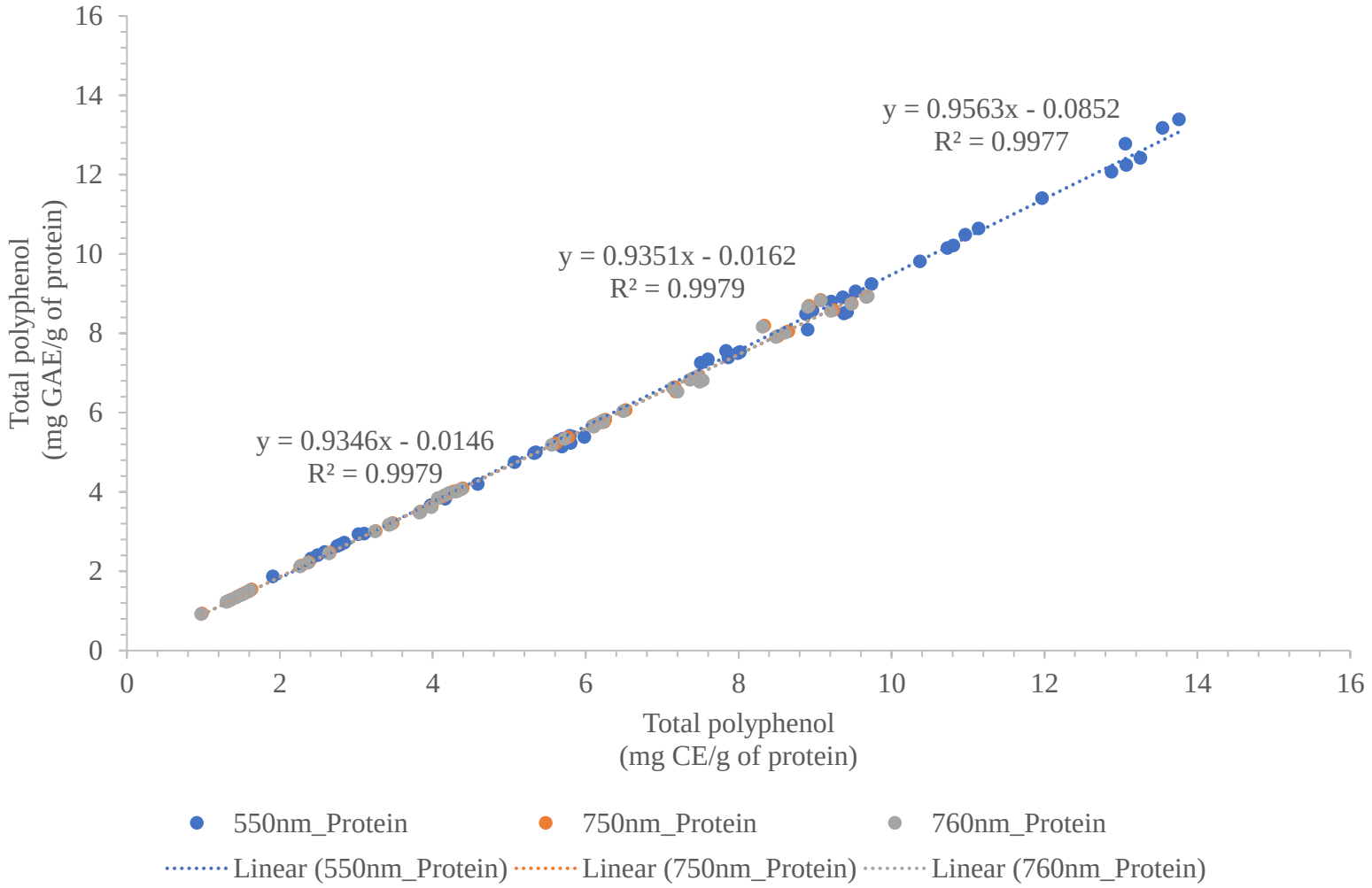

Figure 4.3. *Comparison of* ***total polyphenol content*** *reported as between mg CE/g of protein and mg GAE/g of protein for untreated and treated whole yellow field pea flour, protein concentrate, 80% protein isolate, and 90% protein isolate* ***(protein basis).***

Table 4.8. Effect of different processing methods on the **total polyphenol content (quantified as CE)** of whole yellow field pea flour, protein concentrate, coarse fraction, 80% protein isolate, and 90% protein isolate **(DM basis).**

	550 nm		**750 nm**		**760 nm**	
Sample Description	**Corrected Polyphenol (mg CE/g of sample, DM)[1]**	**Relative Change (%)[2]**	**Corrected Polyphenol (mg CE/g of sample, DM)[1]**	**Relative Change (%)[2]**	**Corrected Polyphenol (mg CE/g of sample, DM)[1]**	**Relative Change (%)[2]**
Whole Pea Flour						
Untreated	3.05^{a}	0.00	2.25^{a}	0.00	2.25^{a}	0.00
Boiled	1.84^{e}	-39.67	1.01^{e}	-55.11	1.00^{e}	-55.56
Autoclaved	2.31^{c}	-24.26	1.42^{d}	-36.89	1.40^{d}	-37.78
Micronized	2.51^{b}	-17.70	1.73^{c}	-23.11	1.73^{c}	-23.11
Baked	2.09^{d}	-31.48	1.47^{d}	-34.67	1.46^{d}	-35.11
HHP-treated	2.62^{b}	-14.10	2.03^{b}	-9.78	2.02^{b}	-10.22
Pooled SE	**0.0026**	/	**0.0019**	/	**0.0019**	/
***P*-value**	**<0.0001**	/	**<0.0001**	/	**<0.0001**	/
Protein Concentrate						
Untreated	4.50^{a}	0.00	3.61^{a}	0.00	3.61^{a}	0.00
Baked	2.95^{c}	-34.44	2.24^{c}	-37.95	2.24^{c}	-37.95
HHP-treated	3.86^{b}	-14.22	3.01^{b}	-16.62	3.00^{b}	-16.90
Coarse fraction	1.86^{d}	-58.67	1.21^{d}	-66.48	1.21^{d}	-66.48
Pooled SE	**0.0071**	/	**0.0037**	/	**0.0035**	/
***P*-value**	**<0.0001**	/	**<0.0001**	/	**<0.0001**	/

Data are expressed as mean of three replicates (two measurements per replicate). Mean values followed by different superscript letters within the column of each yellow field pea type indicate statistically significant differences between treatments ($p < 0.05$) using one-way ANOVA along with Tukey's multiple comparison test.

[1]Values were corrected for a 99% catechin purity.

[2] Untreated sample of each category is considered as a baseline. The relative change of each treated sample was calculated based on the baseline.

Abbreviations: CE = Catechin equivalent; DM = Dry matter; SE = Standard error.

Table 4.8. Continued.

Sample Description	550 nm		750 nm		760 nm	
	Corrected Polyphenol (mg CE/g of sample, DM)[1]	**Relative Change (%)[2]**	**Corrected Polyphenol (mg CE/g of sample, DM)[1]**	**Sample Description**	**Corrected Polyphenol (mg CE/g of sample, DM)[1]**	**Relative Change (%)[2]**
80% Protein Isolate						
Untreated	4.78[a]	0.00	3.19[a]	0.00	3.19[a]	0.00
Baked	3.47[c]	-27.41	1.99[c]	-37.62	1.98[c]	-37.93
HHP-treated	4.28[b]	-10.46	2.77[b]	-13.17	2.75[b]	-13.79
Pooled SE	**0.0306**	**/**	**0.0125**	**/**	**0.0125**	**/**
***P*-value**	**0.0003**	**/**	**<0.0001**	**/**	**<0.0001**	**/**
90% Protein Isolate						
Untreated	2.66[a]	0.00	1.40[a]	0.00	1.39[a]	0.00
Baked	2.00[b]	-24.81	1.08[b]	-22.86	1.06[b]	-23.74
HHP-treated	2.39[ab]	-10.15	1.33[ab]	-5.00	1.32[ab]	-5.04
Pooled SE	**0.0326**	**/**	**0.0131**	**/**	**0.0127**	**/**
***P*-value**	**0.0123**	**/**	**0.0290**	**/**	**0.0286**	**/**

Data are expressed as mean of three replicates (two measurements per replicate). Mean values followed by different superscript letters within the column of each yellow field pea type indicate statistically significant differences between treatments ($p < 0.05$) using one-way ANOVA along with Tukey's multiple comparison test.

[1]Values were corrected for a 99% catechin purity.

[2] Untreated sample of each category is considered as a baseline. The relative change of each treated sample was calculated based on the baseline.

Abbreviations: CE = Catechin equivalent; DM = Dry matter; SE = Standard error.

Table 4.9. Effect of different processing methods on the **total polyphenol content (quantified as CE)** of whole yellow field pea flour, protein concentrate, coarse fraction, 80% protein isolate, and 90% protein isolate **(protein basis).**

	550 nm		**750 nm**		**760 nm**	
Sample Description	**Corrected Polyphenol (mg CE/ g of protein)[1]**	**Relative Change (%)[2]**	**Corrected Polyphenol (mg CE/ g of protein)[1]**	**Relative Change (%)[2]**	**Corrected Polyphenol (mg CE/ g of protein)[1]**	**Relative Change (%)[2]**
Whole Pea Flour						
Untreated	13.07^{a}	0.00	9.61^{a}	0.00	9.61^{a}	0.00
Boiled	7.65^{d}	-41.47	4.20^{e}	-56.30	4.15^{e}	-56.82
Autoclaved	9.54^{c}	-27.01	5.84^{d}	-39.23	5.79^{d}	-39.75
Micronized	10.64^{b}	-18.59	7.35^{c}	-23.52	7.32^{c}	-23.83
Baked	9.02^{c}	-30.99	6.33^{d}	-34.13	6.30^{d}	-34.44
HHP-treated	11.36^{b}	-13.08	8.80^{b}	-8.43	8.77^{b}	-8.74
Pooled SE	**0.0790**	/	**0.0489**	/	**0.0493**	/
***P*-value**	**<0.0001**	/	**<0.0001**	/	**<0.0001**	/
Protein Concentrate						
Untreated	9.23^{b}	0.00	7.39^{b}	0.00	7.41^{b}	0.00
Baked	5.71^{d}	-38.14	4.34^{d}	-41.27	4.34^{d}	-41.43
HHP-treated	7.96^{c}	-13.76	6.20^{c}	-16.10	6.18^{c}	-16.60
Coarse fraction	13.45^{a}	+45.72	8.78^{a}	+18.81	8.76^{a}	+18.22
Pooled SE	**0.0558**	/	**0.0483**	/	**0.0495**	/
***P*-value**	**<0.0001**	/	**<0.0001**	/	**<0.0001**	/

Data are expressed as mean of three replicates (two measurements per replicate). Mean values followed by different superscript letters within the column of each yellow field pea type indicate statistically significant differences between treatments ($p < 0.05$) using one-way ANOVA along with Tukey's multiple comparison test.

[1]Values were corrected for a 99% catechin purity.

[2] Untreated sample of each category is considered as a baseline. The relative change of each treated sample was calculated based on the baseline.

Abbreviations: CE = Catechin equivalent; DM = Dry matter; SE = Standard error.

Table 4.9. Continued.

Sample Description	550 nm		750 nm		760 nm	
	Corrected Polyphenol (mg CE/ g of protein)[1]	**Relative Change (%)[2]**	**Corrected Polyphenol (mg CE/ g of protein)[1]**	**Relative Change (%)[2]**	**Corrected Polyphenol (mg CE/ g of protein)[1]**	**Relative Change (%)[2]**
80% Protein Isolate						
Untreated	5.82[a]	0.00	3.89[a]	0.00	3.88[a]	0.00
Baked	4.24[c]	-27.15	2.44[c]	-37.28	2.42[c]	-37.63
HHP-treated	5.25[b]	-9.79	3.39[b]	-12.85	3.38[b]	-12.89
Pooled SE	**0.0485**	**/**	**0.0200**	**/**	**0.0200**	**/**
***P*-value**	**0.0003**	**/**	**<0.0001**	**/**	**<0.0001**	**/**
90% Protein Isolate						
Untreated	2.99[a]	0.00	1.58[a]	0.00	1.56[a]	0.00
Baked	2.27[b]	-24.08	1.22[b]	-22.78	1.21[b]	-22.44
HHP-treated	2.71[ab]	-9.36	1.51[ab]	-4.43	1.49[ab]	-4.49
Pooled SE	**0.0433**	**/**	**0.0172**	**/**	**0.0168**	**/**
***P*-value**	**0.0151**	**/**	**0.0337**	**/**	**0.0333**	**/**

Data are expressed as mean of three replicates (two measurements per replicate). Mean values followed by different superscript letters within the column of each yellow field pea type indicate statistically significant differences between treatments ($p < 0.05$) using one-way ANOVA along with Tukey's multiple comparison test.

[1]Values were corrected for a 99% catechin purity.

[2] Untreated sample of each category is considered as a baseline. The relative change of each treated sample was calculated based on the baseline.

Abbreviations: CE = Catechin equivalent; DM = Dry matter; SE = Standard error.

Table 4.10. Effect of different processing methods on the **total polyphenol content (quantified as GAE)** of whole yellow field pea flour, coarse fraction, protein concentrate, 80% protein isolate, and 90% protein isolate **(DM basis).**

	550 nm		**750 nm**		**760 nm**	
Sample Description	**Corrected Polyphenol (mg GAE/ g of sample, DM)[1]**	**Relative Change (%)[2]**	**Corrected Polyphenol (mg GAE/ g of sample, DM)[1]**	**Relative Change (%)[2]**	**Corrected Polyphenol (mg GAE/ g of sample, DM)[1]**	**Relative Change (%)[2]**
Whole Pea Flour						
Untreated	2.86[a]	0.00	2.07[a]	0.00	2.07[a]	0.00
Boiled	1.78[f]	-37.76	0.95[e]	-54.11	0.94[e]	-54.59
Autoclaved	2.20[d]	-23.08	1.32[d]	-36.23	1.31[d]	-36.71
Micronized	2.37[c]	-17.13	1.61[c]	-22.22	1.60[c]	-22.71
Baked	2.00[e]	-30.07	1.37[d]	-33.82	1.36[d]	-34.30
HHP-treated	2.50[b]	-12.59	1.89[b]	-8.70	1.88[b]	-9.18
Pooled SE	**0.0021**	**/**	**0.0015**	**/**	**0.0016**	**/**
***P*-value**	**<0.0001**	**/**	**0.0001**	**/**	**<0.0001**	**/**
Protein Concentrate						
Untreated	4.09[a]	0.00	3.27[a]	0.00	3.27[a]	0.00
Baked	2.76[c]	-32.52	2.09[c]	-36.09	2.08[c]	-36.39
HHP-treated	3.63[b]	-11.25	2.78[b]	-14.98	2.78[b]	-14.98
Coarse fraction	1.81[d]	-55.75	1.19[d]	-63.61	1.18[d]	-63.91
Pooled SE	**0.0052**	**/**	**0.0029**	**/**	**0.0027**	**/**
***P*-value**	**<0.0001**	**/**	**<0.0001**	**/**	**<0.0001**	**/**

Data are expressed as mean of three replicates (two measurements per replicate). Mean values followed by different superscript letters within the column of each yellow field pea type indicate statistically significant differences between treatments ($p < 0.05$) using one-way ANOVA along with Tukey's multiple comparison test.

[1]Values were corrected for a 95.3% gallic acid purity.

[2] Untreated sample of each category is considered as a baseline. The relative change of each treated sample was calculated based on the baseline.

Abbreviations: GAE = Gallic acid equivalent; DM = Dry matter; SE = Standard error.

Table 4.10. Continued.

Sample Description	550 nm		750 nm		760 nm	
	Corrected Polyphenol (mg GAE/ g of sample, DM)[1]	**Relative Change (%)[2]**	**Corrected Polyphenol (mg GAE/ g of sample, DM)[1]**	**Relative Change (%)[2]**	**Corrected Polyphenol (mg GAE/ g of sample, DM)[1]**	**Relative Change (%)[2]**
80% Protein Isolate						
Untreated	4.32[a]	0.00	2.91[a]	0.00	2.90[a]	0.00
Baked	3.19[b]	-26.16	1.87[c]	-35.74	1.85[c]	-36.21
HHP-treated	4.01[a]	-7.18	2.56[b]	-12.03	2.55[b]	-12.07
Pooled SE	**0.0228**	/	**0.0097**	/	**0.0097**	/
***P*-value**	**0.0003**	/	**<0.0001**	/	**<0.0001**	/
90% Protein Isolate						
Untreated	2.55[a]	0.00	1.32[a]	0.00	1.31[a]	0.00
Baked	1.94[b]	-23.92	1.02[b]	-22.73	1.01[b]	-22.90
HHP-treated	2.29[ab]	-10.20	1.25[ab]	-5.30	1.24[ab]	-5.34
Pooled SE	**0.0274**	/	**0.0110**	/	**0.0108**	/
***P*-value**	**0.0115**	/	**0.0285**	/	**0.0282**	/

Data are expressed as mean of three replicates (two measurements per replicate). Mean values followed by different superscript letters within the column of each yellow field pea type indicate statistically significant differences between treatments ($p < 0.05$) using one-way ANOVA along with Tukey's multiple comparison test.

[1]Values were corrected for a 95.3% gallic acid purity.

[2] Untreated sample of each category is considered as a baseline. The relative change of each treated sample was calculated based on the baseline.

Abbreviations: GAE = Gallic acid equivalent; DM = Dry matter; SE = Standard error.

Table 4.11. Effect of different processing methods on the **total polyphenol content (quantified as GAE)** of whole yellow field pea flour, protein concentrate, coarse fraction, 80% protein isolate, and 90% protein isolate **(protein basis).**

Sample Description	550 nm		750 nm		760 nm	
	Corrected Polyphenol (mg GAE/ g of protein)[1]	**Relative Change (%)[2]**	**Corrected Polyphenol (mg GAE/ g of protein)[1]**	**Relative Change (%)[2]**	**Corrected Polyphenol (mg GAE/ g of protein)[1]**	**Relative Change (%)[2]**
Whole Pea Flour						
Untreated	12.24[a]	0.00	8.87[a]	0.00	8.86[a]	0.00
Boiled	7.38[e]	-39.71	3.95[e]	-55.47	3.91[e]	-55.87
Autoclaved	9.07[d]	-25.90	5.44[d]	-38.67	5.40[d]	-39.05
Micronized	10.06[c]	-17.81	6.81[c]	-23.22	6.79[c]	-23.36
Baked	8.62[d]	-29.58	5.88[d]	-33.71	5.86[d]	-33.86
HHP-treated	10.84[b]	-11.44	8.19[b]	-7.67	8.16[b]	-7.90
Pooled SE	**0.0661**	**/**	**0.0409**	**/**	**0.0412**	**/**
***P*-value**	**<0.0001**	**/**	**<0.0001**	**/**	**<0.0001**	**/**
Protein Concentrate						
Untreated	8.38[b]	0.00	6.71[b]	0.00	6.71[b]	0.00
Baked	5.35[d]	-36.16	4.05[d]	-39.64	4.04[d]	-39.79
HHP-treated	7.47[c]	-10.86	5.73[c]	-14.61	5.72[c]	-14.75
Coarse fraction	13.11[a]	+56.44	8.58[a]	+27.87	8.55[a]	+27.42
Pooled SE	**0.0415**	**/**	**0.0369**	**/**	**0.0376**	**/**
***P*-value**	**<0.0001**	**/**	**<0.0001**	**/**	**<0.0001**	**/**

Data are expressed as mean of three replicates (two measurements per replicate). Mean values followed by different superscript letters within the column of each yellow field pea type indicate statistically significant differences between treatments ($p < 0.05$) using one-way ANOVA along with Tukey's multiple comparison test.

[1]Values were corrected for a 95.3% gallic acid purity.

[2] Untreated sample of each category is considered as a baseline. The relative change of each treated sample was calculated based on the baseline.

Abbreviations: GAE = Gallic acid equivalent; DM = Dry matter; SE = Standard error.

Table 4.11. Continued.

Sample Description	550 nm		750 nm		760 nm	
	Corrected Polyphenol (mg GAE/ g of protein)[1]	**Relative Change (%)[2]**	**Corrected Polyphenol (mg GAE/ g of protein)[1]**	**Relative Change (%)[2]**	**Corrected Polyphenol (mg GAE/ g of protein)[1]**	**Relative Change (%)[2]**
80% Protein Isolate						
Untreated	5.25^{a}	0.00	3.54^{a}	0.00	3.53^{a}	0.00
Baked	3.90^{b}	-25.71	2.28^{c}	-35.59	2.26^{c}	-35.98
HHP-treated	4.91^{a}	-6.48	3.14^{b}	-11.30	3.13^{b}	-11.33
Pooled SE	**0.0363**	/	**0.0156**	/	**0.0155**	/
***P*-value**	**0.0003**	/	**<0.0001**	/	**<0.0001**	/
90% Protein Isolate						
Untreated	2.87^{a}	0.00	1.49^{a}	0.00	1.47^{a}	0.00
Baked	2.20^{b}	-23.34	1.15^{b}	-22.82	1.14^{b}	-22.45
HHP-treated	2.60^{ab}	-9.41	1.42^{ab}	-4.70	1.40^{ab}	-4.76
Pooled SE	**0.0364**	/	**0.0146**	/	**0.0143**	/
***P*-value**	**0.0143**	/	**0.0334**	/	**0.0330**	/

Data are expressed as mean of three replicates (two measurements per replicate). Mean values followed by different superscript letters within the column of each yellow field pea type indicate statistically significant differences between treatments ($p < 0.05$) using one-way ANOVA along with Tukey's multiple comparison test.

[1]Values were corrected for a 95.3% gallic acid purity.

[2] The untreated sample of each category is considered as a baseline. The relative change of each treated sample was calculated based on the baseline.

Abbreviations: GAE = Gallic acid equivalent; DM = Dry matter; SE = Standard error.

Table 4.12. Effect of different processing methods on the **condensed tannin (CT)** content of whole yellow field pea flour, protein concentrate, coarse fraction, 80% protein isolate, and 90% protein isolate.

Sample Description	DM Basis		Protein Basis	
	Corrected CT (mg CE/ g of sample, DM)[1]	**Relative Change (%)[2]**	**Corrected CT (mg CE/ g of protein)[1]**	**Relative Change (%)[2]**
Whole Pea Flour				
Untreated	0.024[b]	0.00	0.102[b]	0.00
Boiled	N/A	N/A	N/A	N/A
Autoclaved	0.026[b]	+8.33	0.108[b]	+3.92
Micronized	0.037[b]	+58.33	0.155[b]	+59.80
Baked	0.146[a]	+508.33	0.629[a]	+516.67
HHP-treated	0.147[a]	+512.50	0.634[a]	+521.57
Pooled SE	**0.0003**	**/**	**0.0051**	**/**
***P*-value**	**<0.0001**	**/**	**<0.0001**	**/**
Protein Concentrate				
Untreated	0.099[b]	0.00	0.203[b]	0.00
Baked	0.108[b]	+9.09	0.210[b]	+3.45
HHP-treated	0.207[a]	+109.09	0.426[ab]	+109.85
Coarse fraction	0.073[b]	-26.26	0.528[a]	+160.10
Pooled SE	**0.0008**	**/**	**0.0097**	**/**
***P*-value**	**0.0023**	**/**	**0.0083**	**/**

Data are expressed as mean of three replicates (six measurements per replicate) and presented on the DM and protein bases. Mean values followed by different superscript letters within the column of each yellow field pea type indicate statistically significant differences between treatments ($p < 0.05$) using one-way ANOVA along with Tukey's multiple comparison test.

[1]Values were corrected for a 99% catechin purity.

[2] Untreated sample of each category is considered as a baseline. The relative change of each treated sample was calculated based on the baseline.

Abbreviations: CT = Condensed tannin; DM = Dry matter; CE = Catechin equivalent; SE = Standard error; N/A = Not applicable.

Table 4.12. Continued.

Sample Description	DM Basis		Protein Basis	
	Corrected CT (mg CE/ g of sample, DM)[1]	**Relative Change (%)[2]**	**Corrected CT (mg CE/ g of protein)[1]**	**Relative Change (%)[2]**
80% Protein Isolate				
Untreated	0.394[b]	0.00	0.479[b]	0.00
Baked	0.588[a]	+49.24	0.719[a]	+50.10
HHP-treated	0.590[a]	+49.75	0.723[a]	+50.94
Pooled SE	**0.0007**	/	**0.0011**	/
***P*-value**	**0.0002**	/	**0.0002**	/
90% Protein Isolate				
Untreated	0.096[c]	0.00	0.109[c]	0.00
Baked	0.311[a]	+223.96	0.353[a]	+223.85
HHP-treated	0.239[b]	+148.96	0.272[b]	+149.54
Pooled SE	**0.0004**	/	**0.0005**	/
***P*-value**	**<0.0001**	/	**<0.0001**	/

Data are expressed as mean of three replicates (six measurements per replicate) and presented on the DM and protein bases. Mean values followed by different superscript letters within the column of each yellow field pea type indicate statistically significant differences between treatments ($p < 0.05$) using one-way ANOVA along with Tukey's multiple comparison test.

[1]Values were corrected for a 99% catechin purity.

[2] Untreated sample of each category is considered as a baseline. The relative change of each treated sample was calculated based on the baseline.

Abbreviations: CT = Condensed tannin; DM = Dry matter; CE = Catechin equivalent; SE = Standard error; N/A = Not applicable.

4.5 CONCLUSION OF STUDY 1

This study demonstrated the effects of both thermal and non-thermal processing methods on key ANFs in yellow field pea flour, protein concentrate, 80% protein isolate, and 90% protein isolate. The thermal treatments proved highly effective in reducing TIA, lectins, PA, and TPC across all the pea samples, largely due to heat-induced denaturation of these compounds, though the treatments increased the amount of CT, with the exception of boiling, which totally eliminated CT. Specifically, boiling consistently achieved the greatest reductions in all ANFs of flour. Baking was also particularly effective in the reduction of ANFs for both flour and protein derivatives. On the other hand, HHP had a more modest effect on reduction in TIA, PA, and TPC in most samples, but unexpectedly increased lectin and CT contents, particularly in the flour and 90% protein isolate. The coarse fraction demonstrated comparable concentrations of ANFs to those of the protein concentrate, but polyphenolic and CT contents were higher in the coarse fraction when expressed on a protein basis. These findings highlight the significance of processing methods in overcoming the constraints of ANFs to enhance the utilization of pea proteins and promote the adoption of sustainable plant-based protein ingredients for researchers and food processors. A limitation of this study is the lack of specificity in detecting certain reactive forms of lectins and PA that demonstrate anti-nutritional effects. Future research should explore alternative non-thermal processing conditions and their synergistic effects in further reducing the ANFs without compromising the nutritional value of yellow field pea products or other pulse products. Additionally, more precise detection methods should be developed to target and evaluate different forms of ANFs.

CHAPTER 5: IMPACT OF DIFFERENT PROCESSING METHODS ON IN VITRO PROTEIN QUALITY AND REACTIVE LYSINE OF YELLOW FIELD PEAS AND THEIR DERIVATIVES (STUDY 2)

5.1 ABSTRACT

Background: Yellow field peas (*Pisum sativum* L.) are an affordable, nutritious, and sustainable source of plant-based proteins, though the limited amino acid profile and lower protein digestibility hinder their nutritional performance, resulting in a lower protein quality when compared to animal-based proteins. Advances in processing methods offer the potential to enhance the amino acid composition, protein digestibility and overall protein quality of yellow field peas and their derivatives. However, lysine, an indispensable amino acid (IAA), is particularly vulnerable to damage from chemical modifications during food processing, further negatively affecting the nutritional value of processed foods.

Objectives: This study investigated the effect of thermal (boiling, autoclaving, micronization, and baking) and non-thermal (high-hydrostatic pressure, HHP) treatments on yellow field pea flour. The impact of baking and HHP treatments on pea-derived products (protein concentrate, 80% protein isolate, and 90% protein isolate) was also examined. A starch-rich byproduct (coarse fraction) separated through air classification along with the protein-enriched fine fraction (protein concentrate) was also evaluated. The study of interest focused on amino acid profiles, *in vitro* protein digestibility, and *in vitro* protein quality, which was assessed using *an in vitro* Protein Digestibility Corrected Amino Acid Score (*in vitro* PDCAAS), and reactive lysine content (measured by an OPA fluorometric assay).

Results: Lysine (LYS) and leucine (LEU), two indispensable amino acids (IAAs), were present in notably high concentrations in the pea samples. Boiling significantly improved IAAs in the flour, while HHP reduced IAAs across all samples on a DM basis. Tryptophan (TRP) with amino acid scores (AAS) of 0.91 – 0.97, was the most limiting amino acid in the untreated and treated flour and protein concentrate samples, as well as the air-classified coarse fraction. In the baked protein concentrate, and untreated and treated protein isolates, sulfur-containing amino acids (AAS of 0.84

– 0.91) were the first limiting amino acids. The *in vitro* protein digestibility (IVPD), as determined by the true fecal protein digestibility (TPD1) method, showed higher protein digestibility values in both protein isolate samples (95.77% – 97.33%) when compared to the coarse fraction, flour, and protein concentrate samples (88.90% – 94.60%). A significant improvement in *in vitro* PDCAAS was observed only for the boiled flour, with a value of 91.57%. However, all other processing methods showed unchanged or reduced patterns for the pea samples. Furthermore, reactive lysine content showed comparability to total lysine in untreated flour and protein concentrate but showed significant reductions after boiling and baking. Boiling, in particular, resulted in a reduction of over 70% in reactive lysine content in the flour. In contrast, non-thermal HHP treatment preserved more reactive lysine, with minimal reductions of only up to 4.73% observed in both flour and 90% protein isolate.

Conclusions: Boiling significantly enhanced the *in vitro* PDCAAS of yellow field pea flour; however, the *in vitro* PDCAAS was less likely to be affected by other processing treatments. Reactive lysine content was preserved more effectively under non-thermal HHP treatment.

Significance and Novelty: This study demonstrates optimizing processing methods has the potential to improve the nutritional quality of yellow field pea samples and preserve reactive lysine, as well as the need for accurate reactive lysine assays. The outcomes provide insights for researchers and food processors to select appropriate approaches for developing more nutritious, and sustainable plant-based products.

5.2 INTRODUCTION

Dietary proteins are essential macronutrients that provide the indispensable amino acids necessary for maintaining human health and well-being. Increasing dietary protein intake may be critical for achieving optimal health benefits, particularly in populations such as older adults (FAO/WHO, 2013, Hertzler et al., 2020). Traditionally, animal-based proteins derived from meat, dairy, and eggs, serve as a primary source of high-quality protein with a complete amino acid profile and high protein digestibility (Nosworthy & House, 2017; Sá & House, 2024; Herreman et al., 2020). There is a growing global interest in plant-based proteins as a sustainable surrogate for animal-based proteins in response to increasing demand for economically viable, environmentally friendly, and health-conscious dietary options (Government of Canada, 2024; Bessada et al., 2019). As a study by Berrazaga et al. (2020) mentioned, plant-based protein sources contribute approximately 60% of the total global dietary protein intake, with the remaining 40% coming from animal-based protein sources.

Legumes, including soybeans, peas, chickpeas, and lentils, are major sources of plant-based proteins, offering higher protein content compared to other plant sources like cereals and tubers (Nosworthy et al., 2023; Lumsden et al., 2024). Yellow peas, in particular, are widely cultivated for their protein content (23 - 25% on a dry matter basis on average) along with relatively balanced indispensable amino acids (IAAs). They also provide valuable nutrients such as carbohydrates, total dietary fibers, and essential micronutrients such as B-complex vitamins, potassium, and phosphorus (Bessada et al., 2019; Stone et al., 2019; Frias et al., 2011; Nosworthy et al., 2017a; Nosworthy et al., 2017c; Rogers et al., 2024; Shanthakumar et al., 2022). Yellow pea proteins are commercially processed into a variety of ingredient forms, including yellow pea flour, protein concentrates, and protein isolates, to enhance their functionality and application in food products (Zhang et al., 2024; Bessada et al., 2019).

However, the utilization of plant-based protein sources including yellow peas in food systems is limited. They possess an incomplete amino acid profile, particularly low in sulfur-containing amino acids, and lower protein digestibility, resulting in lower protein quality when compared to animal-based protein sources (Nosworthy et al., 2017a). Traditional thermal processing methods, such as boiling, microwave heating, and extrusion, can improve the nutritional bioavailability and quality of pulses by altering their physical and chemical structures (Sánchez-Velázquez et al., 2021; Khattab et al., 2009; Ma et al., 2017). Emerging non-thermal techniques, such as high-hydrostatic pressure (HHP), offer the ability to preserve thermosensitive nutrients while maintaining sensory characteristics and nutritional quality of foods without the need for heat application (Gharibzahedi & Smith, 2021; Lee et al., 2018).

In the evaluation of protein quality, a widely used method is the Protein Digestibility Corrected Amino Acid Score (PDCAAS) method, incorporating two essential elements: amino acid profile and protein digestibility, established as a standard approach in the United States and has recently been accepted in Canada (Wiggins et al., 2019; Health Canada, 2023). Assessment of protein digestibility via *in vitro* methods has the advantages of time/cost-effectiveness and no ethical concerns, indicating this option is a valuable alternative to traditional *in vivo* methods (Mansilla et al., 2020).

Lysine, an indispensable amino acid, is particularly susceptible to chemical alteration by various food processing methods, with the extent of impact varying depending on processing conditions (Rutherfurd, 2015; Rutherfurd & Moughan, 1997; Van Lieshout et al., 2019; Moughan & Rutherfurd, 2008). During processing treatments such as heating or storage, the ε-amino group of lysine can readily interact with other compounds (especially reducing sugars), leading to the formation of unreactive, nutritionally unavailable lysine, and reducing the nutritional value of the products (Rutherfurd, 2015). Reactive lysine, also referred to as nutritionally available lysine,

represents lysine with free or unmodified ε-amino groups, and serves as a critical indicator for evaluating the true nutritional value of processed proteins (Rutherfurd, 2015; Barba et al., 2013). Conventional analytical methods may inaccurately measure the true nutritionally available lysine content in processed foods by including lysine that has reverted from early Maillard products during acid hydrolysis. As this reverted lysine is no longer bioavailable, these methods tend to overestimate the nutritionally available lysine, leading to an inflated assessment of its true nutritional value (Rutherfurd, 2015; Rutherfurd & Moughan, 2007).

Therefore, this study aims to investigate the effect of both thermal (i.e., boiling, autoclaving, micronization, and baking) and non-thermal (HHP) treatments on yellow field peas, as well as the effect of baking and HHP on yellow pea protein concentrate, and both the 80% and 90% protein isolates, with regard to protein quality via *in vitro* PDCAAS method and reactive lysine analysis. Results will be expressed on a dry matter (DM) and protein bases, except for protein digestibility and quality measurements. The coarse fraction (starch-rich byproduct) obtained through air classification of whole yellow field pea flour, alongside fine fraction (protein concentrate), will be also included in this study.

5.3 MATERIALS AND METHODS

5.3.1 Chemicals

All the chemicals used in Study 2 were purchased from the suppliers given in **Table 5.1**. The common reagents, including NaOH, HCl, methanol and 2-octanol were sourced from Fisher Scientific (Ottawa, ON, Canada). RO water was utilized for the IVPD assay, while Milli-Q (MQ) water was employed throughout all other analyses in this study.

Table 5.1. Information of chemicals used for all analyses in Study 2.

Analysis	Chemical	Supply	Catalog NO.
Amino acid hydrolysis	Commercial organic pea protein isolate 870 (P870)	Puris	/
	Soy flour NIST® Standard Reference Material 3234	NIST	3234
	Bovine serum albumin (BSA)	Sigma-Aldrich	A7030
	High nitrogen casein (80 mesh)	Dyets Inc.	400601
	Phenol	Sigma-Aldrich	P5566
	L-Norvaline	Sigma-Aldrich	N7627
	Waters Corp AccQ-Tag Ultra Derivatization Kit	Fisher Scientific	50-782-039
	Waters Corp AccQ-Tag Ultra Eluent A	Fisher Scientific	50-782-041
	Formic acid, 88%	Fisher chemical	A118P-4
	Acetonitrile	Fisher chemical	A996-4
	Hydrogen peroxide	Fisher Scientific	H325-500
	Sodium metabisulfite	Fisher chemical	S244-500
	Barium hydroxide octahydrate	Thermo Scientific	203140010
	Orthophosphoric acid, 85%	Fisher chemical	A242-1
	Glacial acetic acid	Sigma-Aldrich	695092
	1,1,1-trichloro-2-methyl-2-propanol hemihydrate	Sigma-Aldrich	112054
	Ethanolamine	Sigma-Aldrich	398136
	L-tryptophan	MP Biomedicals	103151

Table 5.1. Continued.

Analysis	Chemical	Supply	Catalog NO.
IVPD	Trypsin from porcine pancreas (Type IX-S, lyophilized powder, 13,000–20,000 BAEE units/mg protein)	Sigma-Aldrich	T0303
	α-Chymotrypsin from bovine pancreas (Type II, lyophilized powder, ≥40 units/mg protein)	Sigma-Aldrich	C4129
	Protease from Streptomyces griseus (Type XIV, ≥3.5 units/mg solids)	Sigma-Aldrich	P5147
Reactive lysine (OPA assay)	High nitrogen casein (80 mesh)	Dytes, Inc.	400600
	Sodium tetraborate	Sigma-Aldrich	221732
	Trichloroacetic acid	Sigma-Aldrich	T6399
	Sodium dodecyl sulfate	Sigma-Aldrich	L3771
	Phthaldialdehyde	Sigma-Aldrich	P1378
	β-mercaptoethanol	Sigma-Aldrich	M3148

5.3.2 Sample Procurement and Preparation

The untreated, boiled, autoclaved, micronized, baked and HHP-treated yellow field pea flours, as well as the untreated, baked, and HHP-treated protein concentrates, 80% protein isolates, and 90% protein isolates prepared and used in Study 1, were employed for Study 2. Additionally, the coarse fraction collected through air classification of the whole yellow field pea flour was also included in the analyses.

5.3.3 Processing Treatments and Post-Processing Procedures

As outlined in Study 1, all unprocessed and processed samples were subjected to a two-stage milling process, with final particle sizes standardized by passing through a 200 µm mesh. To achieve representative and uniform sampling for subsequent analyses, most of the samples were homogenized and sub-sampled using the CQM, except for the HHP-treated samples, which were excluded from this homogenization process due to their inadequate sample weight. All prepared samples were then stored in a freezer at -20°C until analysis.

5.3.4 In Vitro Protein Quality

5.3.4.1 Amino acid composition

The amino acid composition analyses were carried out in singlet for each sample. Three standardized methodologies were used to ensure that the amino acid profiles were accurately determined. HIS and sulfur-containing amino acids (including CYS and MET) were quantified through the performic acid oxidized amino acid hydrolysis method following the Association of Official Analytical Chemists (AOAC) official method 985.28. The remaining amino acids, with the exception of TRP, were determined by the regular amino acid hydrolysis method outlined in

AOAC official method 982.30 (AOAC, 1995). TRP was analyzed using the alkaline hydrolysis method as specified by the International Organizations for Standardization 13904:2005 (E) (ISO, 2016).

For the three amino acid hydrolysis methods, both flour samples and standards were weighed according to the specifications outlined in **Table 5.2**, with the sample weights determined based on their protein content. Additionally, reference external standards with known weights and amino acid profiles, including soy flour (NIST), high nitrogen casein, pea protein isolate (P870), and/or bovine serum albumin (BSA), were applied alongside the unknown experimental samples under the same conditions. One external standard was included for every ten samples. For the regular and performic acid oxidized amino acid hydrolyses, corn starch was added in quantities equivalent to those of high protein samples (>35% protein) and certain reference standards (i.e., casein, P870, and BSA). In contrast, no corn starch was added for the alkaline hydrolysis.

Table 5.2. Standard and sample weight requirements for amino acid hydrolysis analyses.

No.	Standard and sample description	Starch addition (Yes/No)	Weight range (g)
1	NIST standard	No	0.0200-0.0300
2	P870 standard	Yes	0.0150-0.0200
3	BSA standard	Yes	0.0100-0.0150
4	Casein standard	Yes	0.0150-0.0200
5	Samples with <35% protein	No	0.0450-0.0550
6	Samples with 35-65% protein	Yes	0.0200-0.0350
7	Samples with 65-90% protein	Yes	0.0150-0.0200
8	Samples with 90-100% protein	Yes	0.0100-0.0150
9	Soybean samples	No	0.0200-0.0300

*No corn starch is added for alkaline hydrolysis.

5.3.4.1.1 Regular amino acid hydrolysis

Samples and external standards were weighed into 11 mL Pyrex glass tubes in accordance with the guidelines outlined in **Table 5.2**. Approximately 20 µL of 2-octanol was added to each tube with a Teflon-lined screw neck to minimize bumping during the hydrolysis process. The weighed samples in the tubes were then combined with 4 mL of 0.1% phenol, and 1.25 mM norvaline in 6 N HCl solution (dissolve 0.14644 g of L-norvaline and 1 g of phenol in 1 L 6 N HCl), which served as an internal standard. To prevent oxidation, the hydrolysis tubes were then purged with nitrogen gas for approximately eight seconds before being snugly capped. The sealed hydrolysis tubes were placed in copper sleeves within a metal rack. The rack was then positioned in a suitable container and covered with a steel sheet. This is intended to minimize the risk of breakage and to prevent any external contamination. The entire setup was incubated in a forced-air oven at 110°C ± 2°C for 24 hr. Once the tubes had cooled to room temperature, the resulting hydrolysates were neutralized by adding 4 mL of 25% NaOH and then poured into 50 mL beakers to further adjust the digest to between pH 5.5 and 6.0. Subsequently, the suspensions were transferred to 50 mL volumetric flasks and brought to the volume with MQ water. Approximately 1 mL of each hydrolysate was filtered into a labelled cryovial through a syringe equipped with a 13 mm 0.22 µm Nylon Luer lock filter and was then frozen at -20°C.

Before pre-column derivatization with the Waters AccQ·Tag Ultra Derivatization Kit, it is important to ensure the filtrates are thawed to room temperature and that a derivatization block is pre-heated to 55°C. To prepare the standard stock, 80 µL of Waters amino acid standard and 80 µL of 2.5 mM norvaline were mixed and brought to a final volume of 1 mL with MQ water to achieve a concentration of 200 µM. This stock standard is then used to prepare a series of dilutions at concentrations of 10, 50, 100, and 200 µM to establish a calibration curve for quantitative analysis. In the derivatization step, 70 µL of AccQ-tag borate buffer, 10 µL of a series of

calibration standards/external standards/filtrated samples, and lastly 20 µL of AccQ-Tag reagent were added into 300 µL limited volume polypropylene vials and vortexed for eight seconds. All the vials were placed on the derivatization block and incubated at 55℃ for 10 min. Once the derivatization process was completed, each vial was inspected to ensure that there were no air bubbles present. The vials were then either stored in a dark environment overnight or placed directly in the autosampler rack of the UPLC system for analysis.

The subsequent chromatographic analysis was conducted in conjunction with mobile phase A (diluting AccQ-Tag Ultra Eluent A reagent 20 times) and mobile phase B (2% formic acid in acetonitrile). Both mobile phases were filtered through a 0.22 µm filter prior to their connection to a Shimadzu Nexera UPLC system (Shimadzu Corporation, Kyoto, Japan) equipped with a Waters AccQ C18 column (100 mm × 2.1 mm, 1.7 µm particle size) and a Shimadzu Nexera SIL-30AC autosampler. Approximately 1 µL of the prepared samples was injected into the UPLC system and analyzed under the specific conditions: a flow rate of 0.7 mL/min, a column oven temperature maintained at 51℃, UV detection at 260 nm, and an overall run time of 17 min. The Lab Solutions software (Shimadzu, Kyoto, Japan) was utilized to process the data from the UPLC runs. The amino acid profiles were calculated based on the hydrated form of amino acids.

5.3.4.1.2 Performic acid oxidized amino acid hydrolysis

For the quantification of sulfur-containing amino acids (MET and CYS), as well as HIS, all experimental samples and external standards underwent an initial oxidation step with performic acid prior to acid hydrolysis. To maintain optimal reactivity, the performic acid solution was freshly prepared by mixing 0.1% phenolic in 88% formic acid with 30% hydrogen peroxide at a volume ratio of 9:1. After mixing, this solution was allowed to react for at least one hr and stored in a refrigerator. Approximately 20 µL of 2-octanol and 2 mL of the prepared performic acid

solution were pipetted into 8-inch glass tubes containing pre-weighed protein samples and external standards according to the aforementioned criteria in **Table 5.2**. The glass tubes were then sealed with glass stoppers and refrigerated overnight at 4°C to allow the oxidation process to take place. During this incubation period, MET and CYS were oxidized to methionine sulfone and cysteic acid, respectively, ensuring their stability throughout the subsequent hydrolysis process. The next morning, each tube received 0.35 g of sodium metabisulfite to halt the oxidation reaction. The tubes were then gently swirled to ensure thorough mixing, with this process repeated at intervals of 5 min, 30 min, and 2 hr. After swirling again, the mixture in each tube was hydrolyzed with 2 mL of 2.5 mM norvaline in concentrated HCl on a digestion block at 110°C ± 2°C for 18 hr. Following the hydrolysis process, samples were allowed to cool down for a few minutes and then were treated with 7.6 mL of 25% NaOH. The subsequent steps aligned with the post-hydrolysis procedures specified in the regular amino acid method (**Section 5.3.4.1.1**), including pH adjustment, filtration, derivatization and further analysis using UPLC. However, a key difference between the regular and oxidized amino acid hydrolysis methods is that, for the oxidized assay, the standard stock was prepared by diluting 80 µL of the oxidized amino acid standard to a final volume of 1 mL. The UPLC settings for the oxidized assay were as follows: a flow rate of 0.4 mL/min, column oven temperatures of 60°C for MET and 40°C for both CYS and HIS, fluorescence detection with excitation at 266 nm and emission at 473 nm, and a total run time of 30 min.

5.3.4.1.3 Alkaline hydrolysis

Protein samples and external standards were weighed into disposable borosilicate glass tubes according to the specified weights provided in **Table 5.2**. A digestion blank loaded with only reagents was required in the alkaline hydrolysis assay. To each glass tube containing either a pre-

weighed sample, external standard, or blank, was added 2.1 g of barium hydroxide octahydrate and 3.5 mL of MQ water. The tubes were then capped by glass stoppers and autoclaved at 110°C for 20 hr. Following hydrolysis, the tubes were removed from the autoclave once the temperature had dropped to 80°C. All hydrolysates were then immediately mixed with 7.5 mL of MQ water and swirled thoroughly. After the solutions had cooled to room temperature, they were transferred into 100 mL beakers before adding 1.25 mL of 0.5 M orthophosphoric acid and 2 mL of 6 N HCl. Additionally, 1 mL of 0.0005 g/mL pre-thawed tryptophan solution, prepared using 0.1 N HCl, was added to the tube designated as the digestion blank. Subsequently, each solution was adjusted to the range of pH 2.95 to 3.20. Following the addition of 20 mL of absolute methanol, the solutions were diluted to a final volume of 100 mL with MQ water in a 100 mL graduate cylinder. Approximately 1mL of the final solutions was squeezed through a syringe fitted with a 13 mm 0.22 µm Luer lock filter into an amber glass GC vial and then kept in a -20°C freezer for further quantification using the UPLC. The mobile phase (i.e., TRP buffer) for UPLC was made by mixing 5.72 mL of glacial acetic acid and 100 mL of 1.0% 1,1,1-trichloro-2-methyl-2-propanol in methanol with 1800 mL of MQ water. The mobile phase was adjusted to a pH of 5.0 with ethanolamine, followed by dilution to a final volume of 2 L in a graduated cylinder. This buffer was filtered through a 0.22 µm Nylon filter paper before use. The UPLC system connected to the TRP buffer was allowed to equilibrate on the column for 120 min at 28°C. Approximately 5 µL of the food samples, external standards, and digestion blank were injected into the UPLC system. Key operational parameters of the UPLC used for alkaline assay included a flow rate of 1.0 mL/min, a C18 Luna column (125 x 4 mm, 3 µm particle size), fluorescence detection with excitation at 280 nm and emission at 356 nm, and a total run duration of approximately 34 min.

5.3.4.2 Amino acid score

According to House et al. (2010), the AAS for each unprocessed and processed sample was calculated using Equation 5.1:

$$AAS = \frac{mg\ of\ amino\ acid\ per\ gram\ of\ protein\ (test\ protein)}{mg\ of\ corresponding\ amino\ acid\ per\ gram\ of\ protein\ (reference\ pattern)} \quad \text{(Eq. 5.1)}$$

The resulting IAA profile of the experimental samples was expressed in mg of amino acid per gram of protein, while the amino acid composition of the reference protein used as the denominator in the AAS calculation was based on FAO/WHO (1991) recommendations for preschool age (2- to 5-year-old) children. The reference values for each IAA were as follows: HIS, 19; ILE, 28; LEU, 66; LYS, 58; MET + CYS, 25; PHE + TYR, 63; THR, 34; TRP, 11; VAL, 35 (amino acid, mg/g protein). The AAS for each sample ultimately was determined by identifying the IAA with the lowest ratio relative to the reference pattern, which is referred to as the first limiting amino acid (LAA).

5.3.4.3 In vitro protein digestibility

In vitro protein digestibility (IVPD) was assessed using the pH-drop method as originally described by Hsu et al. (1977), with some modifications from Tinus et al. (2012) and Franczyk (2018). Protein samples and casein were measured in triplicate, where the casein was used as a standard. In brief, each sample containing 62.5 mg ± 3 mg of crude protein was weighed and then hydrated in a 20 mL beaker with 10 mL of RO water for approximately 1 hr. During the hydration period, the samples were agitated at a moderate speed (e.g., 300 rpm) and maintained at 37°C ± 1°C on a hot plate. Meanwhile, a multi-enzyme cocktail composed of 1.6 mg/mL trypsin, 3.1 mg/mL chymotrypsin, and 1.3 mg/mL protease was freshly prepared and dissolved in RO water. This enzyme cocktail was subjected to stirring at a moderate speed and heated to 37°C ± 1°C until

complete solubilization was achieved. Once both the enzyme cocktail and the sample suspensions reached the target temperature of 37°C ± 1°C, they were adjusted to pH 8.0 ± 0.05 using either 1 M NaOH or 1 M HCl. The enzyme cocktail was then rapidly cooled in an ice bath to a temperature range of 0 – 4°C before the assay. When the initial pH of the sample suspensions was stabilized and recorded, 1 mL of the prepared enzyme cocktail was added to each sample under continuous agitation at 37°C ± 1°C. The final pH value was recorded 10 min after the addition of the enzyme cocktail. The change in pH was used for subsequent calculation of the IVPD of the samples according to the following equations described by Hsu et al. (1977), Franczyk et al. (2024), and Mendes et al. (2016):

$$Apparent\ fecal\ protein\ digestibility\ (AFPD, \%) = 65.66 + 18.10 \times \Delta pH10min \quad \text{(Eq. 5.2)}$$

$$True\ fecal\ protein\ digestibility\ 1\ (TPD\ 1, \%) = 76.15 + 15.26 \times \Delta pH10min \quad \text{(Eq. 5.3)}$$

$$True\ fecal\ protein\ digestibility\ 2\ (TPD\ 2, \%) = 93.1359\ (1 - e\text{^}(-3.4138) \times \Delta pH10min) \quad \text{(Eq. 5.4)}$$

Where

ΔpH_{10min} represents the change in pH after 10 min of enzyme addition.

5.3.4.4 In vitro protein digestibility corrected amino acid score

In vitro protein digestibility corrected amino acid score (*in vitro* PDCAAS) was calculated by multiplying the AAS of the first limiting amino acid (LAA) by the values of IVPD for each sample via the following equations (Nosworthy et al., 2018).

$$AFPD - PDCAAS\ (\%) = AAS\ of\ the\ first\ LAA \times AFPD\% \quad \text{(Eq. 5.5)}$$

$$TPD1 - PDCAAS\ (\%) = AAS\ of\ the\ first\ LAA \times TPD1\% \quad \text{(Eq. 5.6)}$$

$$TPD2 - PDCAAS\ (\%) = AAS\ of\ the\ first\ l\ LAA \times TPD2\% \quad \text{(Eq. 5.7)}$$

5.3.5 Reactive lysine analysis

Reactive lysine was quantified at Agriculture and Agri-Food Canada (AAFC, Guelph, ON, Canada) using the *ortho*-phthaldialdehyde (OPA) fluorometric method in accordance with the procedure of Barba et al. (2013) with minor modifications, as shown in **Figure 5.1**. Prior to the OPA assay, blanks, samples, and interferences were prepared and incubated overnight. Briefly, flour samples were weighed in duplicate in Falcon 14-mL polypropylene round-bottom tubes, with the weight adjusted according to their protein content. Specifically, 25 mg of flour was weighed for samples with greater than 50% protein, while 50 mg of flour was for samples with less than 25% protein. Each sample was thoroughly mixed with 10 mL of 0.1 M sodium tetraborate buffer, which was prepared by dissolving 10.061 g sodium tetraborate in MQ water and adjusting the final volume to 500 mL. The buffer was stirred at 220 rpm at 50°C to ensure complete solubilization. The sample solutions were allowed to fully hydrate in this buffer for 2 hr at room temperature using a rotator set at 20 rpm. Following the hydration process, an aliquot of 0.14 mL of the hydrated samples was further diluted to a final volume of 0.2 mL with 0.1 M sodium tetraborate buffer, in duplicate, in 1.2 mL of 8 tube strips capped with 8 closure strips that were purchased from VWR (Mississauga, ON, Canada). To eliminate any potential interferences from non-protein nitrogen compounds such as free amino acids, peptides, and amines, 0.7 mL of the hydrated samples were treated with 0.3 mL of 25% (w/v) Trichloroacetic acid (TCA) in 1.5 mL of microcentrifuge tubes. This acid precipitation step selectively precipitates proteins while leaving non-proteinaceous interferences in the supernatant (Morales et al., 1995; Ferrer et al., 2003). This mixture was then vortexed vigorously for 1 min, followed by centrifugation at the highest speed (16,100×g) for 15 min at 4°C to separate the precipitated proteins from the supernatant. Afterwards, a total of 0.20 mL of the resulting supernatants were transferred to strip tubes in duplicate. For the preparation of the blank, only 0.20 mL of 0.1 M sodium tetraborate buffer was added directly to

the strip tubes. Subsequently, 0.80 mL of 7.5% (w/v) sodium dodecyl sulfate (SDS) solution was dispensed into all prepared strip tubes, including those containing the samples, interferences, or blanks using a multi-channel pipette and capped. The strip tubes were vortexed for 1 min to thoroughly mix the contents before being incubated at 4°C in the refrigerator for 12 to 18 hr.

The next day, an OPA-absolute methanol solution was first made up by completely dissolving 100 mg of OPA solid in 2.5 mL of absolute methanol in an amber bottle. This was followed by freshly preparing 100 mL of the OPA reagent in a separate amber bottle by combining 50 mL of 0.1 M sodium tetraborate buffer, 5 mL of 20% (w/v) SDS, 42.8 mL of MQ water, 0.2 mL of β-mercaptoethanol, and 2 mL of the prepared OPA-absolute methanol solution. This reagent was stirred at 200 rpm at room temperature for at least one hr to allow stabilization. A standard curve was constructed by a 1 mg/mL casein stock solution, which was prepared by fully dissolving 10 mg of casein in 10 mL of 0.1 M sodium tetraborate buffer. This stock solution was further diluted to a final volume of 1 mL using 0.1 M sodium tetraborate buffer in the strip tubes, yielding a series of standard concentrations: 0, 0.2, 0.4, 0.6, 0.8, and 1.0 mg/mL of casein. Meanwhile, the strip tubes, which had been incubated overnight, were subjected to sonication in an ultrasonic bath for 15 min at 25°C. Then, 0.03 mL of each of the sonicated standards, blanks, samples, and interferences were promptly transferred to 96 well flat-bottom microplates in triplicate using the multichannel pipette. To each well, 0.17 mL of the freshly prepared OPA reagent was added with the multi-channel pipette, following the measurement sequence specified by the fluorometer with gently shaking for 10 sec. The fluorescence intensities of the microplates were immediately measured in kinetic mode at 1-min intervals over a total period of 4 min. The measurements were performed using a Microplate reader (Synergy H1, BioTek Instruments, Inc., USA) set to an excitation wavelength of 340 nm and an emission wavelength of 455 nm at 25°C. For each well, the maximum fluorescence intensity recorded among the five-time points was selected for

calculation. The reactive lysine content of each sample was corrected for interference and blank, and quantified based on the casein standard curve, where 1 mg/mL of casein in assay solution was equivalent to 0.06438 mg lysine/mL assay solution. The final results were expressed on both DM and protein bases.

$$Reactive\ lysine\ (g\ lysine/100g\ sample\ or\ \%) = \left(\frac{(Abs_S - Abs_0) - b}{a} - \frac{(Abs_I - Abs_0) - b}{a}\right) \times \frac{10\ mL\ of\ solvent}{S} \times D \div 10$$

(Eq. 5.8)

Where

Abs_S, Abs_I, and Abs_0 = The maximum fluorescence intensities of the sample, interference, and blank, respectively,

b and a = The intercept and slope of the standard curve, respectively,

S = Sample weight (g), and

D = Dilution factor (i.e., (0.14 mL + 0.06 mL + 0.80 mL)/0.14 mL = 7.14).

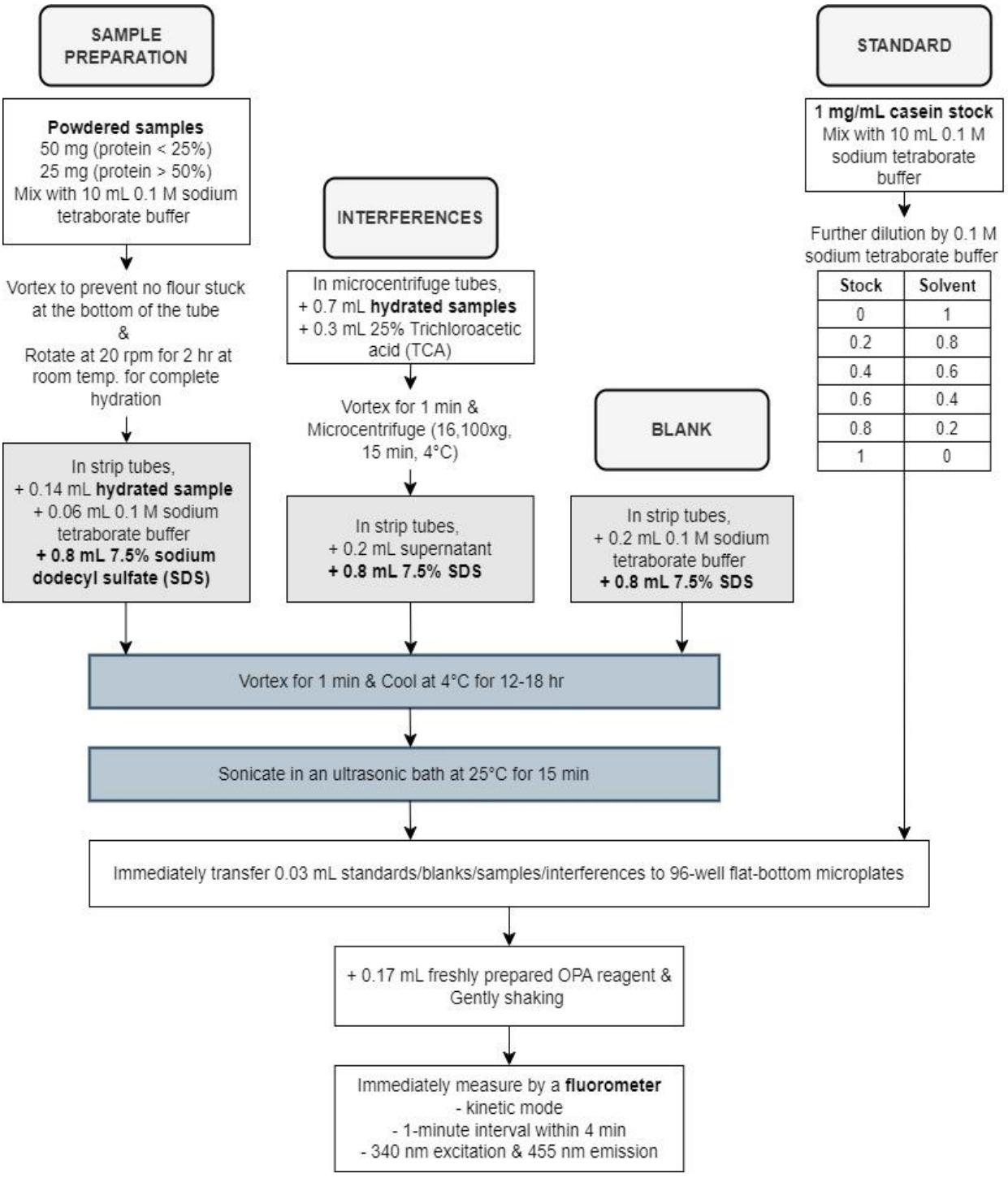

Figure 5.1. *Schematic diagram of the experimental procedure for the determination of reactive lysine.*

5.3.6 Statistical Analysis

Each sample, either unprocessed or processed, was prepared in triplicate. For most analyses, measurements were carried out in triplicate for each sample, with the exception of the amino acid hydrolysis analyses, which were performed in singlet. The data were expressed as the mean of three replicates. Statistical analysis was done using one-way analysis of variance (ANOVA) along with a Tukey's multiple comparison test to determine statistically significant differences between treatments within each type of pea samples (pea flour, protein concentrate, 80% protein isolate, and 90% protein isolate). A pooled standard error (SE) and significance accepted at a probability level of $p < 0.05$ were provided for each sample type. All statistical analyses were conducted using Microsoft Excel 2021, GraphPad Prism, and R.

5.4 RESULTS AND DISCUSSION

5.4.1 In Vitro Protein Quality

5.4.1.1 Amino acid profile

Tables 5.3 and **5.4** present detailed profiles of indispensable and dispensable amino acids in whole yellow field pea flour, protein concentrate, and both 80% and 90% isolates before and after different processing treatments, expressed on a DM basis, including data for the untreated coarse fraction. Among the indispensable amino acids (IAAs), LYS was identified as the most abundant, followed by LEU, in both untreated and treated flours and protein concentrates, with the exception of the boiled flour and baked protein concentrate, where LEU was the predominant IAA. Both untreated and treated 90% isolates exhibited the highest levels of LYS, whereas both untreated and treated 80% isolates had the highest levels of LEU, although they were processed through the same isolation technique. This suggests that the different levels of purification conditions applied can differentially affect amino acid profiles. On the other hand, TRP was consistently the least abundant amino acid in the untreated and treated flours and protein concentrates, as well as in the untreated 90% isolate. This was followed by MET and CYS. In contrast, CYS remained the lowest in both untreated and treated 80% isolates, as well as the baked and HHP-treated 90% isolates, followed by TRP and MET. Additionally, the coarse fraction sample was also highest in LYS and lowest in TRP. In the current study, the levels of most IAAs in the untreated pea flour generally fell within the ranges reported by Frias et al. (2011), Nosworthy et al. (2017a), and Nosworthy et al. (2017c), except for LEU at 1.67%, significantly below the reported range of 1.75 – 1.85 g/100g on a DM basis. Interestingly, IAA contents in the untreated protein concentrate were higher than those reported by Gunawardena et al. (2010), Konieczny et al. (2020), and Çabuk et al. (2018), despite Konieczny et al. (2020) reporting a higher protein

content than observed in our study. Additionally, the amino acid profile of the untreated 90% isolate in the present study demonstrates slight discrepancies compared to the findings of Shi (2022), where the untreated pea isolate (with a protein content of 86.9%) had the highest LEU content, as well as the lowest CYS content followed by TRP and MET. It also reported considerably higher levels of ILE and LEU than those found in the current study, even though they had a comparable protein content. This highlights potential variations in amino acid distributions influenced by factors such as different processing conditions (e.g., milling, air classification parameters or isolation conditions) and pea cultivars. Different processing methods affected the IAA content differentially. Boiling had the most pronounced effect, significantly improving ($p < 0.05$) the levels of IAAs including THR, VAL, ILE, LEU, PHE, and TRP in the pea flour. This is in agreement with the observations of Nosworthy et al. (2017a), who reported the enhancements of these IAAs in boiled yellow split pea flour, except for a decrease in TRP. Additionally, baking significantly increased the TRP level ($p < 0.05$) in the protein concentrate in the present study. Khattab et al. (2009) observed that various processing methods including soaking, boiling, microwave cooking, and autoclaving, positively influenced the total contents of IAAs, which also agreed with those reported by Ma et al. (2017). The observed increase in amino acid levels could be attributed to protein denaturation and unfolding induced by heating. This process makes the proteins more accessible to proteolytic enzymes, thereby promoting the release of amino acids (Ma et al., 2017). However, other processing treatments either maintained or reduced the levels of IAAs in all tested pea samples. Notably, baking resulted in significant reductions ($p < 0.05$) in CYS contents in the flour, protein concentrate, and both 80% and 90% isolates were observed. This could be attributed to the susceptibility of CYS to thermal destruction, particularly under the high temperature employed during baking in this study (Wang & Damodaran, 1990). HHP processing resulted in significant losses ($p < 0.05$) of LYS and VAL in the flour; THR, LYS, VAL, ILE, LEU,

and PHE in the protein concentrate; and LEU in the 90% isolate. Similar reductions in IAAs due to HHP were observed in studies by Deng et al. (2015) and Zhong et al. (2015). These losses could be explained by the effects of high pressures, particularly at 600 MPa, on protein structure. High pressure can induce conformational changes in proteins, such as aggregation, potentially reducing their susceptibility to proteolytic degradation, and thereby limiting the release of amino acids from proteins (Neji et al., 2022; Deng et al., 2015). In short, these findings of IAA highlight the significant influence of different processing treatments on the amino acid profiles of pea-derived products. The extent of amino acid retention or increase varies depending on the processing conditions applied and protein separation methods used to develop the pea products.

Regarding the dispensable amino acids, GLU and ASP were consistently found in higher concentrations observed in all the pea samples, regardless of the processing treatments applied. The finding aligns with the observations of Neji et al. (2022), who reported that these two amino acids are abundant in various legumes such as chickpeas, soybeans, lentils, beans, faba beans, lupins, cowpeas, and peas. On the other hand, PRO was lowest in both untreated and treated flours, protein concentrates, and 90% isolates, as well as in the coarse fraction sample. However, an exception was observed in the 80% isolate samples, where GLY was present at the lowest level.

Processing methods appeared to have a relatively lower impact on dispensable amino acids compared to IAAs. Specifically, SER contents were significantly increased ($p < 0.05$) in the pea flour after both boiling and autoclaving, and in the protein concentrate after baking. Additionally, ALA in flour was notably enhanced ($p < 0.05$) by boiling. These findings are consistent with those reported by Nosworthy et al. (2017a) for boiled yellow split peas, where similar enhancements in SER and ALA were observed. However, Nosworthy et al. (2017a) also noted improvements in most other dispensable amino acids after baking, except for ARG and PRO. This contrasts with the results of the current study, as the majority of dispensable amino acids either remained

unchanged or decreased following baking across all pea sample types. This discrepancy might be due to variations in baking conditions such as temperature, duration, oven type, and preparation procedures. On the other hand, HHP processing had a deleterious effect on several dispensable amino acids. Specifically, HHP treatment significantly reduced ($p < 0.05$) the contents of ARG, GLY, ASP, GLU, ALA, and PRO in the protein concentrate, as well as GLY, ASP, GLU, and ALA in the flour. These losses can be attributed to the structural alterations induced by HHP, similar to its effects on IAAs, which ultimately decrease the availability of amino acids.

Tables 5.5 and **5.6** provide the changes in indispensable and dispensable amino acid profiles on a protein basis for pea flour, protein concentrate, and both 80% and 90% isolates before and after different processing treatments, respectively, as well as data for the untreated coarse fraction. When expressed on a protein basis, the most significant difference was seen in the coarse fraction sample. While its amino acid profile tended to be comparable to that of the protein concentrate, the coarse fraction exhibited significantly lower ($p < 0.05$) contents of ILE, LEU, PHE, SER, ARG, and PRO. Notably, the CYS content in the coarse fraction was significantly higher ($p < 0.05$) than in the protein concentrate. Importantly, the compositions of IAAs, expressed on a protein basis (**Table 5.5**), were further utilized to calculate amino acid scores (AAS) and identify the limiting amino acids (LAA) in accordance with the FAO/WHO (1991) recommendations. These calculations followed the reference pattern established for preschool-aged children (2–5 years), with the reference values for IAAs as follows (mg/g protein): Histidine, 19; Isoleucine, 28; Leucine, 66; Lysine, 58; Methionine + Cysteine, 25; Phenylalanine + Tyrosine, 63; Threonine, 34; Tryptophan, 11; Valine, 35. IAAs with contents below their corresponding reference values and their ratios (the ratio of mg of amino acid per g of test protein to mg of the corresponding amino acid per g of reference protein) less than 1 were classified as LAAs (Nosworthy et al., 2017c). The AAS and LAAs of the samples are detailed in **Tables 5.7** and **5.8**.

Overall, among the IAAs in untreated and treated yellow field peas and their derivative samples on a DM basis, LYS and LEU were the most abundant, while TRP, MET, and CYS were presented in the lowest amounts. For dispensable amino acids, GLU and ASP consistently had the highest concentrations across all the pea samples, whereas PRO and GLY were found in the lowest amounts. Most indispensable and dispensable amino acids showed significant improvements following the boiling treatment but were notably reduced by HHP. While thermal treatments, particularly boiling, can enhance amino acid profiles, it was unexpected that HHP failed to retain or improve amino acid compositions for yellow field pea samples.

Table 5.3. Indispensable amino acid composition of untreated and treated whole yellow field pea flours, protein concentrates, coarse fraction, 80% protein isolates, and 90% protein isolates (%, DM basis).

Sample Description	HIS	THR	CYS	LYS	TYR	MET	VAL	ILE	LEU	PHE	TRP
Whole Pea Flour											
Untreated	0.55^{a}	0.85^{bc}	0.32^{a}	1.72^{ab}	0.73^{a}	0.28^{ab}	1.08^{b}	0.97^{bcd}	1.67^{bc}	1.08^{bc}	0.24^{bc}
Boiled	0.58^{a}	0.90^{a}	0.30^{b}	1.79^{a}	0.73^{a}	0.29^{a}	1.15^{a}	1.05^{a}	1.81^{a}	1.17^{a}	0.26^{a}
Autoclaved	0.58^{a}	0.89^{ab}	0.30^{ab}	1.78^{a}	0.75^{a}	0.28^{ab}	1.13^{ab}	1.02^{ab}	1.74^{ab}	1.14^{ab}	0.25^{ab}
Micronized	0.55^{a}	0.86^{ab}	0.30^{ab}	1.71^{ab}	0.76^{a}	0.28^{ab}	1.10^{ab}	0.98^{bc}	1.69^{b}	1.11^{ab}	0.24^{c}
Baked	0.56^{a}	0.85^{bc}	0.28^{c}	1.65^{bc}	0.68^{a}	0.27^{b}	1.07^{bc}	0.96^{cd}	1.65^{bc}	1.08^{bc}	0.24^{c}
HHP-treated	0.57^{a}	0.81^{c}	0.32^{a}	1.60^{c}	0.71^{a}	0.27^{b}	1.02^{c}	0.91^{d}	1.57^{c}	1.03^{c}	0.23^{c}
Pooled SE	**0.0002**	**0.0003**	**0.0000**	**0.0011**	**0.0017**	**0.0000**	**0.0004**	**0.0004**	**0.0014**	**0.0006**	**0.0000**
***P*-value**	**0.0607**	**0.0005**	**<0.0001**	**<0.0001**	**0.3325**	**0.0108**	**0.0001**	**<0.0001**	**<0.0001**	**0.0003**	**0.0001**
Protein Concentrate											
Untreated	1.22^{a}	1.84^{a}	0.62^{a}	3.76^{a}	1.64^{a}	0.58^{a}	2.33^{a}	2.11^{a}	3.63^{a}	2.39^{a}	0.49^{b}
Baked	1.24^{a}	1.82^{ab}	0.58^{b}	3.51^{b}	1.63^{a}	0.58^{a}	2.33^{a}	2.12^{a}	3.60^{ab}	2.37^{ab}	0.53^{a}
HHP-treated	1.26^{a}	1.76^{b}	0.64^{a}	3.51^{b}	1.61^{a}	0.57^{a}	2.24^{b}	2.01^{b}	3.49^{b}	2.29^{b}	0.50^{b}
Coarse fraction	0.33^{b}	0.50^{c}	0.20^{c}	0.99^{c}	0.43^{b}	0.18^{b}	0.62^{c}	0.55^{c}	0.94^{c}	0.60^{c}	0.14^{c}
Pooled SE	**0.0012**	**0.0005**	**0.0002**	**0.0053**	**0.0024**	**0.0002**	**0.0006**	**0.0007**	**0.0028**	**0.0012**	**0.0002**
***P*-value**	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**

Data are expressed as mean of three replicates (single measurement per replicate). Mean values followed by different superscript letters within the column of each yellow field pea type indicate statistically significant differences between treatments ($p < 0.05$) using one-way ANOVA along with Tukey's multiple comparison test.

Abbreviations: HIS = Histidine; THR = Threonine; CYS = Cysteine; LYS = Lysine; TYR = Tyrosine; MET = Methionine; VAL = Valine; ILE = Isoleucine; LEU = Leucine; PHE = Phenylalanine; TRP = Tryptophan; DM = Dry matter; SE = Standard error.

Table 5.3. Continued.

Sample Description	HIS	THR	CYS	LYS	TYR	MET	VAL	ILE	LEU	PHE	TRP
80% Protein Isolate											
Untreated	1.90^{ab}	2.82^{a}	0.80^{a}	5.90^{a}	2.85^{a}	0.95^{ab}	3.88^{a}	3.63^{a}	6.37^{a}	4.02^{a}	0.84^{a}
Baked	1.86^{b}	2.81^{a}	0.72^{b}	5.85^{a}	2.85^{a}	0.90^{b}	3.85^{a}	3.63^{a}	6.32^{a}	3.99^{a}	0.84^{a}
HHP-treated	2.01^{a}	2.87^{a}	0.83^{a}	5.91^{a}	2.96^{a}	0.99^{a}	3.92^{a}	3.65^{a}	6.44^{a}	4.09^{a}	0.86^{a}
Pooled SE	**0.0032**	**0.0030**	**0.0008**	**0.0116**	**0.0043**	**0.0008**	**0.0061**	**0.0054**	**0.0139**	**0.0049**	**0.0003**
***P*-value**	**0.0352**	**0.3921**	**0.0109**	**0.8073**	**0.1520**	**0.0230**	**0.5926**	**0.9566**	**0.4639**	**0.2864**	**0.3806**
90% Protein Isolate											
Untreated	2.22^{ab}	3.60^{a}	0.98^{a}	7.15^{a}	3.43^{a}	1.02^{ab}	4.44^{a}	3.99^{a}	6.78^{a}	4.45^{a}	0.93^{a}
Baked	2.12^{b}	3.52^{a}	0.87^{b}	6.98^{a}	3.31^{a}	0.98^{b}	4.33^{a}	3.90^{a}	6.64^{c}	4.36^{a}	0.92^{a}
HHP-treated	2.35^{a}	3.63^{a}	0.97^{a}	7.11^{a}	3.33^{a}	1.03^{a}	4.42^{a}	3.96^{a}	6.74^{b}	4.48^{a}	1.02^{a}
Pooled SE	**0.0075**	**0.0031**	**0.0008**	**0.0104**	**0.0077**	**0.0004**	**0.0040**	**0.0053**	**0.0096**	**0.0067**	**0.0081**
***P*-value**	**0.0438**	**0.1108**	**0.0049**	**0.1911**	**0.2763**	**0.0281**	**0.1855**	**0.3362**	**0.2763**	**0.2315**	**0.4160**

Data are expressed as mean of three replicates (single measurement per replicate). Mean values followed by different superscript letters within the column of each yellow field pea type indicate statistically significant differences between treatments ($p < 0.05$) using one-way ANOVA along with Tukey's multiple comparison test.

Abbreviations: HIS = Histidine; THR = Threonine; CYS = Cysteine; LYS = Lysine; TYR = Tyrosine; MET = Methionine; VAL = Valine; ILE = Isoleucine; LEU = Leucine; PHE = Phenylalanine; TRP = Tryptophan; DM = Dry matter; SE = Standard error.

Table 5.4. Dispensable amino acid composition of untreated and treated whole yellow field pea flours, protein concentrates, coarse fraction, 80% protein isolates, and 90% protein isolates (%, DM basis).

Sample Description	SER	ARG	GLY	ASP	GLU	ALA	PRO
Whole Pea Flour							
Untreated	1.08^{b}	1.78^{a}	1.00^{a}	2.71^{a}	3.92^{a}	1.01^{b}	0.94^{ab}
Boiled	1.16^{a}	1.84^{a}	1.01^{a}	2.72^{a}	4.00^{a}	1.06^{a}	0.99^{a}
Autoclaved	1.14^{a}	1.83^{a}	1.01^{a}	2.72^{a}	4.00^{a}	1.06^{ab}	0.96^{a}
Micronized	1.09^{b}	1.82^{a}	1.02^{a}	2.70^{a}	3.94^{a}	1.03^{ab}	0.95^{a}
Baked	1.06^{b}	1.76^{a}	0.99^{ab}	2.65^{ab}	3.88^{a}	1.01^{b}	0.93^{ab}
HHP-treated	1.06^{b}	1.73^{a}	0.94^{b}	2.57^{b}	3.64^{b}	0.94^{c}	0.88^{b}
Pooled SE	**0.0002**	**0.0034**	**0.0005**	**0.0014**	**0.0040**	**0.0003**	**0.0005**
***P*-value**	**<0.0001**	**0.2326**	**0.0064**	**0.0019**	**0.0001**	**<0.0001**	**0.0026**
Protein Concentrate							
Untreated	2.42^{b}	4.07^{a}	2.08^{a}	5.72^{a}	8.43^{a}	2.17^{a}	2.04^{a}
Baked	2.51^{a}	3.97^{ab}	2.08^{a}	5.64^{ab}	8.31^{a}	2.14^{a}	2.05^{a}
HHP-treated	2.37^{b}	3.90^{b}	1.99^{b}	5.57^{b}	7.93^{b}	2.06^{b}	1.95^{b}
Coarse fraction	0.64^{c}	0.99^{c}	0.59^{c}	1.61^{c}	2.31^{c}	0.60^{c}	0.53^{c}
Pooled SE	**0.0008**	**0.0040**	**0.0006**	**0.0032**	**0.0097**	**0.0004**	**0.0005**
***P*-value**	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**

Data are expressed as mean of three replicates (single measurement per replicate). Mean values followed by different superscript letters within the column of each yellow field pea type indicate statistically significant differences between treatments ($p < 0.05$) using one-way ANOVA along with Tukey's multiple comparison test.

Abbreviations: SER = Serine; ARG = Arginine; GLY = Glycine; ASP = Aspartic acid; GLU = Glutamic acid; ALA = Alanine; PRO = Proline; DM = Dry matter; SE = Standard error.

Table 5.4. Continued.

Sample Description	SER	ARG	GLY	ASP	GLU	ALA	PRO
80% Protein Isolate							
Untreated	4.00[a]	6.39[a]	3.17[a]	9.07[a]	13.54[a]	3.37[a]	3.29[a]
Baked	3.99[a]	6.29[a]	3.17[a]	8.96[a]	13.51[a]	3.35[a]	3.29[a]
HHP-treated	4.00[a]	6.47[a]	3.20[a]	9.32[a]	13.61[a]	3.42[a]	3.33[a]
Pooled SE	**0.0060**	**0.0213**	**0.0030**	**0.0702**	**0.0546**	**0.0042**	**0.0028**
***P*-value**	**0.9957**	**0.3535**	**0.6833**	**0.3099**	**0.8746**	**0.4754**	**0.5782**
90% Protein Isolate							
Untreated	4.63[a]	7.03[a]	3.81[a]	10.60[ab]	15.81[a]	4.24[a]	3.79[a]
Baked	4.52[a]	6.87[a]	3.72[a]	10.40[b]	15.44[a]	4.17[a]	3.72[a]
HHP-treated	4.61[a]	7.07[a]	3.78[a]	10.88[a]	15.66[a]	4.27[a]	3.77[a]
Pooled SE	**0.0057**	**0.0180**	**0.0041**	**0.0217**	**0.0435**	**0.0031**	**0.0031**
***P*-value**	**0.2739**	**0.2250**	**0.3136**	**0.0211**	**0.1757**	**0.1400**	**0.3256**

Data are expressed as mean of three replicates (single measurement per replicate). Mean values followed by different superscript letters within the column of each yellow field pea type indicate statistically significant differences between treatments ($p < 0.05$) using one-way ANOVA along with Tukey's multiple comparison test.

Abbreviations: SER = Serine; ARG = Arginine; GLY = Glycine; ASP = Aspartic acid; GLU = Glutamic acid; ALA = Alanine; PRO = Proline; DM = Dry matter; SE = Standard error.

Table 5.5. Indispensable amino acid composition of untreated and treated whole yellow field pea flours, protein concentrates, coarse fraction, 80% protein isolates, and 90% protein isolates (mg/g of protein, as is basis).

Sample Description	HIS	THR	CYS	LYS	TYR	MET	VAL	ILE	LEU	PHE	TRP
Whole Pea Flour											
Untreated	23.53^{a}	36.36^{ab}	13.68^{a}	73.39^{ab}	31.08^{a}	11.90^{a}	46.39^{a}	41.39^{bc}	71.24^{b}	46.39^{ab}	10.46^{ab}
Boiled	24.05^{a}	37.27^{a}	12.35^{bc}	74.27^{a}	30.46^{a}	12.10^{a}	47.76^{a}	43.54^{a}	75.05^{a}	48.56^{a}	10.65^{a}
Autoclaved	23.97^{a}	36.68^{ab}	12.57^{bc}	73.57^{ab}	30.79^{a}	11.54^{a}	46.44^{a}	42.15^{ab}	71.91^{ab}	46.82^{ab}	10.35^{ab}
Micronized	23.39^{a}	36.59^{ab}	12.89^{b}	72.29^{abc}	32.08^{a}	11.83^{a}	46.47^{a}	41.64^{ab}	71.83^{ab}	46.89^{ab}	10.10^{b}
Baked	24.01^{a}	36.49^{ab}	11.93^{c}	70.88^{bc}	29.37^{a}	11.65^{a}	46.17^{a}	41.55^{abc}	71.18^{b}	46.60^{ab}	10.26^{ab}
HHP-treated	24.60^{a}	35.24^{b}	13.80^{a}	69.39^{c}	30.64^{a}	11.72^{a}	44.28^{b}	39.53^{c}	67.90^{c}	44.66^{b}	10.15^{b}
Pooled SE	**0.2767**	**0.3914**	**0.0746**	**1.381**	**2.601**	**0.0783**	**0.3842**	**0.5884**	**1.395**	**0.7492**	**0.0221**
***P*-value**	**0.1482**	**0.0380**	**<0.0001**	**0.0022**	**0.5148**	**0.2634**	**0.0006**	**0.0012**	**0.0003**	**0.0045**	**0.0063**
Protein Concentrate											
Untreated	24.95^{ab}	37.69^{a}	12.66^{b}	77.06^{a}	33.70^{a}	11.85^{ab}	47.77^{a}	43.33^{a}	74.49^{a}	48.95^{a}	9.96^{a}
Baked	24.08^{b}	35.22^{a}	11.22^{c}	68.04^{b}	31.51^{a}	11.20^{b}	45.25^{a}	41.03^{ab}	69.73^{ab}	45.99^{ab}	10.26^{a}
HHP-treated	25.94^{a}	36.20^{a}	13.12^{b}	72.31^{ab}	33.11^{a}	11.73^{b}	46.19^{a}	41.50^{ab}	71.88^{ab}	47.23^{ab}	10.24^{a}
Coarse fraction	23.95^{b}	35.88^{a}	14.70^{a}	71.53^{ab}	31.01^{a}	12.69^{a}	45.18^{a}	39.70^{b}	67.66^{b}	43.62^{b}	10.02^{a}
Pooled SE	**0.4387**	**1.483**	**0.0709**	**5.099**	**1.327**	**0.1247**	**1.275**	**1.684**	**5.561**	**2.666**	**0.1051**
***P*-value**	**0.0209**	**0.1639**	**<0.0001**	**0.0082**	**0.0621**	**0.0058**	**0.0727**	**0.0514**	**0.0371**	**0.0223**	**0.5989**

Data are expressed as mean of three replicates (single measurement per replicate). Mean values followed by different superscript letters within the column of each yellow field pea type indicate statistically significant differences between treatments ($p < 0.05$) using one-way ANOVA along with Tukey's multiple comparison test.

Abbreviations: HIS = Histidine; THR = Threonine; CYS = Cysteine; LYS = Lysine; TYR = Tyrosine; MET = Methionine; VAL = Valine; ILE = Isoleucine; LEU = Leucine; PHE = Phenylalanine; TRP = Tryptophan; SE = Standard error.

Table 5.5. Continued.

Sample Description	HIS	THR	CYS	LYS	TYR	MET	VAL	ILE	LEU	PHE	TRP
80% Protein Isolate											
Untreated	23.08^{ab}	34.38^{a}	9.69^{ab}	71.77^{a}	34.72^{a}	11.55^{ab}	47.28^{a}	44.24^{a}	77.49^{a}	48.93^{a}	10.20^{a}
Baked	22.70^{b}	34.30^{a}	8.81^{b}	71.50^{a}	34.80^{a}	11.04^{b}	47.06^{a}	44.32^{a}	77.15^{a}	48.75^{a}	10.28^{a}
HHP-treated	24.69^{a}	35.20^{a}	10.13^{a}	72.44^{a}	36.24^{a}	12.15^{a}	48.05^{a}	44.69^{a}	78.96^{a}	50.11^{a}	10.51^{a}
Pooled SE	**0.5154**	**0.4522**	**0.1299**	**1.875**	**0.6356**	**0.1159**	**0.9057**	**0.7417**	**2.047**	**0.7198**	**0.0463**
***P*-value**	**0.0317**	**0.2688**	**0.0110**	**0.7020**	**0.1003**	**0.0206**	**0.4527**	**0.8032**	**0.3275**	**0.1839**	**0.2624**
90% Protein Isolate											
Untreated	24.98^{ab}	40.48^{a}	11.01^{a}	80.38^{a}	38.55^{a}	11.42^{ab}	49.92^{a}	44.89^{a}	76.23^{a}	50.05^{a}	10.47^{a}
Baked	23.99^{b}	39.88^{a}	9.84^{b}	79.10^{a}	37.49^{a}	11.07^{b}	49.11^{a}	44.16^{a}	75.24^{a}	49.37^{a}	10.43^{a}
HHP-treated	26.68^{a}	41.22^{a}	11.03^{a}	80.68^{a}	37.74^{a}	11.72^{a}	50.12^{a}	44.95^{a}	76.51^{a}	50.86^{a}	11.53^{a}
Pooled SE	**0.9282**	**0.4263**	**0.1072**	**1.512**	**1.053**	**0.0547**	**0.5325**	**0.6787**	**1.425**	**0.9114**	**1.024**
***P*-value**	**0.0370**	**0.1148**	**0.0064**	**0.3149**	**0.4665**	**0.0374**	**0.2786**	**0.4712**	**0.4419**	**0.2392**	**0.3796**

Data are expressed as mean of three replicates (single measurement per replicate). Mean values followed by different superscript letters within the column of each yellow field pea type indicate statistically significant differences between treatments (p<0.05) using one-way ANOVA along with Tukey's multiple comparison test.

Abbreviations: HIS = Histidine; THR = Threonine; CYS = Cysteine; LYS = Lysine; TYR = Tyrosine; MET = Methionine; VAL = Valine; ILE = Isoleucine; LEU = Leucine; PHE = Phenylalanine; TRP = Tryptophan; SE = Standard error.

Table 5.6. Dispensable amino acid composition of untreated and treated whole yellow field pea flours, protein concentrates, coarse fraction, 80% protein isolates, and 90% protein isolates (mg/g of protein, as is basis).

Sample Description	SER	ARG	GLY	ASP	GLU	ALA	PRO
Whole Pea Flour							
Untreated	46.32^{b}	76.35^{a}	42.66^{a}	115.83^{a}	167.54^{a}	43.30^{a}	40.17^{ab}
Boiled	48.09^{a}	76.36^{a}	42.06^{ab}	112.62^{ab}	165.96^{a}	44.09^{a}	41.05^{a}
Autoclaved	47.03^{ab}	75.26^{a}	41.64^{ab}	112.15^{b}	165.05^{a}	43.51^{a}	39.75^{ab}
Micronized	46.33^{b}	77.19^{a}	43.22^{a}	114.27^{ab}	166.97^{a}	43.62^{a}	40.42^{ab}
Baked	45.84^{b}	75.66^{a}	42.66^{a}	114.29^{ab}	167.03^{a}	43.50^{a}	40.21^{ab}
HHP-treated	46.10^{b}	75.08^{a}	40.50^{b}	111.15^{b}	157.72^{b}	40.92^{b}	38.28^{b}
Pooled SE	**0.2041**	**4.486**	**0.5211**	**1.690**	**4.618**	**0.2757**	**0.6210**
***P*-value**	**0.0006**	**0.8235**	**0.0081**	**0.0090**	**0.0010**	**0.0001**	**0.0192**
Protein Concentrate							
Untreated	49.71^{a}	83.37^{a}	42.67^{a}	117.30^{a}	172.80^{a}	44.40^{a}	41.77^{a}
Baked	48.59^{a}	77.02^{bc}	40.36^{a}	109.33^{a}	161.15^{a}	41.41^{a}	39.78^{ab}
HHP-treated	48.77^{a}	80.34^{ab}	40.91^{a}	114.63^{a}	163.30^{a}	42.37^{a}	40.15^{ab}
Coarse fraction	46.24^{b}	71.81^{c}	42.96^{a}	116.58^{a}	167.22^{a}	43.06^{a}	38.50^{b}
Pooled SE	**0.7110**	**5.604**	**1.938**	**10.65**	**24.75**	**1.727**	**1.422**
***P*-value**	**0.0057**	**0.0019**	**0.1290**	**0.0636**	**0.0854**	**0.1124**	**0.0572**

Data are expressed as mean of three replicates (single measurement per replicate). Mean values followed by different superscript letters within the column of each yellow field pea type indicate statistically significant differences between treatments ($p < 0.05$) using one-way ANOVA along with Tukey's multiple comparison test.

Abbreviations: SER = Serine; ARG = Arginine; GLY = Glycine; ASP = Aspartic acid; GLU = Glutamic acid; ALA = Alanine; PRO = Proline; SE = Standard error.

Table 5.6. Continued.

Sample Description	SER	ARG	GLY	ASP	GLU	ALA	PRO
80% Protein Isolate							
Untreated	48.70[a]	77.83[a]	38.63[a]	110.44[a]	164.84[a]	40.98[a]	40.05[a]
Baked	48.78[a]	76.78[a]	38.67[a]	109.45[a]	165.01[a]	40.92[a]	40.18[a]
HHP-treated	48.99[a]	79.34[a]	39.27[a]	114.21[a]	166.77[a]	41.87[a]	40.82[a]
Pooled SE	**0.9553**	**3.343**	**0.4441**	**11.27**	**8.793**	**0.6510**	**0.4421**
***P*-value**	**0.9345**	**0.2993**	**0.4670**	**0.2638**	**0.6927**	**0.3407**	**0.3771**
90% Protein Isolate							
Untreated	52.07[a]	79.10[a]	42.84[a]	119.23[ab]	177.86[a]	47.71[a]	42.60[a]
Baked	51.28[a]	77.83[a]	42.19[a]	117.90[b]	175.03[a]	47.23[a]	42.12[a]
HHP-treated	52.33[a]	80.23[a]	42.96[a]	123.47[a]	177.72[a]	48.49[a]	42.84[a]
Pooled SE	**0.7750**	**2.402**	**0.5937**	**3.181**	**6.252**	**0.4290**	**0.4412**
***P*-value**	**0.3769**	**0.2432**	**0.4719**	**0.0204**	**0.3606**	**0.1377**	**0.4542**

Data are expressed as mean of three replicates (single measurement per replicate). Mean values followed by different superscript letters within the column of each yellow field pea type indicate statistically significant differences between treatments ($p < 0.05$) using one-way ANOVA along with Tukey's multiple comparison test.

Abbreviations: SER = Serine; ARG = Arginine; GLY = Glycine; ASP = Aspartic acid; GLU = Glutamic acid; ALA = Alanine; PRO = Proline; SE = Standard error.

5.4.1.2 Amino acid score, protein digestibility, and protein quality

Table 5.7 presents the AAS of untreated and treated whole yellow field pea flours, protein concentrates, and both 80% and 90% isolates, as well as the untreated coarse fraction. These scores were calculated based on the IAA compositions provided in **Table 5.5**. Sulfur-containing amino acids (MET + CYS) and TRP were identified as the LAAs in most of the pea samples, with scores below 1. Exceptions to this were observed for sulfur-containing amino acids in the untreated and HHP-treated flours, as well as in the untreated coarse fraction, and TRP in the HHP-treated 90% isolate, where their scores exceeded 1.

The first or most LAA, defined as the amino acid with the lowest score among the nine IAAs, is presented in **Table 5.8**. In the current study, the AASs ranged from 0.92 to 0.97, with TRP identified as the first LAA in both untreated and treated flours. Sulfur-containing amino acids also exhibited limitations in the boiled, autoclaved, micronized, and baked flour samples, with AAS values ranging from 0.94 to 0.99. In the untreated and HHP-treated protein concentrates, TRP was identified as the most LAA, with AAS values of 0.91 and 0.93, respectively, followed by sulfur-containing amino acids, with AAS values of 0.98 and 0.99, respectively. Similarly, the coarse fraction sample obtained by air classification was most limited in TRP, with an AAS of 0.91. However, in baked protein concentrate, sulfur-containing amino acids were the most limiting (AAS of 0.90), followed by TRP (AAS of 0.93). In both untreated and treated 80% and 90% protein isolates, sulfur-containing amino acids were the first limiting amino acids, with AAS values ranging from 0.79 to 0.89 in the 80% isolate samples and 0.84 to 0.91 in the 90% isolate samples. TRP followed as the second most limiting amino acid, with AAS values ranging from 0.93 to 0.96 across all isolate samples, except for the HHP-treated 90% isolate. Compared to the flour and protein concentrate, which exhibited the greatest limitations in TRP, the results indicate

a slight deficiency of sulfur-containing amino acids in both the 80% and 90% protein isolates. This observation is consistent with previous research reported by Shi et al. (2022), which revealed that the protein isolation process potentially results in the removal of sulfur-rich albumin fractions, reducing the levels of sulfur-containing amino acids in the resulting isolates. To support this, Asen et al. (2023) highlighted that pea protein isolates produced via wet fractionation tend to experience greater losses in sulfur-containing amino acids when compared to those produced via dry fractionation methods. The primary reason for this difference lies in the removal of water-soluble albumins, which contain a higher proportion of sulfur-containing amino acids, during the acid precipitation phase of the wet fractionation process. Additionally, cysteine and serine residues can undergo conversion to dehydroalanine, which may further be transformed into lysine during processing, thus contributing to the reduction of sulfur-containing amino acids in the protein isolates (Asen et al., 2023). In the present study, both protein isolates were processed using an aqueous pH-phased method. Interestingly, the untreated 90% protein isolate with a measured protein content of 88.90%, achieved an AAS of 0.90. This score is significantly higher than the AAS value of 0.79 reported by Shi et al. (2022) for a pea protein isolate with an 86.9% protein content that was prepared via a wet method involving the combination of alkaline extraction and pI precipitation. Such differences may be due to variations in extraction methods, processing conditions, and storage practices (Asen et al., 2023). Additionally, Shi et al. (2022) reported pea flour was primarily deficient in TRP (AAS of 0.96), which is in reasonable agreement with the observation of the current study, where the untreated pea flour also identified TRP as the first limiting amino acid, with an AAS of 0.95. A similar observation was made by Stone et al. (2021), who reported TRP as the most LAA in pea flour, with an AAS of 0.85.

In contrast to the findings of the current study, previous research has reported that pea flours, pea protein concentrates, and other pulses are primarily limited by sulfur-containing amino

acids, with TRP being the second most LAA, as reported by Frias et al. (2011), Khattab et al. (2009), Ma et al. (2017), Konieczny et al. (2020), Çabuk et al. (2018), and Zhong et al. (2015). For example, Khattab et al. (2009) identified sulfur-containing amino acids as the most LAA across all investigated samples including Canadian and Egyptian cowpeas, kidney beans, and peas. They also observed slight increases in sulfur-containing amino acid concentrations following different processing methods, including microwave cooking, autoclaving, fermentation, and micronization. Similarly, Nosworthy et al. (2017a) reported that TRP was the first limiting amino acid for extruded (AAS of 0.72) and boiled (AAS of 0.78) yellow split peas. However, their study found sulfur-containing amino acids to be the first LAA in the baked yellow split pea sample (AAS of 0.79), which is inconsistent with the findings of the present study.

Overall, TRP (AAS of 0.91 - 0.97) was identified as the most limiting amino acid in both untreated and treated flour and protein concentrate samples, as well as the coarse fraction sample. Conversely, the sulfur-containing amino acids (AAS of 0.84 - 0.91) were the first LAA in the baked protein concentrate and all 80% and 90% isolate samples. Notably, the AAS values of the yellow field pea samples investigated in the current study were generally higher than those reported in previous studies. In conclusion, a more balanced and nutritionally complete protein profile can be achieved, in which pulse-based proteins including yellow field pea-derived samples emphasize the deficiencies in sulfur-containing amino acids and TRP, making them particularly suitable for complementary pairing with cereals, which are typically low in lysine content.

Given that *in vivo* methods are costly, time-consuming, and the ethical concerns associated with animal use, *in vitro* methods have been increasingly developed and implemented as alternatives to assess protein digestibility, offering relatively comparable reliability to *in vivo* methods (Nosworthy et al., 2017a). Protein digestibility is a critical factor in determining the nutritional quality of food products, as it reflects the extent to which proteins are hydrolyzed and

subsequently absorbed by the human body (Bessada et al., 2019). The *in vitro* protein digestibility (IVPD) in the present study, was determined using the pH-drop multi-enzyme method, which involves a combination of trypsin, chymotrypsin, and peptidase to simulate proteolytic digestion. This method relies on measuring the drop in pH as proteins are hydrolyzed by the enzyme cocktail. The resulting pH decrease is converted into an IVPD value using a regression equation: $Y = 65.66 + 18.10 \times \Delta pH_{10min}$. This method has been widely applied and validated as a predictor of apparent fecal protein digestibility (AFPD) in numerous studies (Hsu et al., 1977; Nosworthy et al., 2017a; Khattab et al., 2009; Ma et al., 2017; Konieczny et al., 2020; Çabuk et al., 2018; Bai et al., 2018; Shi et al., 2022; Zhong et al., 2015). Extensive research has also established a strong correlation between AFPD determined using the pH-drop method and *in vivo* measurements through the rat feeding assay, with a correlation coefficient (R^2) of 0.90, thereby validating its accuracy and reliability as a surrogate measure of protein digestibility (Hsu et al., 1977). Nevertheless, true fecal protein digestibility (TPD) addresses the constraint of AFPD by correcting for endogenous protein losses, thereby providing a more accurate measurement of the actual digestibility of the dietary protein. In the current study, additional key regression equations were utilized to estimate true digestibility values, derived from established *in vivo* data sources: 1) true fecal protein digestibility 1 (TPD1) (Franczyk et al., 2025), 2) true fecal protein digestibility 2 (TPD2) (Mendes et al., 2016). In summary, the regression-based estimation methods provide a comprehensive assessment framework for comparing AFPD with TPD values to more accurately determine protein quality in pulse-based processed food systems.

The effect of various processing methods on the IVPD and *in vitro* protein digestibility-corrected amino acid score (*in vitro* PDCAAS) of yellow field pea and its protein derivatives, is given in **Table 5.8**. Among the flour samples, AFPD values ranged from 82.21% to 87.54%, following the trend: boiling > autoclaving > micronization > baking > HHP > untreated. The TPD1

method provided even higher values, ranging from 90.10% to 94.60%, showing a consistent trend with AFPD. Both AFPD and TPD1 values indicated that thermal and non-thermal treatments significantly increased ($p < 0.05$) the protein digestibility of the pea flour samples, with boiled samples exhibiting the highest digestibility. In contrast, the TPD2 values revealed an opposite pattern, ranging from 89.43% to 90.33% (untreated > HHP > baking > micronization> autoclaving > boiling). The TPD2 values indicated significant reductions ($p < 0.05$) in protein digestibility following both thermal and non-thermal treatments among the flour samples. WHO & FAO (2007) reported a TPD of 88% for mature peas, aligning with the TPD1 and TPD2 values observed for untreated pea flour in the current study. Previous research on boiled and baked yellow split pea flours reported IVPD (i.e., AFPD) values of 86.75% and 82.49%, respectively, while TPD values measured by a rat bioassay were higher, at 89.00% for boiled and 86.77% for baked samples (Nosworthy et al., 2017a). These findings are in agreement with the observations in the current study. However, there was a modest correlation between IVPD and TPD, with an R^2 value of 0.6426, as reported by Nosworthy et al. (2017a). The variability in protein digestibility due to processing conditions has been well-documented. For example, Khattab et al. (2009) demonstrated significant improvements in protein digestibility for cowpea, kidney bean, and pea samples subjected to various treatments. Boiling (87.36% – 98.14%), microwave heating (88.63% – 92.80%), autoclaving (78.95% – 90.26%), soaking (75.96% – 87.45%), and fermentation (73.43% – 85.10%) all demonstrated notable enhancements compared to raw samples (70.53% – 82.29%). In contrast, micronization (67.90% – 80.03%) and roasting (64.92% – 77.59%) resulted in the lowest digestibility results among the tested methods. Interestingly, the untreated protein isolates demonstrated higher AFPD and TPD1 values compared to the flour and protein concentrates, with values of 89.37% and 96.14% for the 80% protein isolates, and 90.38% and 96.99% for the 90% protein isolates, respectively. These results demonstrate the protein digestibility of the protein

isolates is greater than the flour, a phenomenon that has been previously noted in the study of Shi et al. (2022). The lower digestibility of flour may be attributed to the presence of interfering components such as lipids and fibers in the pulse flour, which can hinder enzymatic access to proteins. Protein digestibility can be improved through the fractionation process or protein isolation process (Konieczny et al., 2020). This is also consistent with the findings of the current study, where protein concentrate exhibited significantly higher ($p < 0.05$) AFPD and TPD1 values compared to the coarse fraction that is richer in starch.

Focusing on the pea protein derivatives in different treatments, the AFPD value for the protein concentrate samples ranged from 83.78% to 86.52%, with a significant improvement ($p < 0.05$) observed following HHP treatment. Similarly, the TPD1 values of the protein concentrate samples, ranging from 91.43% to 93.73% (HHP > baking > untreated), were higher than AFPD and TPD2. In contrast, the TPD2 values showed a more stable range, from 89.60% in the HHP-treated sample to 90.07% in the untreated sample. Baking significantly decreased ($p < 0.05$) AFPD and TPD1 values in both 80% and 90% isolate samples, while it significantly increased ($p < 0.05$) TPD2 values in the 90% isolate. Baking had no significant effect ($p > 0.05$) on AFPD, TPD1, or TPD2 values in the protein concentrate and 80% protein isolate. The increments in protein digestibility observed in this study following different processing methods may be due to the breakdown of the protein structures, allowing amino acids and short-chain peptides to be liberated and more accessible to proteolytic enzymes, as stated in Konieczny et al. (2020). HHP by applying high pressure at 600 MPa, especially combined with a mild temperature of 60°C, has also been linked to improvements in protein digestibility by altering the structural integrity of proteins (Linsberger-Martin et al., 2013).

Notably, TPD2 consistently demonstrated an inverse relationship with AFPD and TPD1 across all tested samples. Among the three IVPD methods, AFPD yielded the lowest values for

flour and protein concentrate samples, with the ranking as follows: TPD1 > TPD2 > AFPD. However, for both 80% and 90% isolate samples, TPD2 values were the lowest, with the ranking being TPD1 > AFPD > TPD2. Moreover, the minimal differences observed among TPD2 values across different samples suggest that TPD2 is less influenced by processing treatments compared to TPD1 and AFPD.

Overall, this study highlighted the effectiveness of various processing methods in enhancing the protein digestibility of yellow field pea samples, with boiling emphasized as advantageous, as evidenced by higher values of AFPD and TPD1. The TPD1 method, which accounts for endogenous protein losses, better reflects the significant improvements in protein digestibility across different treatments, thereby contributing to more accurate and higher digestibility values for all untreated and treated pea samples.

With regard to protein quality, significant improvements ($p < 0.05$) were observed only in the boiled flour samples, as indicated by AFPD and TPD1 values of 84.74% and 91.57%, respectively. In contrast, other processing treatments either maintained or reduced protein quality. In particular, baking resulted in a significant decrease ($p < 0.05$) in the protein quality of the 90% isolate. Nosworthy et al. (2017a) showed a strong correlation between *in vitro* and *in vivo* measurements of protein quality, with an R^2 value of 0.9745. When compared to the protein quality observed in the present study, the *in vitro* and *in vivo* PDCAAS values were generally lower in their study, despite reflecting relatively comparable protein digestibility. Specifically, boiled yellow pea split peas demonstrated PDCAAS values of 67.44% (*in vitro*) and 69.19% (*in vivo*), and baked yellow split peas showed values of 65.49% (*in vitro*) and 68.89% (*in vivo*) (Nosworthy et al., 2017a). Similarly, a lower *in vitro* PDCAAS of 67% was reported for pea protein concentrate, which was attributed to its lower AAS (0.84) and lower IVPD (80%) (Çabuk et al., 2018), findings that are consistent with those of Konieczny et al. (2020). Moreover, Shi et al. (2022) demonstrated

that the protein quality of yellow pea flour (71.7%) exceeded that of the pea protein isolate (64.8%). However, in the present study, protein quality based on the TPD1 values showed the untreated flour (85.71%) to have slightly lower protein quality than the 90% protein isolate (87.03%). In the current study, the *in vitro* PDCAAS determined by AAS of LAA and TPD1 consistently yielded the highest values. Nonetheless, the *in vitro* PDCAAS was relatively unaffected by the investigated processing treatments. The study by Bui (2024) indicated that improvements *in vitro* PDCAAS for processed samples were only observed when using the INFOGEST digestion method rather than the pH-drop method, further highlighting the variability in protein quality assessments depending on the methodology used.

In summary, the use of TPD1 has proven to be a preferable and reliable method for assessing the protein quality, offering valuable insights into their nutritional value and quality of yellow field pea samples. Future research could explore alternative methods, such as INFOGEST or other advanced methods, to enhance the accuracy of protein digestibility and quality assessments in processed pulses and their derivatives.

Table 5.7. Amino acid scores of untreated and treated whole yellow field pea flours, protein concentrates, coarse fraction, 80% protein isolates, and 90% protein isolates.

Sample Description	THR	VAL	M+C	ILE	LEU	P+T	HIS	LYS	TRP
Whole Pea Flour									
Untreated	1.07^{ab}	1.33^{a}	1.02^{a}	1.48^{bc}	1.08^{b}	1.23^{a}	1.24^{a}	1.27^{ab}	0.95^{ab}
Boiled	1.10^{a}	1.36^{a}	0.98^{ab}	1.56^{a}	1.14^{a}	1.25^{a}	1.27^{a}	1.28^{a}	0.97^{a}
Autoclaved	1.08^{ab}	1.33^{a}	0.96^{b}	1.51^{ab}	1.09^{ab}	1.23^{a}	1.26^{a}	1.27^{ab}	0.94^{ab}
Micronized	1.08^{ab}	1.33^{a}	0.99^{ab}	1.49^{ab}	1.09^{ab}	1.25^{a}	1.23^{a}	1.25^{abc}	0.92^{b}
Baked	1.07^{ab}	1.32^{a}	0.94^{b}	1.48^{abc}	1.08^{b}	1.21^{a}	1.26^{a}	1.22^{bc}	0.93^{ab}
HHP-treated	1.04^{b}	1.27^{b}	1.02^{a}	1.41^{c}	1.03^{c}	1.20^{a}	1.29^{a}	1.20^{c}	0.92^{b}
Pooled SE	**0.0003**	**0.0003**	**0.0003**	**0.0008**	**0.0003**	**0.0012**	**0.0008**	**0.0004**	**0.0002**
***P*-value**	**0.0380**	**0.0006**	**0.0011**	**0.0012**	**0.0003**	**0.2655**	**0.1483**	**0.0022**	**0.0064**
Protein Concentrate									
Untreated	1.11^{a}	1.36^{a}	0.98^{b}	1.55^{a}	1.13^{a}	1.31^{a}	1.31^{ab}	1.33^{a}	0.91^{a}
Baked	1.04^{a}	1.29^{a}	0.90^{c}	1.47^{ab}	1.06^{ab}	1.23^{ab}	1.27^{b}	1.17^{b}	0.93^{a}
HHP-treated	1.06^{a}	1.32^{a}	0.99^{b}	1.48^{ab}	1.09^{ab}	1.28^{ab}	1.37^{a}	1.25^{ab}	0.93^{a}
Coarse fraction	1.06^{a}	1.29^{a}	1.10^{a}	1.42^{b}	1.03^{b}	1.18^{b}	1.26^{b}	1.23^{ab}	0.91^{a}
Pooled SE	**0.0013**	**0.0010**	**0.0004**	**0.0021**	**0.0013**	**0.0016**	**0.0012**	**0.0015**	**0.0009**
***P*-value**	**0.1640**	**0.0727**	**<0.0001**	**0.0514**	**0.0371**	**0.0207**	**0.0209**	**0.0082**	**0.5976**

Data are expressed as mean of three replicates (single measurement per replicate). Mean values followed by different superscript letters within the column of each yellow field pea type indicate statistically significant differences between treatments ($p < 0.05$) using one-way ANOVA along with Tukey's multiple comparison test. The reference pattern used to determine the amino acid scores was THR, 34; VAL, 35; MET+CYS, 25; ILE, 28; LEU, 66; PHE+TYR, 63; HIS, 19; LYS, 58; TRP, 11.

Abbreviations: THR = Threonine; VAL = Valine; M+C = Methionine + Cysteine; ILE = Isoleucine; LEU = Leucine; P+T = Phenylalanine + Tyrosine; HIS = Histidine; LYS = Lysine; TRP = Tryptophan.

Table 5.7. Continued.

Sample Description	THR	VAL	M+C	ILE	LEU	P+T	HIS	LYS	TRP
80% Protein Isolate									
Untreated	1.01[a]	1.35[a]	0.85[ab]	1.58[a]	1.17[a]	1.33[a]	1.21[ab]	1.24[a]	0.93[a]
Baked	1.01[a]	1.34[a]	0.79[b]	1.58[a]	1.17[a]	1.33[a]	1.19[b]	1.23[a]	0.93[a]
HHP-treated	1.04[a]	1.37[a]	0.89[a]	1.60[a]	1.20[a]	1.37[a]	1.30[a]	1.25[a]	0.96[a]
Pooled SE	**0.0004**	**0.0007**	**0.0006**	**0.0009**	**0.0005**	**0.0006**	**0.0014**	**0.0006**	**0.0004**
***P*-value**	**0.2686**	**0.4525**	**0.0069**	**0.8034**	**0.3275**	**0.1161**	**0.0317**	**0.7017**	**0.2620**
90% Protein Isolate									
Untreated	1.19[a]	1.43[a]	0.90[a]	1.60[a]	1.15[a]	1.41[a]	1.31[ab]	1.39[a]	0.95[a]
Baked	1.17[a]	1.40[a]	0.84[b]	1.58[a]	1.14[a]	1.38[a]	1.26[b]	1.36[a]	0.95[a]
HHP-treated	1.21[a]	1.43[a]	0.91[a]	1.61[a]	1.16[a]	1.41[a]	1.40[a]	1.39[a]	1.05[a]
Pooled SE	**0.0004**	**0.0004**	**0.0004**	**0.0009**	**0.0003**	**0.0009**	**0.0026**	**0.0004**	**0.0085**
***P*-value**	**0.1149**	**0.2785**	**0.0103**	**0.4711**	**0.4418**	**0.4849**	**0.0370**	**0.3150**	**0.3798**

Data are expressed as mean of three replicates (single measurement per replicate). Mean values followed by different superscript letters within the column of each yellow field pea type indicate statistically significant differences between treatments ($p < 0.05$) using one-way ANOVA along with Tukey's multiple comparison test. The reference pattern used to determine the amino acid scores was THR, 34; VAL, 35; MET+CYS, 25; ILE, 28; LEU, 66; PHE+TYR, 63; HIS, 19; LYS, 58; TRP, 11.

Abbreviations: THR = Threonine; VAL = Valine; M+C = Methionine + Cysteine; ILE = Isoleucine; LEU = Leucine; P+T = Phenylalanine + Tyrosine; HIS = Histidine; LYS = Lysine; TRP = Tryptophan.

Table 5.8. Summary of AAS of limiting amino acid (LAA), LAA, *in vitro* protein digestibility (%), and *in vitro* protein digestibility-corrected amino acid score (*in vitro* PDCAAS, %) of untreated and treated whole yellow field pea flours, protein concentrates, coarse fraction, 80% protein isolates, and 90% protein isolates.

Sample Description	AAS of LAA	LAA	AFPD	TPD1	TPD2	AFPD_PDCAAS	TPD1_PDCAAS	TPD2_PDCAAS
Whole Pea Flour								
Untreated	0.95	TRP	82.21^{e}	90.10^{e}	90.33^{a}	78.20^{bc}	85.71^{b}	85.93^{ab}
Boiled	0.97	TRP	87.54^{a}	94.60^{a}	89.43^{e}	84.74^{a}	91.57^{a}	86.57^{a}
Autoclaved	0.94	TRP	86.35^{b}	93.60^{b}	89.63^{d}	81.22^{ab}	88.03^{ab}	84.30^{abc}
Micronized	0.92	TRP	85.51^{bc}	92.89^{bc}	89.77^{cd}	78.48^{bc}	85.25^{b}	82.39^{c}
Baked	0.93	TRP	84.42^{cd}	91.97^{cd}	89.96^{bc}	78.76^{bc}	85.80^{b}	83.93^{abc}
HHP-treated	0.92	TRP	83.88^{d}	91.51^{d}	90.05^{b}	77.40^{c}	84.44^{b}	83.09^{bc}
Pooled SE	/	/	**0.1648**	**0.1172**	**0.0047**	**1.680**	**1.866**	**1.460**
***P*-value**	/	/	**<0.0001**	**<0.0001**	**<0.0001**	**0.0001**	**0.0004**	**0.0082**
Protein Concentrate								
Untreated	0.91	TRP	83.78^{b}	91.43^{b}	90.07^{b}	75.85^{a}	82.77^{a}	81.52^{a}
Baked	0.90	MET+CYS	84.26^{b}	91.83^{b}	89.99^{b}	75.59^{a}	82.37^{a}	80.71^{a}
HHP-treated	0.93	TRP	86.52^{a}	93.73^{a}	89.60^{c}	80.52^{a}	87.24^{a}	83.40^{a}
Coarse fraction	0.91	TRP	80.78^{c}	88.90^{c}	90.57^{a}	73.60^{a}	80.99^{a}	82.51^{a}
Pooled SE	/	/	**0.1587**	**0.1128**	**0.0046**	**9.274**	**10.64**	**8.895**
***P*-value**	/	/	**<0.0001**	**<0.0001**	**<0.0001**	**0.1098**	**0.1833**	**0.7169**

Protein digestibility results are expressed as mean of three replicates (three measurements per replicate), while AAS are expressed as mean of three replicates (single measurement per replicate). Mean values followed by different superscript letters within the column of each yellow field pea type indicate statistically significant differences between treatments ($p < 0.05$) using one-way ANOVA along with Tukey's multiple comparison test.

Abbreviations: AAS =Amino acid score; AFPD = *in vitro* apparent fecal protein digestibility; TPD = *in vitro* true fecal protein digestibility; PDCAAS = protein digestibility-corrected amino acid score.

Table 5.8. Continued.

Sample Description	AAS of LAA	LAA	AFPD	TPD1	TPD2	AFPD _PDCAAS	TPD1 _PDCAAS	TPD2 _PDCAAS
80% Protein Isolate								
Untreated	0.85	MET+CYS	89.37^{ab}	96.14^{ab}	89.12^{ab}	75.94^{ab}	81.69^{ab}	75.72^{ab}
Baked	0.79	MET+CYS	88.93^{b}	95.77^{b}	89.19^{a}	70.61^{b}	76.04^{b}	70.82^{b}
HHP-treated	0.89	MET+CYS	90.03^{a}	96.70^{a}	89.01^{b}	80.24^{a}	86.18^{a}	79.32^{a}
Pooled SE	/	/	**0.1278**	**0.0908**	**0.0037**	**5.184**	**5.805**	**4.353**
***P*-value**	/	/	**0.0249**	**0.0249**	**0.0249**	**0.0061**	**0.0062**	**0.0072**
90% Protein Isolate								
Untreated	0.90	MET+CYS	90.38^{a}	96.99^{a}	88.95^{b}	81.10^{a}	87.03^{a}	79.82^{a}
Baked	0.84	MET+CYS	89.71^{b}	96.43^{b}	89.06^{a}	75.01^{b}	80.62^{b}	74.46^{b}
HHP-treated	0.91	MET+CYS	90.78^{a}	97.33^{a}	88.88^{b}	82.63^{a}	88.60^{a}	80.91^{a}
Pooled SE	/	/	**0.0655**	**0.0466**	**0.0019**	**3.431**	**3.975**	**3.489**
***P*-value**	/	/	**0.0063**	**0.0063**	**0.0063**	**0.0053**	**0.0060**	**0.0116**

Protein digestibility results are expressed as mean of three replicates (three measurements per replicate), while AAS are expressed as mean of three replicates (single measurement per replicate). Mean values followed by different superscript letters within the column of each yellow field pea type indicate statistically significant differences between treatments ($p < 0.05$) using one-way ANOVA along with Tukey's multiple comparison test.

Abbreviations: AAS =Amino acid score; AFPD = *in vitro* apparent fecal protein digestibility; TPD = *in vitro* true fecal protein digestibility; PDCAAS = protein digestibility-corrected amino acid score.

5.4.2 Reactive Lysine Analysis

The comprehensive comparison of total and reactive hydrated lysine contents (as is basis) across various yellow field pea-derived products, including flour, protein concentrate, coarse fraction, and both 80% and 90% protein isolates, subjected to different processing treatments, along with the untreated coarse fraction, is detailed in **Table 5.9**. For the untreated samples, this study showed minimal differences between total and reactive lysine contents in both untreated flour and protein concentrate. Specifically, the untreated flour exhibited a high percentage of reactive lysine, giving 93.60% of the total lysine remaining in its reactive form, with specific total and reactive lysine contents of 1.57% and 1.47%, respectively. In the untreated protein concentrate, the reactive lysine content even exceeded the total lysine content, reaching 106.83%. These findings suggest that the majority of lysine in these samples was unmodified and readily bioavailable for nutritional utilization, as no processing-induced modifications or losses have occurred. Such observations are consistent with previous studies, which have reported a high proportion of reactive lysine relative to total lysine in untreated samples. Rutherfurd & Moughan (1997) explored that with the use of the guanidination method for reactive lysine, untreated skim milk powder had identical total and reactive lysine contents, both measured at 38.1 mg/g air dry weight. Similarly, in untreated peas, total lysine content was reported as 15.1 mg/g air dry weight and reactive lysine content was 14.9 mg/g air dry weight, indicating that 98.68% of the total lysine was in the reactive form. Additionally, Lalitha & Singh (2020) reported that the percentages of reactive lysine in horsegram protein concentrate with a protein content of 78.3%, increased from 65% to 91% of total lysine when derived from the horsegram flour with a protein content of 22.8%. This notable increase in reactive lysine percentages could be attributed to differences in the inherent composition and structural characteristics of the proteins, as well as variations in

processing methods and analytical techniques employed in the studies. Conversely, substantial reductions in reactive lysine in the present study were observed in the untreated 80% and 90% protein isolates, where only 24.69% and 38.25% of the total lysine are reactive, respectively. These reductions indicated that a large proportion of lysine's ε – amino groups in the protein isolate samples may have been chemically modified or become bound to other compounds, or damaged during the protein isolation process, potentially due to pH variations and other factors (Moughan & Rutherfurd, 2008). This renders them nutritionally unavailable and could negatively impact the protein quality of the isolates. However, this is inconsistent with the findings of Hodgkinson et al. (2022), in which the total lysine of whey protein isolate contained 100% reactive lysine, meaning that the lysine structure was unmodified throughout the isolation process.

In the current study, the reactive lysine content in the coarse fraction unexpectedly exceeded the total lysine content, reaching 126.05% of the total lysine content. A similar anomaly was observed in the untreated protein concentrate. The unexpected result, where reactive lysine content surpasses or does not align with total lysine content in untreated samples, may arise from using distinct analytical methods for quantifying total and reactive lysine. In previous studies, the determinations of both total and reactive lysine often relied on regular amino acid analysis. Specifically, for assessing reactive lysine, the guanidination method was employed preceding the regular amino acid hydrolysis to selectively target the ε – amino group of lysine for accurate quantification (Rutherfurd & Moughan, 2007; Rutherfurd et al., 2012). These studies have reported comparable results for total and reactive lysine in various unprocessed samples (Rutherfurd & Moughan, 2007; Rutherfurd et al., 2012). In the current study, total lysine was quantified via regular amino acid analysis, which accounts for all lysine residues present in a sample (reactive lysine and non-reactive (reverted) lysine), while reactive lysine, indicative of the nutritionally available form, was assessed through the OPA fluorometric method (Rutherfurd, 2015; Barba et

al., 2013). Thus, the methodological differences in lysine measurement may pose challenges that complicate direct comparisons between total and reactive lysine, as well as between the findings of the current study and those of previous studies.

The comparison between total lysine and reactive lysine can be used as a key indicator for assessing the extent to which lysine has been bound or modified during food processing, in other words, the amount of lysine that remains available in its reactive form with free ε – amino groups (Rutherfurd, 2015). Among the flour samples in the present study, boiling had the most detrimental effect on lysine, leaving only 26.57% of the total lysine in the reactive form, followed by baking (55.87% of the total lysine). Autoclaving and micronization as thermal treatments had a comparatively milder impact on reactive lysine, which was 69.48% and 75.66% in the total lysine, respectively. In contrast, HHP treatment showed the highest retention of reactive lysine, representing 95.77% of the total lysine. Similarly, the baked protein concentrate showed a significant reduction in reactive lysine content, retaining only 55.50% of the total lysine, while the HHP-treated protein concentrate preserved a considerably higher proportion of reactive lysine, with 71.33% of the total lysine. Both 80% and 90% protein isolate samples demonstrated reduced reactive lysine percentages following baking, accounting for only 19.12% and 30.53% of the total lysine, respectively. These percentages were lower compared to untreated (24.69% and 38.25%, respectively) and HHP-treated (21.50% and 30.63%, respectively) samples. These findings underscore the fact that most thermal processing methods lead to a significant loss of lysine, while HHP as a non-thermal treatment, is more effective in preserving reactive lysine content. As reported in previous studies (Rutherfurd & Moughan, 1997; Kim et al., 2012; Fontaine et al., 2007; Hodgkinson et al., 2022), the proportion of reactive lysine can vary significantly depending on the processing method or processing conditions (e.g., time and temperature), which largely attributed

to the occurrence of the Maillard reaction, which can modify lysine and reduce its nutritional availability.

The effect of different processing treatments on both total and reactive lysine contents (expressed on the DM and protein bases) in yellow field pea flour, protein concentrate, and both 80% and 90% protein isolates, including data for the untreated coarse fraction, is present in **Table 5.10**. The total lysine content increased proportionally with increasing protein content and was relatively stable across all processing treatments. Specifically, when expressed on a DM basis, the total lysine content was found to be 0.99% in the coarse fraction, 1.60 – 1.79% in the flour samples, 3.51 – 3.76% in the protein concentrate samples, 5.85 – 5.91% in the 80% protein isolate samples, and 6.98 – 7.15% in the 90% protein isolate samples. Significant changes ($p < 0.05$) in the total lysine content were only found in the HHP-treated flour, as well as in the baked and HHP-treated protein concentrates, with decreases of 6.58%, 6.64%, and 6.59%, respectively, when compared to their respective untreated samples. After conversion to a protein basis, the total lysine contents of all untreated and treated pea samples ranged from 6.80% to 8.07%, with no statistically significant differences, except for the HHP-treated flour (6.94%) and baked protein concentrate (6.80%), both of which were significantly lower ($p < 0.05$) when compared to the corresponding untreated samples. Overall, total lysine content was not substantially altered or enhanced by different processing methods. Consequently, measuring total lysine alone does not fully capture the true nutritional value and quality of processed products, underscoring the need to assess reactive lysine for an accurate evaluation of bioavailable lysine content.

In contrast to the observed trends for total lysine, a different pattern was evident for reactive lysine as its susceptibility to chemical modifications did not change significantly with increased protein purity in the tested samples. Among the untreated samples, the highest reactive lysine content on a DM basis was observed in the protein concentrate, with a value of 4.02%, followed

by the 90% protein isolate at 2.73%. In contrast, the flour and 80% protein isolate demonstrated comparatively lower reactive lysine contents, which were 1.61% and 1.46%, respectively. However, after adjusting to the protein basis, the reactive lysine contents in the flour and protein concentrate increased to 6.87% and 8.23%, respectively. Only minor changes were discovered in the reactive lysine contents of both 80% and 90% protein isolates, which reached 1.77% and 3.07%, respectively. Focusing on the protein isolates produced through the aqueous pH-phased method under different degrees of isolation conditions, the untreated 90% protein isolate demonstrated higher reactive lysine content than the untreated 80% isolate on both DM and protein bases. This may be due to the fact that the higher protein purity in the 90% isolate has more reactive lysine while removing more non-protein components. Additionally, in comparison to the reactive lysine content in the protein concentrate, the coarse fraction with a lower protein content exhibited a lower reactive lysine content of 1.25% on a DM basis. However, when expressed on a protein basis, its reactive lysine content increased to 9.02%, showing no significant difference ($p > 0.05$) compared to the protein concentrate.

Different processing treatments had a remarkable effect on the reactive lysine content of the yellow field pea samples. Among the flour samples, reactive lysine content varied widely, ranging from 0.48% to 1.61% on a DM basis and 1.97% to 6.87% on a protein basis. The ranking of reactive lysine content across treatments, consistent on both DM and protein bases, was as follows: boiling < baking < autoclaving < micronization < HHP < untreated. The most significant reduction ($p < 0.05$) in reactive lysine content was observed in the boiled flour, with a substantial decrease of at least 70.32% on both unit bases. This considerable loss raises concerns about maintaining the protein quality of the final products subjected to boiling. In contrast, the thermal treatments of autoclaving and micronization induced a relatively minimal effect on reactive lysine reduction in the flour, resulting in non-significant decreases ($p < 0.05$) of up to 25.55% and 20.40%,

respectively, on both bases. Baking, which employs dry heat, had a considerably reduced effect on the reactive lysine content in the pea flour and its derivatives. In the baked flour, reactive lysine content was significantly reduced ($p < 0.05$) by up to 42.72% on both bases, though this effect was less severe than that of boiling. Furthermore, baking resulted in significant losses ($p < 0.05$) in reactive lysine across all sample types, with reductions observed at up to 54.13% in the protein concentrate, 23.09% in the 80% protein isolate, and 22.04% in 90% protein isolate across both DM and protein bases. The findings above are in close agreement with those of previous studies, which consistently demonstrate that reactive lysine is significantly more susceptible to degradation by processing than total lysine (Fontaine et al., 2007; Rutherfurd & Moughan, 1997; Van Rooijen et al., 2014; Kim et al., 2012; Hodgkinson et al., 2022). For example, Rutherfurd & Moughan (1997) reported that both total and reactive lysine contents decreased with increased processing time for skim milk powder and with elevated processing temperature for field peas. Specifically, after autoclaving skim milk powder at 121°C for up to 10 min, the total lysine contents decreased by up to 33%, while the reactive lysine contents decreased by up to 82%. Similarly, field peas subjected to autoclaving at 165°C for 15 min experienced maximum reductions of 42% in total lysine and 64% in reactive lysine. Therefore, the reactive lysine contents were consistently lower compared to total lysine contents in processed products, and both decreased with increasing severity of the processing conditions. This observation is also aligned with the findings of Kim et al. (2012), which showed that the difference between total and reactive lysine progressively increased with prolonged heating time. This phenomenon is likely attributed to lysine degradation during thermal processing, primarily driven by the formation of Maillard reaction products, as well as protein denaturation and aggregation (Moughan & Rutherfurd, 2008). Overall, the impact of processing conditions on the denaturation of both total and reactive lysine is significant, with reactive lysine exhibiting a greater susceptibility to thermal treatments.

HHP treatment, as a non-thermal process, appears to have a protective effect on reactive lysine when compared to thermal treatments. On both a DM and protein bases, HHP treatment resulted in statistically significant ($p < 0.05$) reductions in the reactive lysine contents of the protein concentrate and 80% protein isolate, with decreases of up to 37.64% and 12.66%, respectively. In contrast, smaller and non-significant reductions ($p > 0.05$) in reactive lysine content were observed in the flour and 90% protein isolate subjected to HHP treatment, with decreases of up to 4.36% and 4.73%, respectively. HHP treatment exerted a relatively lower impact on reactive lysine when compared to thermal treatments, likely due to its non-thermal nature in protecting lysine from chemical interactions, suggesting that HHP is more effective at preserving reactive lysine and further maintaining the nutritional quality of processed food products (Aganovic et al., 2021). Additionally, HHP-induced preservation in reactive lysine could be also attributed to its ability to suppress the progression of the Maillard reaction beyond the initial stages. While HHP can accelerate early interactions between reducing sugars and amino compounds (such as lysine), leading to the formation of Amadori rearrangement products (ARP), however, it does not significantly promote the later stage of the Maillard reaction. This limitation arises because HHP alone does not generate enough heat to drive the Maillard reaction to the formation of more advanced Maillard reaction products, which are responsible for browning, flavor compounds, and lysine degradation, particularly under a low-pH environment (pH < 8) (Aganovic et al., 2021; Moreno et al., 2002). Moreover, the progression of the Maillard reaction can also be influenced by other factors such as oxygen availability, water activity, and temperature (Moreno et al., 2002). As such, the reactive lysine can be preserved in a form that remains nutritionally available following HHP treatment. However, drawing direct comparison with existing literature is challenging due to the scarcity of research investigating the effects of HHP on reactive lysine content in pulses and their derivatives.

Overall, the reactive lysine was more sensitive to degradation from heat and other processing conditions compared to total lysine, whereas HHP treatment was more effective in retaining reactive lysine contents when compared to other thermal methods. The present study highlights the significant differences and complexities in how various processing treatments impact total and reactive lysine, with these effects highly influenced by specific processing parameters applied, such as temperature, exposure time, and moisture levels. The observed reductions in the ratio of reactive lysine to total lysine following the use of various processing methods suggest that a portion of the lysine quantified through regular amino acid analysis may not be nutritionally available. Furthermore, the differences between total and reactive lysine contents reveal how each processing method influences lysine availability, and by extension, the overall protein quality of the food products. Given these findings, it is crucial to have specific assays for accurately measuring reactive lysine, as the regular amino acid analysis may not provide a complete picture of the nutritional utilization of lysine.

Table 5.9. Comparison of total and reactive hydrated lysine contents of whole yellow field pea flours, protein concentrates, coarse fraction, 80% protein isolates, and 90% protein isolates before and after processing (as is basis).

Sample Description	Total Lysine Content (%)	Reactive Lysine Content (%)	Reactive Lysine in Total Lysine (%)
Whole Pea Flour			
Untreated	1.57^{bc}	1.47^{a}	93.60
Boiled	1.73^{a}	0.46^{c}	26.57
Autoclaved	1.70^{a}	1.18^{ab}	69.48
Micronized	1.62^{b}	1.23^{ab}	75.66
Baked	1.60^{b}	0.90^{b}	55.87
HHP-treated	1.52^{c}	1.46^{a}	95.77
Pooled SE	**0.0008**	**0.0214**	/
***P*-value**	**<0.0001**	**<0.0001**	/
Protein Concentrate			
Untreated	3.45^{a}	3.68^{a}	106.83
Baked	3.17^{b}	1.76^{bc}	55.50
HHP-treated	3.32^{ab}	2.37^{b}	71.33
Coarse fraction	0.96^{c}	1.21^{c}	126.05
Pooled SE	**0.0043**	**0.0728**	/
***P*-value**	**<0.0001**	**<0.0001**	/

Data are expressed as mean of three replicates (single measurement per replicate for total lysine; three measurements per replicate for reactive lysine) and presented on as is basis. Mean values followed by different superscript letters within the column of each yellow field pea type indicate statistically significant differences between treatments ($p < 0.05$) using one-way ANOVA along with Tukey's multiple comparison test.
Abbreviation: SE = Standard error.

Table 5.9. Continued.

Sample Description	Total Lysine Content (%)	Reactive Lysine Content (%)	Reactive Lysine in Total Lysine (%)
80% Protein Isolate			
Untreated	5.64[a]	1.39[a]	24.69
Baked	5.67[a]	1.08[b]	19.12
HHP-treated	5.67[a]	1.22[b]	21.50
Pooled SE	**0.0105**	**0.0037**	**/**
***P*-value**	**0.8752**	**0.0025**	**/**
90% Protein Isolate			
Untreated	6.79[a]	2.60[a]	38.25
Baked	6.77[a]	2.07[b]	30.53
HHP-treated	6.83[a]	2.50[a]	36.62
Pooled SE	**0.0155**	**0.0168**	**/**
***P*-value**	**0.8392**	**0.0053**	**/**

Data are expressed as mean of three replicates (single measurement per replicate for total lysine; three measurements per replicate for reactive lysine) and presented on an as is basis. Mean values followed by different superscript letters within the column of each yellow field pea type indicate statistically significant differences between treatments ($p < 0.05$) using one-way ANOVA along with Tukey's multiple comparison test.
Abbreviation: SE = Standard error.

Table 5.10. Comparison of total lysine and reactive hydrated lysine of untreated and treated whole yellow field pea flours, protein concentrates, coarse fraction, 80% protein isolates, and 90% protein isolates (DM and protein bases).

Sample Description	DM Basis				Protein Basis			
	Total Lysine (%)	**Relative Change (%)[1]**	**Reactive Lysine (%)**	**Relative Change (%)[1]**	**Total Lysine (%)**	**Relative Change (%)[1]**	**Reactive Lysine (%)**	**Relative Change (%)[1]**
Whole Pea Flour								
Untreated	1.72^{ab}	0.00	1.61^{a}	0.00	7.34^{ab}	0.00	6.87^{a}	0.00
Boiled	1.79^{a}	+4.44	0.48^{c}	-70.32	7.43^{a}	+1.20	1.97^{c}	-71.25
Autoclaved	1.78^{a}	+4.04	1.24^{ab}	-22.78	7.36^{ab}	+0.25	5.11^{ab}	-25.55
Micronized	1.71^{ab}	-0.57	1.29^{ab}	-19.66	7.23^{abc}	-1.49	5.47^{ab}	-20.40
Baked	1.65^{bc}	-4.08	0.92^{b}	-42.72	7.09^{bc}	-3.42	3.97^{b}	-42.25
HHP-treated	1.60^{c}	-6.58	1.54^{a}	-4.36	6.94^{c}	-5.45	6.64^{a}	-3.32
Pooled SE	**0.0011**	/	**0.0237**	/	**0.0138**	/	**0.4140**	/
***P*-value**	**<0.0001**	/	**<0.0001**	/	**0.0022**	/	**<0.0001**	/
Protein Concentrate								
Untreated	3.76^{a}	0.00	4.02^{a}	0.00	7.71^{a}	0.00	8.23^{a}	0.00
Baked	3.51^{b}	-6.64	1.95^{bc}	-51.50	6.80^{b}	-11.71	3.78^{b}	-54.13
HHP-treated	3.51^{b}	-6.59	2.50^{b}	-37.64	7.23^{ab}	-6.16	5.16^{b}	-37.38
Coarse fraction	0.99^{c}	-73.69	1.25^{c}	-68.96	7.15^{ab}	-7.18	9.02^{a}	+9.54
Pooled SE	**0.0053**	/	**0.0847**	/	**0.0510**	/	**0.7006**	/
***P*-value**	**<0.0001**	/	**<0.0001**	/	**0.0082**	/	**0.0002**	/

Data are expressed as mean of three replicates (single measurement per replicate for total lysine; three measurements per replicate for reactive lysine) and presented on the DM and protein bases. Mean values followed by different superscript letters within the column of each yellow field pea type indicate statistically significant differences between treatments ($p < 0.05$) using one-way ANOVA along with Tukey's multiple comparison test.

[1] Untreated sample of each category is considered as a baseline. The relative change of each treated sample was calculated based on the baseline.

Abbreviations: DM = Dry matter; SE = Standard error.

Table 5.10. Continued.

Sample Description	DM Basis				Protein Basis			
	Total Lysine (%)	**Relative Change (%)[1]**	**Reactive Lysine (%)**	**Relative Change (%)[1]**	**Total Lysine (%)**	**Relative Change (%)[1]**	**Reactive Lysine (%)**	**Relative Change (%)[1]**
80% Protein Isolate								
Untreated	5.90[a]	0.00	1.46[a]	0.00	7.18[a]	0.00	1.77[a]	0.00
Baked	5.85[a]	-0.69	1.12[b]	-23.09	7.15[a]	-0.38	1.37[b]	-22.83
HHP-treated	5.91[a]	+0.27	1.27[b]	-12.66	7.24[a]	+0.93	1.56[b]	-12.08
Pooled SE	**0.0116**	**/**	**0.0040**	**/**	**0.0188**	**/**	**0.0059**	**/**
***P*-value**	**0.8073**	**/**	**0.0020**	**/**	**0.7017**	**/**	**0.0020**	**/**
90% Protein Isolate								
Untreated	7.15[a]	0.00	2.73[a]	0.00	8.04[a]	0.00	3.07[a]	0.00
Baked	6.98[a]	-2.34	2.13[b]	-22.04	7.91[a]	-1.60	2.42[b]	-21.45
HHP-treated	7.11[a]	-0.53	2.60[a]	-4.73	8.07[a]	+0.38	2.96[a]	-3.88
Pooled SE	**0.0104**	**/**	**0.0205**	**/**	**0.0151**	**/**	**0.0263**	**/**
***P*-value**	**0.1911**	**/**	**0.0048**	**/**	**0.3149**	**/**	**0.0054**	**/**

Data are expressed as mean of three replicates (single measurement per replicate for total lysine; three measurements per replicate for reactive lysine) and presented on the DM and protein bases. Mean values followed by different superscript letters within the column of each yellow field pea type indicate statistically significant differences between treatments ($p < 0.05$) using one-way ANOVA along with Tukey's multiple comparison test.

[1] The untreated sample of each category is considered as a baseline. The relative change of each treated sample was calculated based on the baseline.

Abbreviations: DM = Dry matter; SE = Standard error.

5.5 CONCLUSION OF STUDY 2

This research was undertaken to show the effect of thermal and non-thermal methods on the *in vitro* protein quality and reactive lysine content in yellow field peas and their derivatives. TRP was identified to be the first LAA in the untreated and treated flour and protein concentrate samples, as well as the coarse fraction while sulfur-containing amino acids were the most limited in the baked protein concentrate and in both untreated and treated 80% and 90% protein isolates. Processing methods, especially boiling and HHP, significantly improved IVPD (determined using the TPD1 method), particularly in the flour. However, among all investigated samples, *in vitro* PDCAAS values were largely unaffected by both thermal and non-thermal treatments, with only boiling significantly improving the overall protein quality of the flour, which was not found in the derivatives. Conversely, non-thermal HHP treatment did not enhance nutritional quality but was effective in preserving reactive lysine. It is obvious that the high reactive lysine contents in untreated pea flour and protein concentrate remain unmodified and bioavailable. Thermal treatments drastically reduced the nutritional availability of lysine in the pea samples particularly when exposed to boiling and baking, likely diminishing overall protein quality. The extent of lysine modification varied depending on the processing methods, with reactive lysine showing greater susceptibility to thermal degradation when compared to total lysine. In contrast, HHP demonstrated superior preservation of reactive lysine, maintaining higher lysine bioavailability. The specialized OPA fluorometric assay proved to be reliable and efficient, offering significant advantages in simplicity, high sensitivity, and rapid throughput, making it an ideal method for large-scale analysis of reactive lysine. These results are significant for researchers and food processing industries aiming to optimize processing methods for protein quality improvement and reactive lysine retention for the development of sustainable plant-based products. Monitoring food processing conditions is essential to prevent excessive lysine degradation and maintain protein

quality. One significant limitation of this study is the potential inaccuracies in determining protein quality in the processed samples, suggesting that need to explore alternative methods or other advanced methods to more precisely evaluate protein digestibility and quality in processed pulses and their derivatives.

CHAPTER 6: GENERAL CONCLUSION

In conclusion, the effects of both thermal and non-thermal processing methods on ANFs, protein quality, and reactive lysine content in whole yellow field pea flour, protein concentrate, and protein isolates were investigated in this study.

In the first part of the study, all investigated thermal treatments were highly effective in reducing TIA, lectins, PA, and TPC across all samples. The application of boiling was the most effective hydrothermal method for eliminating heat-labile and heat-stable ANFs in the pea flour. Baking also showed notable efficacy in reducing ANFs in the flour and its derivatives. In contrast, HHP demonstrated a more modest reduction in ANFs but unexpectedly increased lectin and CT contents, especially in the flour and 90% protein isolate.

The second part of the study demonstrated that boiling significantly improved IVPD and was the only method that significantly enhanced *in vitro* PDCAAS in the pea flour. However, other processing methods had minimal effects on *in vitro* PDCAAS values across all the tested pea samples. Reactive lysine content, which was high in the untreated pea flour and protein concentrate, was significantly reduced by thermal treatments, particularly boiling and baking. In contrast, HHP effectively preserved reactive lysine, maintaining higher lysine bioavailability.

For the significance of this study, yellow field peas and their derivatives have the potential to become excellent protein sources with higher reactive lysine content and reduced concentrations of ANFs when subjected to appropriate processing treatments. While thermal methods are beneficial for reducing ANFs and improving IVPD and overall protein quality, HHP represents a promising alternative for preserving reactive lysine and maintaining overall protein quality. Therefore, HHP could be adopted and utilized in the food industry to meet consumer demands for healthier, more sustainable, and minimally processed protein sources. The significance of reactive

lysine determination lies in its critical role as a more accurate indicator of actual nutritionally available lysine in processed foods rather than total lysine content.

Limitations of the study include potential inaccuracies in specifically identifying the reactive forms of ANFs that exert anti-nutritional effects, as well as possible inaccuracies in assessing protein digestibility for processed samples. Additionally, the degree of the relationship between ANFs and protein quality was not explored in this study, presenting an area for future investigation.

CHAPTER 7: FUTURE DIRECTIONS

Future studies should aim to comprehensively evaluate the impact of different processing methods with different processing conditions (by adjusting exposure time, temperature, and pre-treatment conditions) on both nutritional and anti-nutritional profiles of a broader range of pulses and their derivatives. This will provide clearer guidelines for optimizing processing methods and conditions to improve health outcomes. In particular, further optimization of HHP conditions such as combining high pressure with mild heating or other treatments, could synergistically enhance the reduction of different ANFs. This approach could increase the versatility and efficiency of HHP as a processing technique, expanding its applicability across a wide range of food products.

Regarding reactive lysine, future research should focus on establishing standardized protocols for its measurement across various food products and processing conditions. This includes optimizing food processing methods to retain reactive lysine and developing strategies to compensate for lysine deficiencies in plant-based protein sources (e.g., combining pulses with cereals) based on the results measured by specialized assays such as the OPA fluorometric assay or other advanced alternative methods. Such efforts will contribute to improving the overall protein quality of various processed plant-based food products and ensure they meet the nutritional demands of consumers.

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APPENDIX I

Table A1. Potential reasons for ANF results.

ANF	Untreated	Thermal-treated	HHP-treated
TIA (Heat-labile)	**Concentrate > flour:** linked to higher protein content; starch removal via air classification **Lower in isolates:** isolation process	**Decreased:** heat-induced protein denaturation; disruption of bonds and binding sites of TIA	**Decreased:** structural changes/denaturation and subsequent aggregation of proteins through extreme pressure
Lectins (Heat-labile)		**Deceased:** degradation into subunits; formation of heat-induced soluble aggregates	**Increased:** facilitated the release of intracellular proteins
PA (Water-soluble)	**Concentrate > flour:** linked to higher protein content, as PA occurs as globoid crystals within protein bodies **Lower in isolates:** isolation process	**Decreased:** leaching during hydration; phytase activation; and hydrothermal degradation	**Decreased:** cellular disruption, enzyme activation, and enzymatic hydrolysis into lower inositol phosphates
Polyphenols (Water-soluble)	**Lower in isolates:** due to the isolation process, which increases protein purity and removes non-protein fractions. **Coarse fraction > Concentrate:** linked to the retention of seed coat	**Decreased:** leaching of water-soluble polyphenolics; activation of polyphenol oxidase; and hydrothermal degradation	**Decreased:** cellular disruption, enzyme activation, and enzymatic degradation
CT (Water-soluble)	**Small or undetectable CT:** associated with the light-colored seed coat of yellow peas	**Decreased by boiling:** water-soluble CT leaching or degradation **Increased by other treatments:** variations in leaching dynamics; thermally induced transformations	**Increased:** cellular disruption and release of bound CT