THE EVALUATION OF FACTORS INFLUENCING THE PROTEIN DIGESTIBILITY AND QUALITY OF PULSE-BASED INGREDIENTS AND PRODUCTS

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FORWARD

This thesis was prepared in a manuscript-based format. As the first author, I contributed original research presented in this thesis as three manuscripts. These contributions include Chapters 3 and 4 (manuscripts I and II) which were both published in Cereal Chemistry in 2025. The final research chapter, Chapter 5 (manuscript III), was submitted to the Journal of Food Composition and Analysis following thesis distribution. Research findings from these chapters were presented at various conferences in both oral and poster formats, as described below in reverse chronological order.

Oral Presentations

- Work from Chapters 3 to 5 was presented in a talk titled "Protein quality evaluation through *in vitro* determination in pulses and cereals" at the Guelph Research and Development Centre, Agriculture and Agri-Food Canada (Virtual), July 2022.
- Work from Chapters 3 and 4 was presented as a talk titled "The influence of Bühler milling on protein quality of pulse fractions" at the Manitoba Sustainable Protein Research Symposium (Virtual), June 2022.

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- Findings from Chapter 5 were presented at multiple conferences, with some data presented separately and others overlapping across presentations. These included the following: "The impact of amino acid reference patterns on the *in vitro* protein quality of pre-treated pulse products" was presented at the American Oil Chemists' Society Annual Meeting, Montreal, QC, April 2024; "Amino acid scoring patterns alter the *in vitro* protein quality of pre-treated chickpea (Cicer arietinum) products" was presented at the International Symposium on Dietary Protein for Human Health, Utrecht, Netherlands,

September 2023; "*In vitro* protein digestibility: The effect of anti-nutritional factors, methodology, and regression on pre-treated chickpea bread, pasta, and extrudates" was presented at the Manitoba Sustainable Protein Research Symposium, Winnipeg, MB, June 2023.

- For Chapter 3, the poster titled "Effect of milling and pre-treatments on *in vitro* protein digestibility and quality of green lentil and yellow pea" was presented at the American Oil Chemists' Society Plant Protein Science and Technology Forum (Virtual), October 2021.

CONTRIBUTIONS OF AUTHORS

Chapter 1 & 2: The literature review, including all writing, research, updates, and content development, was completed by Adam J. Franczyk. Critical feedback was provided by James D. House, Martin Nyachoti, Pam Ismail, and Sijo Joseph at the time of the proposal.

Chapter 3: Adam J. Franczyk conceived and designed the study, carried out the research for each component, analyzed the data, and wrote and reviewed each draft of the manuscript. Ashok Sarkar conceived the milling trial design including pre-treatments. Nguyen Bui and Jiayi Chen assisted all collections of *in vitro* protein digestibility data and amino acid hydrolysis, which was analyzed by Jason Neufeld. Sample preparation, including pre-treatments and milling, was completed by Lindsey Boyd. Michael Nickerson was the nominated principal investigator, and James D. House, Elaine Sopiwnyk, and Jitendra Paliwal were co-investigators involved in conceiving and designing the study, reviewing data analysis, and approving the final draft of the manuscript.

Chapter 4: Adam J. Franczyk conceived and designed the study, carried out the research for each component, analyzed the data, and wrote and reviewed each draft of the manuscript. Ashok Sarkar conceived the milling trial design, including pre-treatments. Nguyen Bui and Jiayi Chen assisted in all collections of *in vitro* protein digestibility data and amino acid hydrolysis, which was analyzed by Jason Neufeld. Sample preparation, including pre-treatments and milling, was completed by Lindsey Boyd. Michael Nickerson was the nominated principal investigator, and James D. House, Elaine Sopiwnyk, and Jitendra Paliwal were co-investigators involved in

conceiving and designing the study, reviewing data analysis, and approving the final draft of the manuscript.

Chapter 5: Adam J. Franczyk conceived and designed the study, carried out the research for each component, analyzed the data, and wrote and reviewed each draft of the manuscript. Nguyen Bui and Jiayi Chen assisted with all collections of *in vitro* protein digestibility data and amino acid hydrolysis, which was analyzed by Jason Neufeld. Assistance in methodological design, collection and calculation of trypsin inhibitors and polyphenols were also aided by Jiayi Chen. Sample preparation, including pre-treatments and milling, was completed by Lindsey Boyd. Michael Nickerson was the nominated principal investigator, and James D. House, Elaine Sopiwnyk, and Jitendra Paliwal were co-investigators involved in conceiving and designing the study, reviewing data analysis, and approving the final draft of the manuscript. With the loss of James D. House at the time of manuscript preparation, Rotimi E. Aluko assisted with reviewing data analysis and approving the final draft of the manuscript.

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DEDICATION

I would like to dedicate this thesis to the memory of Dr. James D. House.

“It’s only wafer-thin.”

– Monty Python's The Meaning of Life

In loving memory of my father, Chester Franczyk.

“My father didn’t tell me how to live. He lived and let me watch him do it.”

– Clarence Budington Kelland

And to my wife Jenikka Lumbera

For your patience when I had none, your encouragement when I doubted, and your love—always given in the way only you know how. Not just through this thesis, but always.

“I would rather spend one lifetime with you than face all the ages of this world alone.”

– The Fellowship of the Ring

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THESIS ABSTRACT

The growing shift towards plant-based diets has increased demand for pulses—such as lentils, peas, beans, and chickpeas—as sustainable, affordable protein sources. However, their nutritional potential is constrained by deficiencies in certain indispensable amino acids and by reduced protein digestibility, impacted by intrinsic and extrinsic factors. Accurate evaluation of pulse protein quality is essential for substantiating protein content claims and guiding product development. While *in vivo* assays remain the regulatory standard, they are costly, time-consuming, and ethically constrained. *In vitro* digestion models offer practical, scalable alternatives, yet their application across diverse pulse products remains underexplored.

This thesis examined how processing strategies and compositional factors influence *in vitro* protein digestibility and quality in pulse-based ingredients and chickpea-fortified foods. Three integrated studies were conducted: (1) evaluation of mechanical scouring and moisture conditioning effects on protein quality in green lentil and yellow pea flours; (2) extension of these treatments to navy beans and chickpeas to assess pulse class-dependent responses; and (3) assessment of food processing methods and product matrices in chickpea-based breads, pastas, and extruded products.

Across studies, pre-milling treatments had limited and pulse class-dependent effects, with small improvements observed in lentils but negligible impact in peas, chickpeas, and navy beans. Differences in protein quality among flours were driven mainly by variations in protein content and amino acid composition. In chickpea-fortified foods, thermal processing methods (particularly roasting and micronization) reduced phytate and trypsin inhibitors and altered protein secondary structures, modestly improving *in vitro* digestibility and amino acid scores.

Critically, while processing treatments influenced protein quality metrics, assessment of protein content claims showed products achieving equivalent regulatory thresholds regardless of pre-treatment. This contrast highlights a disconnect between underlying nutritional differences and consumer-facing protein content claims, emphasizing the importance of considering both compositional factors and processing effects when interpreting protein quality outcomes.

These findings support the use of *in vitro* digestion models for evaluating protein quality in pulse-derived foods and underscore the need for holistic, context-specific evaluation to optimize plant protein nutritional value and support transparent labeling.

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LIST OF ABBREVIATIONS

AA – Amino Acid
AAS – Amino Acid Score
AID – Apparent Ileal Digestibility
ALA – Alanine
ANF – Antinutritional Factor
AOAC – American Association of Analytical Chemists
APD – Apparent Protein Digestibility
ARG – Arginine
ASP – Asparagine
CF – Crude Fat
CP – Crude Protein
CYS – Cysteine
DIAAS – Digestible Indispensable Amino Acid Score
DM – Dry Matter
FAO – Food and Agriculture Organization
GLU – Glutamine
GLY – Glycine
HIS – Histidine
IAA – Indispensable Amino Acid
ILE – Isoleucine
IVPD – *in vitro* Protein Digestibility
LEU – Leucine
LYS – Lysine
MET – Methionine
NIST – National Institute of Standards and Technology
OPA – o-Phthaldialdehyde
PDCAAS – Protein Digestibility Corrected Amino Acid Score
PER – Protein Efficiency Ratio
PHE – Phenylalanine
PRO – Proline
SER – Serine
SID – Standardized Ileal Digestibility
THR – Threonine
TID – True Ileal Digestibility
TIM – TNO Gastro-Intestinal Model
TPD – True Protein Digestibility
TRP – Tryptophan
TYR – Tyrosine
VAL – Valine
WHO – World Health Organization

CHAPTER 1. INTRODUCTION

Protein, composed of amino acids, serves as the primary dietary source of nitrogen, supporting essential metabolic functions. Some amino acids are indispensable (IAAs) to human nutrition, meaning they must be supplied through the diet or synthesized at rates sufficient to meet metabolic demands. To promote both human health and environmental sustainability, Canada's food guide now encourages consuming plant-based proteins more often. Pulses—the dry seeds of peas, beans, lentils, and chickpeas—are an important source of plant-based dietary protein. However, limitations in certain IAAs, including tryptophan and the sulfur-containing amino acids methionine and cysteine, often constrain the protein quality of these crops. Assessing whether a dietary protein can supply adequate IAAs requires measuring its quality. Several methodological approaches exist to evaluate protein quality across established regulatory and scientific frameworks. Both Canada and the United States continue to permit the use of the protein efficiency ratio (PER) method for evaluating protein quality in infant foods. For general food products, Canada allows both PER and the protein digestibility corrected amino acid score (PDCAAS), while the United States relies primarily on PDCAAS. This method estimates a protein's digestibility and absorption across the digestive tract relative to human IAA requirements. The joint 1991 report from the World Health Organization (WHO) and Food and Agriculture Organization (FAO) recommended replacing PER with PDCAAS. In the decades since, limitations of PDCAAS in accurately assessing protein quality have been recognized. As a result, more recent recommendations have shifted toward the digestible indispensable amino acid score (DIAAS), which evaluates the digestibility of individual amino acids at the end of the small intestine. This method provides a more accurate measure of IAA availability for human metabolism and growth.

All of these established methods rely on animal models to assess protein quality. To address this limitation, *in vitro* (non-animal) digestion models have been developed to estimate protein and amino acid digestibility. These models vary in complexity, with static systems including pH-drop assays and simulated gastro-intestinal digestion protocols (e.g. pepsin-pancreatin methods and INFOGEST), while dynamic systems (e.g. TIM-1) provide more advanced simulations of digestion. Despite their potential, many *in vitro* models were originally developed for evaluating animal feed or have been tested on only a limited range of food proteins, restricting their validation for human nutrition assessment.

Plant-based proteins pose additional challenges for *in vitro* evaluation due to intrinsic antinutritional factors such as phenolic compounds, phytate, and trypsin inhibitors, limiting their protein digestibility. Extrinsic factors introduced through processing—including heat treatment, Maillard reaction products, and protein structural changes—can further reduce digestibility. Protein content, amino acid composition, digestibility, and overall protein quality are also influenced by processing methods such as milling, heating, and germination. These processes can alter the physical structure of proteins, disrupt antinutritional factors, and modify amino acid availability.

The aim of this doctoral research is to evaluate static *in vitro* digestion models for predicting *in vivo* protein digestibility in a variety of pulse flours and pulse-based products. This research will examine the effects of different milling pre-treatments and product types. Examination of both intrinsic and extrinsic factors will be applied to offer a holistic evaluation of how these factors influence *in vitro* estimates of protein digestibility and protein quality.

CHAPTER 2. LITERATURE REVIEW

2.1: An Overview of Pulses

Pulses, like other pod-plants share the same botanical lineage as other leguminous families, *Fabaceae* or *Leguminosae* (McCrory et al., 2010). Their separation from these other families (Figure 2.1) is defined by their dry grain classification, relative to oilseed (soy and peanut), vegetable (green beans and green peas), and sowing legume crops (clover and alfalfa)(FAO, 1994). Common pulses include, beans (*Phaseolus spp.*), peas (*Pisum spp.*), lentils (*Lens spp.*) and chickpeas (*Cicer arietinum*), although The United Nations Food and Agriculture Organization (FAO) recognizes a total of 11 commodity pulse crops (FAO, 1994). Economically, pulse crops constitute an important export in Canada, with a value of nearly $2.2 billion in 2011 (Government of Canada, 2014a). Although, the consumption of pulses has been a staple crop around the globe for over 10,000 years (Caracuta et al., 2015), only 13% of Canadians include pulses in their diet (Mudryj et al., 2012). Aside from potential reduction in cardiometabolic related diseases (S. J. Kim et al., 2016; McCrory et al., 2010; Mudryj et al., 2014; Rebello et al., 2014), the inclusion of at least one cup of pulses can enhance micronutrient intakes for thiamin, vitamin B6, folate, iron, magnesium, phosphorus and zinc as observed relative to non-consumers (Mudryj et al., 2012). Due to the high protein content (18-25%; dry weight basis) of pulses, overall protein intakes also improved in these consumers. Communicating protein claims to consumers, however, is not solely based on quantity, but also quality, which differs by jurisdiction and corresponding policy implementation (Marinangeli & House, 2017). The protein quality aspect of a food reflects both the content of nitrogen and IAAs relative to the digestibility, or availability of the IAAs and nitrogen for routine metabolic purposes. The digestibility of conventionally (stovetop, boiled) cooked pulses is relatively high

(≥80%), in addition to supplying an excellent source of most IAAs (mg/g protein basis), with the exception of limited tryptophan or the sulfur containing amino acids cysteine and methionine (Boye et al., 2010; El-Adawy, 2002; Nosworthy et al., 2018a, 2018b, 2020; Nosworthy, Franczyk, Medina, et al., 2017). Pairing pulses with cereal grains can balance these limiting amino acids, which makes them an excellent alternative to animal-based proteins that may be high in saturated fat. This pairing is also sought to support communities (e.g. South-Central Asia and East Africa) where children suffer from protein-energy-malnutrition, or to improve food security (Boye et al., 2010). Regionally, the protein digestibility is also lower in these communities, relative to North America, which is in part due to the content of antinutritional factors (ANF), such as tannins, phytate and protease inhibitors present in pulses and other plant-based proteins (S. G. Gilani et al., 2012). Emerging evidence over the past several decades has established the applicability of pulses, or pulse-based fractions in food applications, such as pastas, breads, meat-based/imitation products, extruded and baked snacks, among others (Boye et al., 2010). Processing techniques utilized in these applications can also reduce ANFs, and improve the protein quality of pulses (Sá et al., 2019). Pulse fraction solubility, water binding capacity, fat binding capacity, gelation, emulsifying and foaming properties have also been explored (Boye et al., 2010).

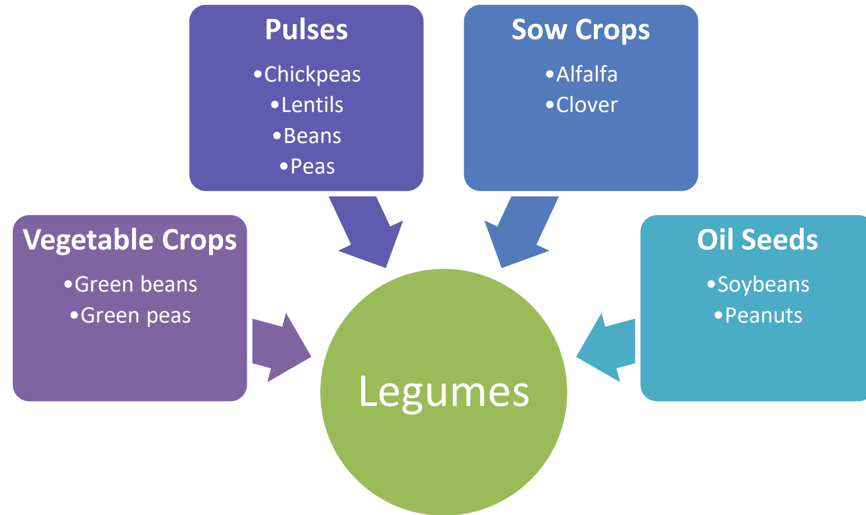

Figure 2.1. Legume sub-types including pulses adapted from McCrory et al. (2010)

2.2: Motivations for Protein Quality Assessment

Adequate evaluation of protein quality is believed to be vital for the support of health and well-being of human populations, with additional regulatory and economic implications (FAO/WHO, 1991, 2013). It is estimated that the world population will increase by approximately 2 billion, to 9.7 billion by the year 2050, putting additional constraints on food production and land utilization. These motivations garnered a joint WHO and FAO commission in 1989 which put forth sufficient scientific basis for the adoption of the Protein Digestibility Corrected Amino Acid Score (PDCAAS) method, as a successor to the antiquated Protein Efficiency Ratio (PER) method to assess protein quality (FAO/WHO, 1991). One key issue underlying the PER was identified as over-estimating the quality of animal-based, and under-estimating plant-based proteins. This is due to the relative differences in amino acid requirements of rats to humans, in which there is significantly higher metabolic need for cysteine, methionine, isoleucine and valine, in addition to the non-indispensable (in humans) amino acid arginine (FAO/WHO, 1991; National Research Council US Subcommittee on Laboratory Animal

Nutrition, 1995). Values garnered by the PER reflect the amount of feed consumed relative to weight gain in rats, adjusted relative to the value for casein, standardized to a set value of 2.5. Inferences from PER values are difficult to ascertain, such that single protein sources could not be combined to calculate new PER values, or that greater PER values at a value of 2.0 did not deduce the protein was twice as good as a PER value of 1.0. In addition to nitrogen balance studies in humans, a rat surrogate remained comparatively expensive and time consuming to complete. These challenges to the PER method also influenced the commissions decision to put forth the PDCAAS, as a reasonable replacement.

2.3: The Protein Digestibility Corrected Amino Acid Score

The PDCAAS reflects both the measure of bioavailability and bioaccessibility, the digestibility of a protein, corrected for the IAA content of the test article relative to a reference pattern of IAAs required for normal human metabolism (e.g. growth, repair & maintenance). Weanling rats are the recommended subjects utilized to assess the digestibility component, measured from the nitrogen remaining in feces. Test articles are formulated into diets which constitute a set content of crude protein (N; Nitrogen x 6.25; 10%) requirements in rats. The fecal assessment is representative of either the apparent or true fecal protein digestibility (APD and TPD). The APD represents the nitrogen remaining after total tract digestion, whereas the TPD includes the additional correction for endogenous losses (e.g. digestive enzymes, enterocytes, microbiota…) gathered from rats fed a nitrogen-free diet. The amino acid composition of the test article, relative to a selected age group amino acid reference pattern (Table 2.1), is also used to calculate a chemical score. The most limiting amino acid as a result of calculating chemical scores for each IAA, is selected as the amino acid score (AAS). The product of the TPD and the AAS represent the PDCAAS, as a value of 0 to 1, or otherwise

multiplied by 100. The PDCAAS for individual ingredients may also be used to estimate the protein quality for mixed ingredients without additional assays, which may be subject to truncation (maximum value of 100).

Table 2.1. Four reference amino acid scoring patterns (mg/g protein) based on the 1991 and 2013 FAO/WHO recommendations for different age groups

	THR	VAL	SAA	ILE	LEU	AAA	HIS	LYS	TRP
2 to 5 years[a]	34	35	25	28	66	63	19	58	11
0 to 6 months[b]	44	55	33	55	96	94	21	69	17
6 months to 3 years[b]	31	43	27	32	66	52	20	57	8.5
> 3 years[b]	25	40	23	30	61	41	16	48	6.6

THR = Threonine; VAL = Valine; SAA = Methionine and Cysteine; ILE = Isoleucine; LEU = Leucine; AAA = Phenylalanine and Tyrosine; HIS = Histidine; LYS = Lysine; TRP = Tryptophan

[a]Amino acid scoring pattern from 1989 (FAO/WHO, 1991)

[b]Amino acid scoring pattern from 2013 (FAO/WHO, 2013)

2.4: Protein Content Claims within Canada and The United States

The Protein Efficiency Ratio (PER) can still be used to assess protein content claims in Canada and remains required for infant formula in both Canada and the United States. In Canada, protein content claims are communicated through front-of-package labeling, with claim language defined by the protein rating system (Government of Canada, 1981). Foods can be labeled as a "source" (protein rating ≥20) or an "excellent source" (protein rating ≥40) (Government of Canada, 2014b). These claims are calculated using the product of the adjusted PER value and either a known (Schedule K) or estimated (Schedule M) reasonable level of protein intake for the specific food item (Marinangeli & House, 2017).

Alternatively, protein rating can be calculated using the Protein Digestibility-Corrected Amino Acid Score (PDCAAS), based on a single IAA amino acid reference pattern for preschool aged children (FAO/WHO, 1991). The PER can also be estimated from a PDCAAS value by multiplying by 2.5. When PDCAAS is used, the thresholds for content claims are adjusted: foods providing a protein rating of ≥8 qualify as a "source," while those at ≥16 qualify as an "excellent source." Challenges associated with the PER method, including its reliance on animal testing and limited applicability to multi-source protein products, have been widely noted (Marinangeli & House, 2017; Wiggins et al., 2019). Additionally, the absence of a validated *in vitro* method for directly assessing PER limits its broader applicability (Krul et al., 2024; Mansilla et al., 2020). In light of these limitations, transitioning to a PDCAAS-based system in Canada was proposed and has since been adopted.

In contrast, the United States has fully adopted PDCAAS for evaluating protein content claims for over three decades, following its adoption in 1993 (Boye et al., 2012). Similar to Canada, the single IAA reference pattern representing the needs of preschool children (ages 2–5)

is used for claim calculations (FAO/WHO, 1991). The reference amount customarily consumed (RACC) for each food is defined by the US Food and Drug Administration (US Food and Drug Administration, 2019). Protein claims are calculated as the product of the food's PDCAAS value and the protein content within the RACC. This corrected protein level is expressed as a percentage of the Daily Value (%DV), based on a 50 g reference intake. In the US, foods providing 10–19.9% DV can be labeled as a "good source" of protein, while those providing ≥20% DV qualify as an "excellent source." For example, using a RACC of 110 g—typical for foods such as dinners, desserts, fruits, vegetables, soups, and ready-to-serve items—Figure 2.2 illustrates how varying PDCAAS values and protein levels affect claim eligibility. Values on or above the green line indicate a "good source" claim, while those on or above the blue line meet the threshold for an "excellent source" claim.

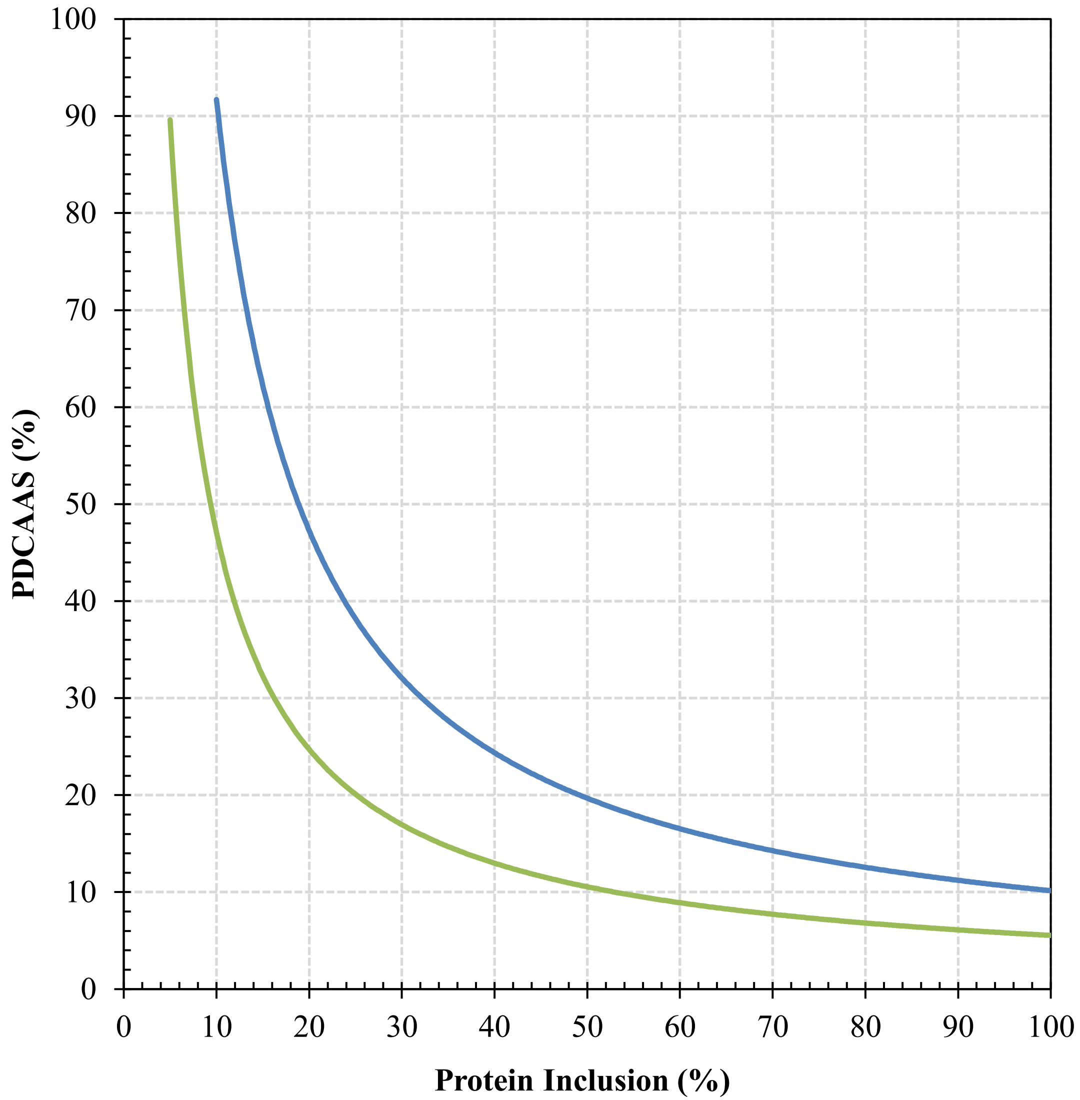

Figure 2.2. Potential for a good (green) or excellent (blue) protein claim based on a protein digestibility-corrected amino acid score value between 0 and 100 (%) relative to the protein inclusion (%) within a 110g reference amount customarily consumed

2.5: Potential Future of Protein Quality

Twenty years after the introduction of PDCAAS, recommendations from the FAO and WHO shifted to support the adoption of the Digestible Indispensable Amino Acid Score, or DIAAS, as outlined in their 2013 report (FAO/WHO, 2013). Several key issues prompted the shift in recommendations. First, the focus moved from crude protein to indispensable amino acids (IAAs) as the primary nutritional concern. Second, emphasis was placed on assessing amino acid digestion at the pre-colonic (ileal) level, rather than using total tract (fecal) digestibility, to better reflect actual amino acid absorption. Additionally, the bioavailability of amino acids—particularly lysine—requires separate consideration due to potential losses from Maillard reaction products, which can reduce lysine availability. For digestibility studies, humans are now the preferred subjects, followed by pigs, and then rats. In these studies, IAA content is measured from the terminal ileum, which represents the final site of amino acid absorption and is least affected by endogenous amino acid losses. Digestibility assessment of each IAA involves three independent methods at the ileal level: apparent ileal digestibility, standardized ileal digestibility, and true ileal digestibility, referred to as AID%, SID%, and TID%, respectively (Stein et al., 2007; Wolfe et al., 2016). These values are calculated based on corrections for IAA losses. AID applies no correction for endogenous losses, while SID adjusts for basal endogenous losses and TID accounts for total endogenous losses. Basal losses represent diet-independent losses, typically measured using nitrogen-free or amino acid-defined diets. Total losses include both basal losses and additional food-specific endogenous losses, which result from factors such as dietary fibre or antinutritional compounds. Due to limited availability of ileal digestibility values for foods prepared for human consumption, the use TPD (fecal) may substitute the digestibility component for each IAA in the calculation of DIAAS. The relative

amount of each digestible IAA is compared to a reference amino acid pattern, and the lowest ratio determines the DIAAS value for the test article, expressed either as a value (e.g., 1.2) or as a percentage (e.g., 120%). Unlike PDCAAS, DIAAS values are not truncated at 1.0 (or 100%), even when assessing mixed protein sources, allowing proteins that exceed the reference requirements to retain their full calculated value. Reference patterns have since been updated to include different age groups and corresponding amino acid requirements (FAO/WHO, 2013), with specific patterns available for infants (birth to 6 months), young children (6 months to 3 years), and older children, adolescents, and adults (Table 2.1). Although DIAAS is considered the most accurate method for assessing protein quality in humans, no jurisdiction has formally adopted it in its complete form. In principle, a simplified version—using TPD, updated reference patterns, and eliminating truncation—could be implemented under the DIAAS framework, even without evaluating individual amino acid digestibility at the ileal level. However, neither this approach nor targeted updates to PDCAAS, such as applying new reference patterns or removing truncation, have been adopted to date.

2.6: Challenges to the assessment of Protein Digestibility

Both PDCAAS and DIAAS are *in vivo* measurements conducted in living organisms. These methods face several challenges, including correcting for endogenous amino acid losses, accounting for species-specific digestive differences, and managing sample collection. Protein source can further influence results, as plant-based proteins often show significant differences between total tract and ileal digestibility values (Rutherfurd et al., 2015). Digestibility differences between plant- and animal-based proteins are partly attributed to antinutritional factors (ANFs), which can inhibit proteolytic enzymes, promote the formation of insoluble or digestion-resistant protein fractions, and increase colonic fermentation (Day, 2013; S. G. Gilani

et al., 2012; Sá et al., 2019; Schaafsma, 2012; Young & Pellett, 1994). The role of ANFs and other plant-based factors will be addressed in later sections.

Ileal digestibility is generally considered a better measure of IAA availability than total tract digestibility, as it reflects amino acid absorption prior to the onset of extensive microbial fermentation (FAO/WHO, 2013). Total tract digestibility (i.e. TPD), by contrast, assesses overall nitrogen losses across the digestive system (M. Fuller, 2012). After leaving the terminal ileum, approximately 90% of nitrogen is utilized by colonic microbes before excretion (Blachier et al., 2007; FAO/WHO, 2013; M. Fuller, 2012). While microbial activity also occurs within the small intestine, its influence is comparatively minor (Boisen & Eggum, 1991).

Current protein quality assessments do not consider the microbiome as a significant source of IAAs for growth or maintenance. However, some exceptions challenge this assumption. Passive diffusion of certain amino acids, such as L-arginine, from the colon into circulation has been demonstrated in swine (Binder, 1970), although similar evidence for IAAs is lacking (Darragh et al., 1994). While cross-species generalizations are common in the literature, recent findings in rats reveal amino acid transporters in the colon and caecum, with higher transcript expression than in the small intestine (Chen et al., 2020). Species differences also extend to endogenous amino acid losses, with rats showing losses approximately 45% higher than humans, which affects digestibility assessments of plant proteins (Bodwell et al., 1980; Deglaire & Moughan, 2012). Endogenous loss corrections differ depending on whether nitrogen-free or peptide-based diets are used during testing (Moughan & Rutherfurd, 1990), and results vary across species (Deglaire & Moughan, 2012; Hendriks et al., 2002). Digestibility values can also differ within a species based on the age of the organism, particularly for plant-based proteins

(Deglaire et al., 2009; M. F. Fuller & Tomé, n.d.; G. S. Gilani & Sepehr, 2003; Rutherfurd & Moughan, 2003).

Collecting ileal digestate poses significant technical challenges and is unsuitable for routine analysis. In humans, collection typically relies on subjects fitted with ileostomy bags or naso-ileal tubes. These methods are affected by host health and microbiota overgrowth in the terminal ileum, which can alter digestate composition (Moughan, 2003). Swine are recommended as the preferred model organism, using surgically fitted ileal cannulas for direct sample collection. However, swine studies require large quantities of the test article and involve more complex housing, handling, and care requirements compared to rodent models. When material or resources are limited, rats serve as a secondary model (FAO/WHO, 2013), although collection still requires euthanizing the animal to access terminal ileal contents. Reports of coprophagy in rat studies may also be under-reported. Younger, growing rats—commonly used in digestibility studies—demonstrate fecal consumption more frequently than older animals (S. Ikeda et al., 1999). Standard housing conditions, such as wire-bottom cages, may not fully prevent this behavior or accurately account for all fecal losses, potentially compromising the accuracy of digestibility measurements.

Given the combined influence of species differences, endogenous losses, and protein-specific factors, *in vivo* digestibility values are inherently variable. Beyond these technical challenges, using live subjects introduces additional limitations related to cost, time, and ethical considerations, which make routine assessment impractical. To address these challenges, *in vitro* methods have been developed as alternatives for estimating protein digestibility in the calculation of PDCAAS and DIAAS. However, *in vitro* results also display significant variability and often yield inconsistent correlations with *in vivo* data, likely influenced in part by the

aforementioned biological factors. Some authors have suggested that *in vitro* digestibility may, in fact, represent a form of "real" digestibility—a digestibility coefficient not influenced by endogenous secretions or host-specific physiological factors (Boisen & Moughan, 1996; Cone & Van Der Poel, 1993). Nonetheless, as will be discussed in the following section, there is recognition that under certain conditions, *in vitro* methods can achieve strong correlations with *in vivo* outcomes.

2.7: *In vitro* Digestion Models

Over the past several decades, various *in vitro* methods have been developed to simulate monogastric digestion. These approaches generally fall into two categories: static mono-compartmental systems and dynamic mono- or multi-compartmental systems. Static systems replicate digestion using relatively simple and fixed parameters, such as pH, temperature, time, sequence of digestive phases, and the addition of digestive enzymes. Some systems, however, incorporate additional features like immobilized surface enzymes, active filtration (e.g., dialysis), removal of digestion products, or even microbial inputs (Bohn et al., 2018; Boisen & Eggum, 1991; Butts et al., 2012; Guerra et al., 2012; Hur et al., 2011; Savoie, 1994). Most dynamic models incorporate all of these parameters, along with simulated peristaltic movements, timed digestive secretions, and computer-controlled adjustments of digestion conditions such as variable pH, secretion rates, fed or fasted state, species, and age (Dupont et al., 2019). The applicability of these digestion models has been extensively reviewed in the context of both livestock and human nutrition (Alegría et al., 2015; Boisen & Eggum, 1991; Butts et al., 2012; Dupont et al., 2018; Guerra et al., 2012; Hur et al., 2011; Moughan, 1999; Savoie, 1994; Swaisgood & Catignani, 1991). The following subsections will focus primarily on

methodologies used to investigate *in vitro* protein digestibility (IVPD) and their associations with *in vivo* protein digestibility outcomes.

2.7.1: *Static Mono-compartmental in vitro Protein Digestion Models*

Single incubation and reaction models, such as the pH-drop method (Hsu et al., 1977) and the pH-stat method (Pedersen & Eggum, 1983), offer quick and simple approaches for assessing IVPD. Both methods adjust a protein solution to a set pH before digestion with a proteolytic enzyme mix (e.g., trypsin, chymotrypsin, peptidases), which causes a drop in pH as hydrolysis proceeds. The pH-stat method differs by maintaining the pH at a constant point through titration with NaOH, while both approaches ultimately estimate either APD or TPD using regression equations based on rat *in vivo* data.

The pH-drop method demonstrated high correlation with *in vivo* digestibility for both plant and animal proteins, achieving $r = 0.90$ ($n = 23$) for APD (Hsu et al., 1977). Some human studies also suggest that the pH-drop method can reliably estimate both APD and TPD, with correlations of $r \geq 0.77$ for plant proteins and $r \geq 0.85$ for animal proteins when considered separately (Bodwell et al., 1980). Similarly, the pH-stat method has produced excellent correlations with TPD across a variety of protein types with $r = 0.95$ for plant proteins ($n = 13$), $r = 0.98$ for animal proteins ($n = 10$), $r = 0.97$ for mixed proteins ($n = 9$), and $r = 0.96$ overall ($n = 30$) (Pedersen & Eggum, 1983). Further analysis of sixteen protein sources, including plant, animal, and mixed proteins, confirmed high reproducibility of the pH-stat method across six laboratories (Mcdonough et al., 1990).

Previous recommendations from the FAO/WHO for assessing PDCAAS put forward the pH-stat method (FAO/WHO, 1991). Compared to the pH-drop method, the pH-stat is better

suited for overcoming the buffering capacity of certain proteins and for estimating TPD. However, in a more recent evaluation using additional TPD data, the pH-drop method (r = 0.93) showed a stronger correlation than the pH-stat method (r = 0.84) across eighteen plant and animal proteins (Mendes et al., 2016).

Multiple incubation and reaction models, which sequentially digest proteins, have also been developed to simulate digestion parameters found in monogastric animals. These models typically focus on replicating digestion within the stomach and small intestine, commonly referred to as pepsin-pancreatin, gastro-intestinal or two-step digestion models. In these systems, proteins are first solubilized in hydrochloric acid and exposed to pepsin, the primary gastric enzyme. The resulting solution is then neutralized to inactivate pepsin and raise the pH to levels suitable for small intestinal digestion. At this stage, either pancreatin (a pancreas extract containing peptidases, carbohydrases, lipases, elastases and nucleases) or a proteolytic enzyme mix (e.g., trypsin and chymotrypsin) is added to complete digestion. One of the most widely cited pepsin-pancreatin models, used for evaluating protein quality, demonstrated excellent correlations (r = 0.94–0.98) between digestibility results for plant and animal proteins (n = 12) and established biological measures such as biological value, chemical score, essential amino acid index, and the pepsin-pancreatin digest index reported in the literature (Akeson & Stahmann, 1964). Using a similar two-step approach, but substituting pancreatin with jejunal digestion effluent collected from T-cannulated pigs, crude (fecal) digestibility showed high correlations in both ground (r = 0.98, n = 7) and unground diets (r = 0.91, n = 7) (Furuya et al., 1979). Simulated two-step digestion also demonstrated exceptionally high correlations with crude digested protein from pig feeding trials, both in single feeds (r = 0.99, n = 7) and compound feeds (r = 0.95, n = 16) (Babinszky et al., 1990). Additional procedures, such as

digestion under constant dialysis, have also shown potential for estimating protein digestibility (Gauthier et al., 1982). This has been achieved by measuring nitrogen in the dialysate (r = 0.88, n = 8) or by using SDS-PAGE analysis (r = 0.92, n = 6) to assess correlations with APD in rats (Rozan et al., 1997). Estimating AID from pig diets also showed an excellent correlation (r = 0.96, n = 15) when results were corrected for endogenous dry matter losses (Boisen & Ferna´ndez, 1995). However, when these authors tested an additional 48 feed combinations with known *in vivo* digestibility values, the correlation decreased (r = 0.75). A two-step digestion method, incorporating continuous dialysis through a 10,000 Dalton membrane during the second stage, has also been used to estimate amino acid digestibility in poultry. This approach produced excellent correlations with *in vivo* data for cereal grains (r = 0.92), but results were less reliable for soybean meal and corn gluten meal (r = 0.42) (Cave, 1988).

Comparing both pH-drop and pepsin-pancreatin digestion models across 65 raw and processed plant-based proteins against TPD measured in rodents resulted in lower correlations (r = 0.45 and r = 0.56, respectively) than those reported in earlier studies (Franczyk, 2018). In this study, the pepsin-pancreatin method tended to overestimate the digestibility of raw proteins, while the pH-drop method tended to underestimate it. Pulse-based protein concentrates, isolates, baked products, and particularly beans were identified as protein sources for which both methods struggled to accurately estimate TPD. These findings build on earlier observations that specific protein types may influence the strength of *in vitro* and *in vivo* associations, suggesting that certain proteins are inherently more difficult to assess accurately. This may help explain why narrower protein selections in earlier studies produced stronger correlations.

2.7.2: *Dynamic Multi-compartmental in vitro Protein Digestion Models*

Sophisticated *in vitro* systems that simulate the dynamic physiological processes of mammalian digestion have been developed (Dupont et al., 2019). Among nearly a dozen systems reviewed, only the TIM system (TIM-1 or tiny-TIM), developed by TNO Nutrition & Food Research Institute, has produced estimates for IVPD. The development of TIM incorporated several key principles considered essential for simulating *in vivo* digestion effectively: (1) sequential addition of digestive enzymes, (2) maintenance of appropriate pH for enzyme activity and cofactor function (including bile salts), (3) removal of digestion end-products, (4) appropriate mixing at each stage, and (5) realistic digestion times and transit rates (Longland, 1991).

The TIM system models gastro-intestinal digestion through multiple compartments simulating the stomach, duodenum, jejunum, and ileum. Key features include computer-controlled regulation of enzyme and acid secretion rates, peristaltic pumping of chyme, continuous removal of digested products via dialysis, and temperature control using heated water jackets around each compartment. Simulations can be programmed to reflect species-specific parameters, meal composition, and feeding state (e.g., postprandial conditions) prior to digestion. The tiny-TIM and TIM-1 systems have demonstrated strong correlations for protein digestibility outcomes, including TID% in humans ($r = 0.98$) for several animal-based proteins ($n = 7$) (Havenaar et al., 2016), and AID% in pigs ($r = 0.95$) for multiple proteins ($n = 5$) (Minekus, 1998). Estimating TPD using the TIM system has also been demonstrated, although results generally overestimated the TPD of raw, microwaved, baked, boiled, and fried potatoes (Bailey et al., 2023). While dynamic systems like TIM offer promising potential for assessing protein digestibility and quality (Schaafsma, 2005), routine application across a broader range of

proteins—such as pulses—remains limited. These systems are often considered unnecessarily sophisticated and costly for routine assessments (Butts et al., 2012; Krul et al., 2024).

2.8: Standardized *in vitro* Static Methodology and Variables

Proximity of a digestion model's procedures to physiological conditions is generally believed to improve its accuracy in estimating food digestion, and by extension, protein digestion. Based on this assumption, a hierarchy of models can be described: dynamic models (e.g., TIM) are considered the most physiologically relevant, followed by multiple incubation, sequential static models (e.g., pepsin-pancreatin), and finally single incubation static models (e.g., pH-drop), which are considered the least physiologically representative. Further development and validation are likely required to determine whether this generalization holds true—particularly in the context of predicting digestibility for protein content claims, which are currently based on total tract digestion, though future methods may consider ileal digestion as evaluation criteria evolve. At present, the strongest associations in estimating IVPD appear to relate to total tract digestion, with more variable outcomes observed for IVPD or amino acid digestibility predictions based on ileal digestion. Most digestion models with broader protein selection and *in vivo* comparisons have been developed using static methods. These models typically derive *in vivo* associations from livestock diets (e.g., swine and poultry; bovine not reviewed), which include not only cooked foods suitable for human consumption, but also raw ingredients and by-products not typically consumed by humans.

Simulated digestion in these models often lacks clear justification for the selection of critical parameters, including enzyme type (species of origin or specific proteases used), enzyme quantity or ratios (substrate-to-enzyme), composition of digestion fluids (acids, bases, salts, buffers), temperature (based on enzyme selection or species being simulated), digestion time

(physiological transit time or fixed endpoint), and pH (pre- or post-meal, or optimum versus suboptimum for enzyme activity).

Standardized static methodologies for *in vitro* digestion have been proposed using two primary approaches (Brodkorb et al., 2019; Hollebeeck et al., 2013; Minekus et al., 2014), both aiming to improve inter-study comparisons by providing rationale for digestion parameters. These methods diverge in their design: one uses physiologically relevant inputs based on average human digestion parameters, as recognized in the INFOGEST protocol (Brodkorb et al., 2019; Minekus et al., 2014), while the other applies response surface methodology to optimize enzyme inputs for specific substrates such as carbohydrates, lipids, and proteins (Hollebeeck et al., 2013). In the context of protein digestibility assessment, both protocols simulate digestion of an average mixed meal (e.g., ~12% protein, 28% lipid, and 60% carbohydrate). This differs from protocols used in PDCAAS and DIAAS studies, which prepare test diets with fixed protein (10%) and lipid (10%) contents, supplemented with fibre and micronutrients. From a protein quality perspective, enzyme inputs could potentially be reduced relative to general meal simulations, given the low lipid content in such test diets, thereby lowering the need for lipases or bile. Both INFOGEST and the response surface method simulate oral digestion with α-amylase before gastric digestion. While some authors argue oral simulation is unnecessary—citing the presence of α-amylase in pancreatin extracts—others suggest it may facilitate proteolysis by exposing protein embedded in polysaccharide matrices, particularly in plant-based ingredients like pulses.

Downstream protein or amino acid analysis introduces additional complexities requiring standardization, which has recently been adapted for the INFOGEST method in DIAAS estimation (Sousa et al., 2023). Ensuring adequate protein collection from digestate is critical (Boisen & Eggum, 1991). Techniques such as acid precipitation (e.g., using trichloroacetic acid),

refrigeration, filtration, size exclusion chromatography, and centrifugation are employed to recover large peptides (Hwang & Chu, 1996; Ngo, 2014; Rajalingam et al., 2009), which may represent undigested or partially digested protein fractions (Cave, 1988; Pappenheimer et al., 1994; Shahidi & Li, 2013; Yvon et al., 2009). However, these approaches can introduce variability across studies. Nitrogen determination using Dumas combustion or Kjeldahl methods accurately quantifies total nitrogen (Jung et al., 2003; Thompson et al., 2002; Watson & Galliher, 2001), but non-protein nitrogen content may confound digestibility estimates (Mariotti et al., 2008; Tome et al., 2019). Spectrophotometric methods, although faster and cheaper, may yield inaccurate results due to non-specific peptide bond detection (Abdel-Aal, 2008) or inability to quantify specific amino acids (Rutherfurd, 2010). Taken together, current *in vitro* protein digestibility methods appear to follow two primary rationales: (1) the use of physiologically relevant digestion conditions, or (2) optimization for maximal substrate hydrolysis. Historically, the latter approach predominated, with protocols designed to maximize nitrogen or amino acid recovery, often without disclosing the underlying rationale (Boisen & Eggum, 1991; Boisen & Ferna´ndez, 1995; Büchmann, 1979; Gauthier et al., 1986).

In vivo, large endogenous enzyme secretions may compensate for anti-nutritional factors (ANFs) such as trypsin inhibitors, minimizing their impact on total tract digestibility while increasing endogenous losses at the ileum (Cone & Van Der Poel, 1993; Swaisgood & Catignani, 1991). In contrast, *in vitro* models may fail to replicate this compensation, particularly when protocols are optimized for digesting casein, a protein free of ANFs (Gauthier et al., 1986), rather than raw plant ingredients rich in ANFs (Boisen & Ferna´ndez, 1995). It remains unclear whether *in vitro* protocols should adjust enzyme concentrations in relation to the ANF content of test foods. Using physiologically relevant enzyme inputs (approach 1) may offer a more robust

theoretical basis for addressing this uncertainty compared to optimization based solely on substrate hydrolysis (approach 2), as previously suggested (Cone & Van Der Poel, 1993). Given the variable ANF content in human foods, some authors (Moughan, 1999) and animal studies (Kaewtapee et al., 2017) have proposed the use of multi-factorial regression models to accurately estimate the multiple inputs affecting protein digestibility. However, for such models to be meaningful, they must first be anchored to a standardized digestion protocol. Without consistency in method selection and enzyme input rationale, any regression model risks introducing further inter-study variability rather than resolving it. Therefore, selecting and standardizing a digestion protocol—whether physiologically based or optimized—must be considered a prerequisite before developing multi-factorial models to predict protein digestibility.

2.9: Supplementing *In Vitro* in Protein Quality Calculations

The potential for IVPD to supplement TPD in the calculation of PDCAAS has been reviewed (Boye et al., 2012). In this approach, the IVPD value replaces the *in vivo* TPD component, while the same AAS is applied for each test protein. Comparative studies have primarily focused on static methods such as the pH-drop, pH-stat, or pepsin-pancreatin digests. One of the earliest studies to demonstrate the relationship between *in vitro* and *in vivo* calculated PDCAAS used a modified pH-stat method to estimate degree of hydrolysis, alongside the pepsin-pancreatin dialysis cell method to determine total nitrogen digestibility (Rozan et al., 1997). Both the degree of hydrolysis (r = 0.96) and total nitrogen digestibility (r = 0.99) showed excellent correlation with *in vivo* PDCAAS values for various plant-based proteins and casein (Rozan et al., 1997). Similarly, applying the pH drop method yielded strong correlations (r = 0.87–0.99) across a range of pulses—including chickpeas, red and green lentils, yellow and

green split peas, and black, navy, pinto, red kidney, and faba beans—that had been baked, cooked, or extruded (Nosworthy et al., 2018a, 2018b, 2020; Nosworthy, Franczyk, Medina, et al., 2017). Single-ingredient products made from either baked or extruded buckwheat or pinto beans, as well as blends of the two, showed a strong correlation (r = 0.96) between *in vivo* and *in vitro* calculated PDCAAS (Nosworthy, Franczyk, Zimoch-Korzycka, et al., 2017). Continued agreement with the pH drop was also observed in analysis of hemp, several varieties of almonds, and thermally treated potatoes (Bailey et al., 2023; House et al., 2019; Nosworthy et al., 2023). Analysis of chickpea fractions using an assortment of *in vitro* methods to calculate PDCAAS was conducted (Tavano et al., 2016). The study included the pH-drop (r = 0.94), a modified pH-drop (r = 0.96), and a single two-step digestion method with three means of nitrogen analysis; o-phthaldialdehyde (r = 0.90), trinitrobenzenesulfonic acid (r = 0.89) or Kjeldahl (r = 0.93), from which significant ($P \leq 0.05$) correlations with *in vivo* calculated PDCAAS were identified. When comparing pepsin–pancreatin digestion and pH-drop methods across 65 plant-based proteins with low to moderate protein digestibility correlations, adjusting for each test article's respective amino acid score improved the regression (r = 0.92 & 0.91 respectively) results for both methods (Franczyk, 2018).

Although other studies have explored associations between IVPD and protein quality measures in pulses (Khattab et al., 2009), only those reviewed here have included an *in vivo* component. All studies using pH-drop methods calculated IVPD based on APD derived from the original regression equation (Hsu et al., 1977), although alternative equations are available (Bodwell et al., 1980; Mendes et al., 2016; Pedersen & Eggum, 1981). Using an estimate of APD will underestimate digestibility values otherwise applied in PDCAAS calculations, affecting only the magnitude of the results without altering the strength of the relationship between IVPD and

in vivo measures of protein digestibility or quality. Based on the data reviewed, most correlations remained strong, with beans and blends reducing these associations, and chickpea fractions showing poor and non-significant correlations. This may reflect the influence of ANFs that interfere with digestibility estimates (Tavano, da Silva, et al., 2008; Tavano et al., 2016). Casein was consistently used as a high-quality, highly digestible protein standard, which improved correlation strength when included alongside test ingredients. To enhance interpretation, further statistical analyses—such as Bland-Altman plots—should be applied to supplement correlation data (Bland & Altman, 2010), helping to identify proteins that complicate accurate TPD prediction. Overall, establishing strong correlations and generating regression equations from combined *in vivo* and *in vitro* data remain useful strategies for calculating PDCAAS.

2.10: Factors affecting Protein and Amino Acid Digestibility in Pulses

Compounds that interfere with the absorption or utilization of macro- and micronutrients are typically grouped under the umbrella term ANFs. In pulses and cereals, ANFs can be intrinsic to the protein source, associated with structural seed components such as the seed coat (testa), hull, cotyledons (in pulses), or endosperm (in cereals). Compounds such as phytate, tannins, and protease inhibitors (e.g. trypsin inhibitors) are present within these seed fractions but exert their effects differently on *in vivo* and *in vitro* digestibility measures (S. G. Gilani et al., 2012). Mechanical, thermal, chemical, and biological processing can alter the content of ANFs or modify their effects on protein and amino acid digestibility. Depending on the processing conditions, additional extrinsic effects may also occur, including changes to nitrogen and amino acid content, alterations in protein secondary structure, formation of indigestible protein aggregates, and the production of Maillard reaction products (MRP). Pulse class, such as lentils, peas or red kidney beans (Alonso et al., 1998; Parmar et al., 2017; Wang et al., 2009; Wang &

Daun, 2006), may also influence ANF content, the effects of processing, and resulting protein or amino acid digestibility. Specific class differences are not reviewed in this section. Instead, the following subsections will describe and expand on processing inputs, followed by a review of protein and amino acid digestibility in relation to intrinsic and extrinsic factors.

2.10.1: *Processing Overview*

Procedures which describe a variety of milling techniques constitute mechanical processing. Intrinsic constituents of a pulse, such as the seed coat, can be separated through the selection of milling processes and sieves (Thakur et al., 2019). Milling can crush, break, pulverize, and/or centrifuge, producing various particle sizes for a whole flour or a separated component (Wood & Malcolmson, 2021). From this, different fractions may be isolated, allowing for selection of high or low starch, fibre and protein content. Air classification, relative to sieves, is another method which may be utilized to select for fractions, through which milled samples are separated in a spiral air stream (Boye et al., 2010). Protein fractions produced from air classification can produce relatively high protein content products (≥70% DM basis), termed protein concentrates (Boye et al., 2010; Nosworthy & House, 2017). Milling processes which select for smaller particle sizes may be associated with increases in total tract protein digestibility (Wondra et al., 1995), or the elimination of fractions (e.g. hull) with a higher propensity for ANFs (A. J. Jansman et al., 1995). Mechanical processing is frequently a pre- and/or proceeding step following any other processing method, when prepared for food applications or in protein digestibility studies.

Thermal treatments describe a processing step which introduces direct or indirect changes in temperature to a test article. Investigations with pulses include methods such as autoclaving, boiling (or cooking), roasting (or baking), extrusion, micronization/infrared and microwaving

(Boye et al., 2012; Deepa & Hebbar, 2016; Sá et al., 2019). Parameters for all these treatments include time, pressure, temperature, medium (e.g. water and air) and energy input, although they may introduce additional elements (Khattab et al., 2009). Extrusion introduces additional parameters, as the input material is transported along a barrel with one or two screws and forced towards a die at the end of the barrel (Camire, 1991; Day & Swanson, 2013). Heat may be produced by a set or transient inputs, or as a result of friction. The variety of extruder, the dimensions of its barrel, and the screw speed also add shear. Thermal treatment parameters differentially impact protein and amino acid digestibility, in which elements of intrinsic and extrinsic factors measured are altered. Details for these parameters are not reviewed, however, the outcomes relative to individual treatments instead are indicated in the following subsections.

Pre-treatments such as soaking in water or alkaline solutions are often applied prior to thermal processing. These treatments may be classified as biological processing when seeds are alive and germinating, or as chemical processing in the case of osmotic treatments. Chemical processing also includes the production of protein isolates, typically achieved through aqueous alkaline extraction of pulse flour, followed by isoelectric precipitation at lower pH, or alternatively, by ultracentrifugation (Boye et al., 2010). Generally, these isolates yield high-protein products (≥80% DM basis) across a variety of pulse class. Substituting the initial alkaline extraction with acid extraction or using salt solutions based on the salting-in and salting-out phenomena of food proteins, has also been shown to produce high-protein isolates. Germination, a biological processing method, involves introducing moisture and controlling time and light exposure to break seed dormancy. This process activates the embryo, stimulating the production of proteolytic enzymes that begin hydrolyzing proteins and starch, while also reducing antinutritional factors such as trypsin inhibitors (Khattab et al., 2009; Nosworthy & House, 2017;

Sá et al., 2019). Fermentation—also considered a biological processing method—uses micro-organisms such as bacteria and yeast to similarly improve the accessibility of nutrients, such as protein, through proteolytic action.

2.10.2: *Fibre*

Total dietary fibre content in pulses can range from 3% to 30%, with chickpeas at the lower end and beans at the higher end of this range (Boye et al., 2010; Luzardo-Ocampo et al., 2020). Fibre fractions from cereals have been extensively studied for their impact on nutrient absorption, including protein and amino acids, in monogastric animals. In general, increasing fibre content shows a negative association with protein and amino acid digestibility (Pedersen et al., 1989). The negative impact of fibre on protein and amino acid digestibility is attributed to reduced proteolysis, caused by limited enzymatic accessibility throughout the digestive tract and interactions between proteins and polysaccharides (Boisen & Eggum, 1991). Fibre may also indirectly influence digestibility through its effects on gastric emptying, stomach filling, and energy dilution capacity (Sá et al., 2019). Notably, in *ad libitum* feeding conditions—unlike the restricted intake used in protein quality studies—the negative effect of high dietary fibre on protein and amino acid digestibility in swine can be diminished, as animals compensate behaviorally by increasing their total dietary energy intake (Kennelly & Aherne, 1980). Fibre analysis in animal studies, particularly in rodents and swine, typically employs neutral detergent fibre (NDF) and/or acid detergent fibre (ADF) methods to quantify cellulose, lignin, and hemicellulose. In human nutrition, dietary fibre is more broadly defined according to the Codex Alimentarius as:

"*Carbohydrate polymers with ten or more monomeric units, which are not hydrolyzed by the endogenous enzymes in the small intestine of humans…*" (McCleary et al., 2013).

Several methodologies and terminologies are used to describe dietary fibre in human nutrition, including insoluble, soluble, and total dietary fibre. In animal nutrition research, particularly with cannulated swine, early studies using neutral detergent fibre (NDF) and NDF-associated nitrogen explained 92–96% of the variation in ileal digestible crude protein and 70–88% in fecal digestible crude protein across 11 feedstuffs, including cereals, oilseeds (e.g. soy), and animal products (Hall et al., 1988). Similarly, true fecal protein digestibility in rats fed various hemp seed diets showed a strong negative correlation ($r = -0.96$, $P \leq 0.05$) with NDF (on a fat-free basis), with PDCAAS improving when hull fractions were removed (House et al., 2010). Studies using rats to evaluate APD and TPD in diets containing steam-autoclaved, pelleted soy or soy-free (casein-based) diets also identified a strong negative correlation for true digestibility ($r = -0.876$, $P \leq 0.022$), though this was not observed for APD (Taciak et al., 2015). The addition of fibre from barley hulls similarly decreased overall true fecal protein digestibility (Eggum et al., 1984). While the inclusion of lysine had no significant effect on true protein digestibility, supplementation with methionine resulted in a significant reduction in true digestibility. In contrast, analysis of 60 commercial breakfast cereals demonstrated the opposite effect for available lysine, where higher fibre content was associated with lower available lysine, possibly reflecting effects of extrusion processing conditions such as high temperature (Day & Swanson, 2013). In swine studies examining heat-treated full-fat soybeans, a quadratic response in standardized ileal digestibility (SID) was observed (Kaewtapee et al., 2017). Decreases in crude protein and IAAs were associated with increases in neutral detergent insoluble nitrogen, trypsin inhibitor activity, urease activity, and reductions in protein solubility, protein dispersibility index, lysine:crude protein ratio, as well as visual properties like lightness and

redness. A linear decrease in SID values was also reported with declining available lysine:crude protein ratio and increasing NDF content. In pulses such as chickpea, pigeon pea, mung bean, black bean, and soybean, IVPD improved as total dietary fibre content decreased—particularly when fermented or germinated—compared to autoclaving, roasting, or raw controls (Chitra et al., 1996). This inverse relationship between fibre content and IVPD is also observed in bread products made with pulses such as chickpea and lupin (Wu et al., 2017).

2.10.3: *Tannins*

Tannins are water-soluble polyphenolic compounds with highly variable concentrations in pulses (mg/g DM basis), reported as highest in beans (0.3–24.1), followed by peas (0.6–10.5), lentils (3.4–6.1), and chickpeas (0.6–2.7) (S. G. Gilani et al., 2012; Wang et al., 2009). Most of these tannins persist in human-consumed foods, as they are primarily condensed tannins, which are resistant to hydrolysis. Protein and amino acid digestibility in mammals has been shown to be directly reduced by tannins (Robbins et al., 1991), with multiple digestive mechanisms contributing to this effect (Chung et al., 1998; Hagerman et al., 1992; Nikmaram et al., 2017; Prinz & Lucas, 2000; Sá et al., 2019). One mode of action is the ability of tannins to complex with and precipitate proteins in aqueous solutions (Baxter et al., 1997). These interactions extend to digestive enzymes, such as trypsin, which has been shown to bind tannins non-competitively in *in vitro* digestion using buckwheat tannins (K. Ikeda et al., 1991), with some indirect *in vivo* evidence supporting this mechanism (Eggum et al., 1981). The formation of tannin–protein complexes can reduce protein solubility and increase protein cross-linking (S. G. Gilani et al., 2012). In mammals, salivary proline-rich proteins—secreted by species including humans—bind tannins (Bennick, 1982; Manconi et al., 2016), a mechanism that may help mitigate tannin-related inhibition of digestion (A. J. M. Jansman et al., 1994). While this interaction increases

endogenous losses, it may spare dietary proteins from direct protein digestibility reductions *in vivo*. In contrast, IVPD measurements may overestimate the inhibitory effects of tannins, as *in vitro* systems do not simulate the presence of proline-rich proteins, allowing tannins to bind freely to dietary proteins or digestive enzymes. Tannins also appear to preferentially bind proteins rich in histidine, as indicated by studies where inclusion of faba bean hulls in swine diets led to increased endogenous and crude protein losses at the terminal ileum and across the total digestive tract (A. J. Jansman et al., 1995).

A previous literature review suggested that most processing methods are ineffective, expensive, or labor-intensive for reducing tannin content, positioning genetic manipulation as the most effective strategy (S. G. Gilani et al., 2012). This conclusion, however, appears mixed when processing methods and pulse class are considered individually and relative to cereals. Soaking, germination (24, 48, and 72 hours), and extrusion have been shown to effectively reduce condensed tannin content in faba beans (47.7–60.0% reduction) and variably in red kidney beans (24.2–83.8%), compared to raw seeds, with the greatest reductions achieved through dehulling (92.3–93.3%) (Alonso, Aguirre, et al., 2000). Despite its effectiveness in tannin reduction, dehulling alone was less effective than extrusion in improving IVPD. In both humans and rats, APD of black beans improved when the tannin-rich seed coat was removed, whether before or after cooking (de Godínez et al., 1992).

Similarly, soaking followed by boiling or autoclaving reduced tannin content and increased IVPD in chickpeas, lentils, and several beans compared to untreated controls (Rehman & Shah, 2005). In chickpeas, microwave cooking and germination also reduced tannin content and improved IVPD, although germination was less effective than boiling, autoclaving, or microwave cooking (El-Adawy, 2002). In contrast, fermentation (using *Lactobacillus*

fermentum) increased tannin content in soaked or raw beans, though reductions were observed when fermentation followed pre-cooking (Barampama & Simard, 1994). In peas, dehulling and germination (48 and 72 hours) produced small but significant reductions in tannin content across three varieties, while soaking had no effect. Extrusion was the most effective method for reducing tannin content in peas (Alonso et al., 1998; Alonso, Grant, et al., 2000). Similarly, thermal treatments—such as cooking, autoclaving, and microwave cooking—significantly reduced tannin content in vegetable peas (Habiba, 2002).

Overall, extrusion consistently reduces tannin content and increases protein digestibility, measured both *in vivo* and *in vitro*, across all four pulse classes. However, improvements in protein quality may remain limited by the presence of limiting IAAs (Nikmaram et al., 2017; Nosworthy et al., 2018b, 2018a, 2020; Nosworthy, Franczyk, Medina, et al., 2017). While the mechanism by which extrusion reduces tannin availability is not fully understood without pre-treatments, reductions in condensed tannin content appear most influenced by higher moisture content, screw speed, and temperature inputs during extrusion (Nikmaram et al., 2017; Pasqualone et al., 2020).

2.10.4: *Phytate*

In plant seeds, phosphorus is primarily stored as phytate—the mixed cation salt of inositol hexaphosphate, also known as phytic acid (Lott et al., 2000). Similar to tannins, phytic acid negatively affects protein and amino acid digestibility through multiple mechanisms. Phytate can bind directly to proteins or indirectly via cation bridges formed with amino acids such as arginine, lysine, or histidine, resulting in insoluble complexes at low pH (S. G. Gilani et al., 2012). This interference has been shown to reduce pepsin activity by 7% to 25%, as demonstrated in IVPD assays using bovine serum albumin and casein (Knuckles et al., 1985,

1989; Vaintraub & Bulmaga, 1991). At higher pH, typical of the small intestine or oral cavity, phytate can complex with calcium—essential for optimal trypsin and α-amylase activity—forming protein–cation–phytate bridges (Li et al., 1993; Vaintraub & Bulmaga, 1991). Using a pH-stat method with casein (trypsin only), phytate additions at concentrations comparable to those in pulses decreased digestibility by approximately 20% (Lathia & Koch, 1989).

In pulses, phytate is distributed uniformly throughout the cotyledon layer, typical of all eudicots. Considerable variation in phytate content (mg/g DM) exists across raw pulse classes: lentils (6.2–6.7), peas (2.8–7.1), chickpeas (5.4–12.3), and beans (5.0–15.4) (Batista et al., 2010; Chitra et al., 1995; Li et al., 1993; Porres et al., 2002; Vidal-Valverde et al., 1994). Selection for higher protein content in pulses is also generally associated with higher phytate concentrations (Broughton et al., 2003).

Processing methods are relatively ineffective at reducing phytate in pulses, particularly mechanical processing, which may even concentrate phytate (Alonso, Aguirre, et al., 2000; Alonso et al., 1998; Carnovale et al., 1988). This contrasts with cereals, where phytate is concentrated in milled fractions containing the germ, bran, or pericarp (S. G. Gilani et al., 2012). Protein concentrates and isolates from pulses often contain higher phytate content (Carnovale et al., 1988), although IVPD typically increases (Monsoor & Yusuf, 2002). The protein-to-phytate ratio may help explain the variable impact of phytate on protein digestibility (Carnovale et al., 1988). Thermal treatments alone produce modest reductions in phytate content, such as autoclaving lentils (11.3%) or boiling peas (5.7%) (Bishnoi et al., 1994; Porres et al., 2002). Combining soaking with boiling, autoclaving, or microwaving substantially improves phytate reduction in chickpeas (28.9–41.3%) (El-Adawy, 2002). Soaking alone generally has limited effect, with reductions of 4.6–11.3% in peas (Alonso et al., 1998; Bishnoi et al., 1994), and

variable reductions in beans, with 32.7% in faba and 5.7% in red kidney beans (Alonso, Aguirre, et al., 2000). Extrusion achieves the most significant reductions in phytate content, especially in lentils (93.1–99.3%), accompanied by a linear increase in IVPD (70.6–88.6%) with higher extrusion temperatures (140–180°C) and added moisture (14–22%) (Rathod & Annapure, 2016). More modest reductions are reported in beans (16.9–28%), peas (5.9–17.5%), and chickpeas (4.7–17.5%) (Adamidou et al., 2011; Alonso, Aguirre, et al., 2000; Batista et al., 2010; Marzo et al., 2011). The reduction mechanism during extrusion likely involves thermal degradation of phytate and hydrolysis of inositol hexaphosphate, although factors such as moisture, pressure, and residence time also contribute (Nikmaram et al., 2017). Biological processing, particularly germination, consistently produces substantial phytate reductions: peas (44.5%), beans (30.2–60.8%), lentils (80.2%) and chickpeas (56.2%), typically over 3 to 6 days as phytate is metabolized for seedling development (Alonso, Aguirre, et al., 2000; Alonso et al., 1998; Ayet et al., 1997; El-Adawy, 2002; Lott et al., 2000). Fermentation using lactic acid bacteria also reduces phytate content in chickpeas (39.1%) and black beans (30.4%), though outcomes may be influenced by prior dehulling or soaking (Chitra et al., 1996).

The use of phytase enzymes in animal feeds for broilers and pigs has been extensively studied to improve mineral and protein utilization. Strong negative correlations between phytate and protein or amino acid digestibility in these studies, supported by *in vitro* evidence, have reinforced the understanding of phytate's inhibitory effects (S. G. Gilani et al., 2012; Kies, 2005; Selle et al., 2000). Some authors, however, question the consistency of this relationship, citing mixed evidence regarding protein source, digestibility outcomes (*in vivo* and *in vitro*), and processing effects (Rickard & Thompson, 1997). For instance, rat diets containing casein and added phytate (up to 3.45% of diet) or phytate hydrolysates showed no impact on total tract

protein digestibility (Knuckles et al., 1989). Similarly, total tract digestibility of lentils showed no difference between raw and autoclaved samples, despite small reductions in phytate content (Porres et al., 2002). Comparing total tract and ileal digestibility across diets varying in phytate content in rat models may help clarify phytate's role in protein digestibility, given that rats are often used in assessing protein quality for human nutrition.

2.10.5: *Trypsin Inhibitors*

Trypsin inhibitors fall largely into two categories with varying isoforms, the Kunitz or Bowman-Birk inhibitors, which possess specific binding affinity to trypsin and chymotrypsin at independent binding sites (Sarwar Gilani et al., 2012). Within the *in vivo* context, the effects of trypsin inhibitors may or may not directly influence measures of protein digestibility. This is due to the large magnitude of enzyme output during digestion, such as that demonstrated in rodents and swine (Moughan, 1999b). Decreases in growth, increased ileal or fecal endogenous losses, and pancreatic hypertrophy, characterize the impact of these inhibitors (Boisen & Eggum, 1991; Fernández-Quintela et al., 1998; Nyachoti et al., 1997; Sarwar Gilani et al., 2012; Tavano, Silva Junior, et al., 2008). In this context, *in vitro* digestion models can be limited by a set concentration of trypsin, reflected in a direct reduction in IVPD (Hsu et al., 1977), not otherwise seen *in vivo*. Sequential digestion parameters that feature a gastric step may also introduce a variable *in vivo*, such that low pH can reduce trypsin inhibitor activity (TIA) (Boisen & Eggum, 1991). Decreases in TIA content can be associated with an improvement in protein and AA digestibility, however, these associations may also be significantly influenced by decreases in other intrinsic and extrinsic ANFs (S. G. Gilani et al., 2012; Nikmaram et al., 2017; Sá et al., 2019).

The principal location of trypsin inhibitors is throughout the cotyledon within the protein fractions of pulses and legumes (Anderson & Wolf, 1995; Gatel, 1994). Evidence from albumin fractions in chickpeas and peas demonstrated the highest concentration of TIA (Mariotti et al., 2001; Tavano, da Silva, et al., 2008). Human subjects fed pea protein isolates made from fractions of albumin and/or globulin presented with lower real ileal digestibility values for the albumin fraction (Mariotti et al., 2001). Thermal processing is the most effective means to decrease TIA—as these inhibitors are proteins—they become denatured or destroyed after an effective heating temperature and time are achieved, although some heat-resistant trypsin inhibitors can also be present (Clemente et al., 2000). The availability of thiol groups from the endogenous protein reduce TIA, whereas the addition of thiols (e.g. N-acetylcysteine) also reduced the maximum temperature required to decrease TIA (Friedman et al., 1982). Depending on the nature of these protein fractions, heat-resistant trypsin inhibitors may also be present. The reduction in activity can also be influenced by particle size, total TIA content, cultivar and variety (S. G. Gilani et al., 2012).

Pulses can contain varying TIA (mg TIU/g DM), based on the raw content found in a variety of beans (1.41-5.4) (Alonso, Aguirre, et al., 2000; Batista et al., 2010; Gatel, 1994), lentils (4.79-6.4) (Porres et al., 2002; Vidal-Valverde et al., 1994), peas (0.52-12.5) (Alonso et al., 1998; Alonso, Grant, et al., 2000; Gatel, 1994; Vidal-Valverde et al., 2003), and chickpeas (9.4-12.0) (Singh & Jambunathan, 1981). Conventional preparation of pulses through soaking (4 hours) and cooking (boiled in water at 95C for 1 hour) eliminates most trypsin inhibitor content in peas (78.7-81.3%), lentils (100%), beans (91.7-100%) and chickpeas (83.9-88.4%) depending on variety (Shi et al., 2017). Optimization of cooking time with longer soaking periods (24 hours) in chickpea, beans and lentils produced similarly high decreases in TIA (Wang et al.,

2009, 2010). These significant losses in TIA content can be maintained in extrusion processing, which requires more extensive optimization of parameters. Constant water additions (15-12.5 kg/hour) variable decreasing extrusion temperatures throughout the barrel (94-66°C) and differences in heated pre-treatment (70-100°C) produced a range of reduced TIA content in beans (29.7-53.7) and peas (48.5-58.9%) but was sufficient to optimize reduced content in chickpeas (85.8-91.8%) (Adamidou et al., 2011). Higher extrusion temperatures in peas (145-148°C), and beans (152 and 156°C) matched for moisture (25%), however, were capable of tremendously reducing TIA (peas: 94-95% and beans: 86.1-98.9%) (Alonso, Aguirre, et al., 2000; Alonso et al., 1998; Alonso, Grant, et al., 2000). Dehulling slightly decreases TIA content in peas (0.7-5.8%) (Alonso et al., 1998), but increases in beans (9.62-11.6%) (Alonso, Aguirre, et al., 2000). Soaking alone also elicits small decreases in TIA for both peas (1.6-12.0%) (Alonso et al., 1998), beans (4.5-5.5%) (Alonso, Aguirre, et al., 2000) and lentils (11%) (Vidal-Valverde et al., 1994). Heating pulses without soaking (e.g. autoclaving) may be sufficient to manage TIA, but not maximize reduced TIA, as demonstrated in lentils (76% TIA reduction) (Porres et al., 2002). Germinating peas for three days managed TIA variably (27.4-75.5%) (Alonso et al., 1998), depending on variety, and marginally impacted TIA in lentils (6 days; 23-28%) (Vidal-Valverde et al., 1994) and beans (3 days; 25.3-29.0%) (Alonso, Aguirre, et al., 2000).

2.10.6: *Protein Secondary Structure and Aggregates*

The unadulterated (raw) state of a protein can undergo changes to primary, secondary, tertiary, and quaternary structure when the protein is processed, causing changes in the availability, accessibility and content of nitrogen and amino acids. Changes to covalent and non-covalent bonding, hydrolysis of peptide bonds, protein aggregation and loss or modification of amino acids can also occur (Swaisgood & Catignani, 1991). Several methods may be employed

to characterize protein denaturation, such as protein solubility measures (Table 2.2), sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), size exclusion chromatography, Fourier-transform infrared spectrophotometry (FT-IR), circular dichroism spectrophotometry and AA analysis (Hammann & Schmid, 2014; Liu & Hsieh, 2008). The impact on *in vivo* or *in vitro* digestion will be influenced by these changes, due to the increase or decrease in accessibility of proteases to the protein or peptide substrate, resulting in changes in total nitrogen or amino acids remaining in the feces, terminal ileum, or analogous measurement *in vitro*.

Estimating apparent rat fecal nitrogen digestibility utilizing a modified SDS-PAGE technique identified an excellent correlation (r=0.92) with the density of molecular weights in autoclaved soy protein concentrates (Y. A. Kim & Barbeau, 1991). These estimates provided a large range of difference (≥10%) relative to apparent fecal protein digestibility from rats and had relatively no improvement over the pH drop (r=0.95) which also demonstrated a smaller relative difference to *in vivo* (≤5%). This method has not been explored further in literature, likely due to the complexity of the procedure (Butts et al., 2012), nonetheless it positions the importance of protein aggregates relative to protein digestibility. Changes to secondary structure, however, may infer the complexity of protein aggregates that are, or are not, digestible (Carbonaro et al., 1997). This relationship has been supported in soaked and thermally treated (autoclaved) pulse flours, including chickpea, white bean, faba bean and lentils in addition to barley, emmer wheat, milk, mozzarella cheese and chicken meat which identified a strong inverse relationship (r = -0.98) with IVPD and intermolecular β-sheet aggregates (Carbonaro et al., 2012). Extrusion of soy protein isolate between 60°C to 100°C with low moisture (16%) corresponded to significant changes in secondary structure, most notably within β-sheet structures at higher temperatures of

90°C and 100°C (Ma et al., 2018). These results in-part correspond to the decrease in protein quality (through PER) observed when soy-based products are subject to high heat treatments (Rackis, 1974). The structure of protein aggregates within chickpeas also suggests their importance when considering the digestibility of a cooked product, such that a high protein digestibility when raw may not translate into a cooked product (Ribeiro et al., 2017).

Table 2.2. Types of interactions, specific interaction types, associated amino acids, and their corresponding detection reagents

Type of interaction	Specific interaction	Amino acids	Reagents
Covalent	Disulfide bonding	Cysteine-cysteine	Oxidizing or reducing agents, e.g., performic acid, 2-mercaptoethanol, DTT, Na2SO3
Non-covalent neutral	Hydrogen bonding	Asparagine, glutamine, threonine, serine, cysteine	Strong H-bonding agents, e.g., urea, dimethyl formamide, thiourea, SDS
Non-covalent neutral	Hydrophobic interaction	Tyrosine, tryptophan, phenylalanine proline, methionine, leucine, isoleucine, valine, alanine, glycine	Ionic and nonionic detergents, e.g., SDS, thiourea, Triton, CHAPS, sodium salts of long-chain fatty acids
Non-covalent electrostatic	Acid and/or basic hydrophilic	Aspartic acid, lysine, arginine, histidine, glutamic acid	Acids, alkali or salt solutions

As described by Liu & Hsieh (2008)
SDS, sodium dodecyl sulfate; DTT, dithiothreitol; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate

2.10.7: *Maillard Reaction Products*

Heat treatment and storage of protein foodstuffs may also cause endogenous changes to amino acids, causing the formation of Maillard reaction products (MRPs), such as modifying alanine and lysine residues into fructoselysine, lactuloselysine, and lysinoalanine (S. G. Gilani et al., 2012). Enzyme function, such as that of trypsin, has demonstrated reduced accessibility to protein substrates modified by MRPs (Deng et al., 2017; Swaisgood & Catignani, 1991), which may also be responsible for the formation of covalently bonded protein aggregates (Nagaraj et al., 1996; Teodorowicz et al., 2018). Additionally, the presence of glycated amino acids—as a result of racemization or production of MPRs—will also impact the accessibility of trypsin to nearby (relative to MRPs) non-glycated products (Deng et al., 2019). Within extruded lupin (Palanisamy et al., 2019), brown rice, or pinto beans (Sumargo et al., 2016), colour analysis indicating brightness and yellowing (as a measure of MRPs) was associated with decreased IVPD when products were darker and more yellow. Digestibility in pigs also demonstrated a significant decrease in lysine, phenylalanine, cystine, aspartic acid, glycine and most amino acids (-6%), when fed a diet high in MRPs (50% lysine blockage) (Rérat et al., 2002).

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2.12: BRIDGE TO CHAPTER 3.

Chapter 2 reviewed the theoretical and methodological landscape of protein quality assessment in pulses, with particular emphasis on the utility of *in vitro* models and the challenges posed by intrinsic and extrinsic factors. While that discussion encompassed a range of influences on protein digestibility—including antinutritional compounds and structural modifications—the current chapter focuses specifically on how two pre-milling treatments—moisture conditioning and mechanical scouring—affect protein quality in green lentils and yellow peas. These treatments were implemented as hull removal strategies prior to roller milling and assessed for their impact on protein and amino acid composition, IVPD, and IVPDCAAS. By focusing on these defined processing variables, the study offers new insight into how targeted modifications can influence protein digestibility and quality outcomes as assessed by *in vitro*. This work extends previous research on roller milling in the same pulse varieties, which evaluated protein quality alongside functional characteristics (Guldiken et al., 2022). Through the inclusion of pre-treatment variables, the present analysis contributes to a more detailed understanding of processing effects on pulse protein quality.

CHAPTER 3. THE IMPACT OF MECHANICAL SCOURING AND MOISTURE CONDITIONING ON THE *IN VITRO* PROTEIN DIGESTIBILITY AND QUALITY OF ROLLER-MILLED GREEN LENTIL (*LENS CULINARIS*) AND YELLOW PEA (*PISUM SATIVUM*)

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3.1: ABSTRACT

Background and objectives: Milling practices, otherwise refined for specific uses in cereal-based foods, have not been thoroughly developed for pulses. This study investigates whether scouring and moisture conditioning pre-treatments on yellow peas and green lentils can enhance hull removal, and in turn, whether changes in hull removal alter *in vitro* protein digestibility and quality.

Findings: Total by-product losses were significant in green lentils when subject to scouring, which was altered by high moisture addition in yellow peas. The scouring pre-treatment altered both the protein digestibility and amino acid scores of green lentils, which translated to improved protein quality in all streams, but significantly in the break flour stream. Yellow peas similarly demonstrated significant improvements in protein quality from scouring, as a result of altered amino acid scores.

Conclusion: The addition of a scouring procedure can improve the protein quality of yellow peas and green lentils.

Significance and novelty: Pulse milling procedures are rarely evaluated for optimization of protein quality. This research establishes milling pre-treatments which may be used to enhance the protein quality of yellow peas and green lentils.

3.2: INTRODUCTION

Pulses comprise a class of dry edible leguminous seeds, including peas, lentils, beans, and chickpeas. The consumption of pulses fulfills several important roles for people worldwide, particularly as an inexpensive source of protein, but also an important source of energy, fibre, vitamins, and minerals. Canada represents one of the largest producers and exporters of pulses, particularly in peas and lentils (Oduro-Yeboah et al., 2023; Tulbek et al., 2024). Interest in these pulses is also fueled by demand in plant-based proteins, with diversification of proteins being pushed for by consumers. Recent updates to dietary guidelines within Canada have also positioned pulses alongside other protein foods and encourages the consumption of plant-based protein more often (Fernandez et al., 2020). What constitutes a protein food may be more difficult to ascertain for the consumer, which may be supported through front-of-package labelling (Kaur et al., 2017). Communicating these claims for protein requires establishing protein quality in Canada and the US, which is determined by the Protein Digestibility Corrected Amino Acid Score (PDCAAS). This method assesses the digestibility of protein supplied by the test article, in addition to the first limiting amino acid for a given age group. The use of animal bioassays are required to determine the protein digestibility component, where recent *in vitro* analyses have shown good association with these values for peas and lentils in estimating the PDCAAS (Nosworthy et al., 2018; Nosworthy, Franczyk, Medina, et al., 2017). Plant-based foods or ingredients, such as those from pulses, are of particular interest in improving the protein quality of ready-to-eat foods such as breads, rolls, crackers and other baked goods. These foods are frequently cereal-based, and limiting in the amino acid lysine, which is complemented from the inclusion of pulses, which has an abundance of lysine. Individuals within a Canadian population who consume a mid to high proportion of their diet from plant-based proteins do so

from both ready-to-eat and cereal-based foods, demonstrating an increased risk for insufficient protein intake as well as inadequate quality of protein intake (Marinangeli et al., 2021). Consumption of pulses is low in Canada due to their prolonged preparation (soaking and cooking) times and the knowledge required to incorporate them into Western diets (Mudryj et al., 2012; Niva et al., 2017). However, their incorporation into a variety of ready-to-eat foods has shown consumer acceptance (Boye et al., 2010).

Milling pulses into flour, which meets techno-functional properties for flour-based applications, still requires robust development of standards for quality (Scanlon et al., 2018). Roller-milling has demonstrated practical applications in cereal milling (Cappelli et al., 2020), allowing for greater control of desired aspects of flour quality, such as producing additional streams with different particle sizes and removing the outer seed coat (hull). This level of control is appealing in peas and lentils where seed characteristics, such as size and 100-seed weight, are relevant factors in determining the proportion of seed which is inadequately or excessively ground (Thakur et al., 2019). With pulses, greater attention must be paid to the hull fraction, as separation of the hull for some pulse types is challenging due to strong adhesion with the cotyledon layer, bound by gums and pectin, in addition to the interplay of lignin and minerals (Vishwakarma et al., 2018). Hull removal may be warranted for final pulse flour applications due to the presence of antinutritional factors, such as phenolics or condensed tannins in the seed hull, with the highest content in beans, followed by peas, lentils and chickpeas (Gilani et al., 2012). Condensed tannins, for example, are responsible for several phenolic-protein interactions which impact protein hydrolysis, negatively affecting protein digestibility and quality (Ozdal et al., 2013). In this regard, hull removal has been achieved through mechanical scouring, where light abrasion is used to grind the outer seed coat (Vishwakarma et al., 2018). Moisture addition or

conditioning can also loosen the tight bonds of pectin and lignin, further aiding hull removal. Conversely, hull removal may also lead to changes in the overall antinutritional and nutritional profile of pulses. For example, the mechanical removal of the hull can increase phytate content. This antinutritional factor also decreases protein digestibility and quality (Sá et al., 2019), as the proportion of phytate is higher in the cotyledon layer than in the whole milled seed (Saldanha Do Carmo et al., 2022; Wang et al., 2009). Improvements in protein digestibility and quality are generalized across all pulses, where dehulling is used in conjunction with, or is additive in reducing the antinutritional content (i.e. tannins and phytate) following thermal treatments (Patterson et al., 2017). The hull is also a significant contributor to the fibre content of pulses, which may be selectively re-added as an ingredient to food products (Zhong et al., 2018). Specific preparation of seed with effective milling strategies are required to optimize both the techno-functional and the nutritional quality of pulses.

Current works have characterized the impact of roller milling on the techno-functional properties of yellow peas, red lentils and green lentils (Guldiken et al., 2022; Motte et al., 2021; Pulivarthi et al., 2021). Some compositional, functional, and physical characteristics following scouring and moisture pre-treatment from green lentils in this study have been evaluated (Choo et al., 2022). Milling quality standards do not traditionally evaluate protein quality and instead focus on crude protein content for specific end-use applications. In this study, scouring and moisture pre-treatments were applied to both yellow peas and green lentils to assess the extent of change in protein digestibility and quality through *in vitro* methods.

3.3: Materials and Methods

3.3.1: *Pulse samples*

Yellow peas (CDC Spectrum) and green lentils (CDC Greenstar) were harvested in 2018 from Limerick, Saskatchewan, Canada. Seeds were stored at ambient room temperature (22 ± 2°C) and transferred to a freezer (-20°C) once milled.

3.3.2: *Pulse seed pre-treatment*

Descriptions of the experimental pre-treatments involving moisture conditioning and scouring have been previously published for green lentils used in this study (Choo et al., 2022). The same pre-treatment details were applied to yellow peas and are provided in brief. Moisture conditioning was applied at three levels (w/w): no moisture (0%), moderate moisture (0.5%), and high moisture (1%). Initial seed moisture was determined prior to addition by an infrared grain analyzer (Inframatic 9500 Grain and Flour Analyzer, Perten Instruments, Winnipeg, MB). Samples were then either mechanically scoured or left unscoured, resulting in six combinations of seed pre-treatment (Table 3.1) for each flour stream, prepared in duplicate.

Table 3.1. Summary of seed pre-treatments and flour streams in yellow peas or green lentils

Scouring Level (SL)	Moisture Level % (ML)	Flour Stream (FS)
Unscoured (U)	0, 0.5, 1	Break (BK), Middling (MD), Straight Grade (SG)
Scoured (S)	0, 0.5, 1	Break (BK), Middling (MD), Straight Grade (SG)

3.3.3: *Moisture, Scouring and Pre-Break*

The procedures and desired seed moisture were previously described and calculated, in addition to parameters describing scouring on a Bühler MHXA 50/70 (Bühler Group, Uzwil, Switzerland) and the initial pre-break of seeds using the first break (B1) rolls on a Bühler MDDM 1000/250 pilot roller mill (Bühler Group, Uzwil, Switzerland) prior to milling (Choo et al., 2022). Green lentils did not require a pre-break step due to their smaller seed size. Hulls were collected from scouring activities and contributed to total by-product.

3.3.4: *Flour milling and blends*

Pre-broken pulses were milled using a Bühler MLU 202 laboratory mill (Bühler Group, Uzwil, Switzerland). Processing conditions for roller milling including feed rate, sieve size and clearances were previously published (Choo et al., 2022; Guldiken et al., 2022). Yellow pea and green lentil flour blends (break, streams: B1+B2+B3; middling, streams: 1M+2M+3M) were prepared using proportions to the total yield of the milled product from break streams (BK) and middling (MD) streams (Figure 3.7). Straight grade (SG) flour was prepared using proportions to the total yield of milled product from each flour stream. Total milling by-products, including hulls from scouring as well as hulls and shorts from milling, were collected and calculated on a starting weight basis (i.e. before any pulse seed processing). Hulls specifically from milling activities were calculated separately based on a total product basis (i.e. not including scoured materials). Total by-products and hulls were not further assessed in this study.

3.3.5: *Chemicals*

Digestive enzymes were purchased from Sigma-Aldrich (Oakville, ON, Canada), including chymotrypsin (bovine pancreas ≥40 units/mg protein), trypsin (porcine pancreas 13,000-20,000 BAEE units/mg protein) and protease (Streptomyces griseus ≥3.5 units/mg solid). High nitrogen casein (80 mesh) was acquired from Dyets Inc. (Bethlehem, PA, USA). All other ACS grade reagents were acquired from Fisher Scientific (Ottawa, ON, Canada).

3.3.6: *Composition*

Crude protein (N × 6.25) was determined through Dumas combustion for each sample in duplicate on a FP828 LECO nitrogen analyzer (LECO Corp., St. Joseph, MI). The use of 6.25 as the nitrogen-to-protein conversion factor is consistent with guidelines in Canada and the US on protein quality assessment for protein content claim substantiation and nutrition facts tables (House et al., 2024). As the primary objective of this work was to explore the impact of pulse processing steps on the "protein quality" of yellow peas and green lentils, we used the nutritionally relevant nitrogen conversion factor (6.25), as opposed to the biologically relevant factor (generally much lower) in order to allow comparisons to other research. This also allows for direct comparison to national and international databases for pulse nutritional quality that use 6.25. Dry matter was determined for both yellow peas (Table 3.8) and green lentils (Table 3.9) using a standardized protocol (AOAC 925.10).

3.3.7: *Amino acid (AA) composition*

Prior to acidic AA hydrolysis, methionine and cysteine were oxidized to methionine sulfone and cysteic acid through the performic acid procedure (AOAC 994.12). Alkaline

hydrolysis was utilized for tryptophan (ISO 13904) and the remaining AAs were determined using regular hydrolysis (AOAC 982.30). Precolumn derivatization utilizing the AccQ-Tag system (Waters Corporation, Milford, MA, USA) was employed for all AAs except tryptophan (Nosworthy, Franczyk, Zimoch-Korzycka, et al., 2017). Reverse-phase HPLC was used to quantify AAs through UV detection or fluorescent emission. Appropriate reference standards were included in each batch of analysis. Samples were hydrolyzed, analyzed in singlets, and calculated as free AA (% as is, sample basis).

The AA ratios for each test article (pulse flour) are derived from dividing each indispensable AA by its relative abundance, expressed as milligrams of AA for each gram of test article protein, by the relative abundance of each AA from a given reference pattern for ages 2-5 (Food and Agriculture Organization of the United Nations & World Health Organization, 1991). The resulting AA with the lowest ratio—the limiting AA—was selected as the amino acid score (AAS).

$$\mathrm{AAS} = \frac{\text{mg of AA per gram of protein (test article)}}{\text{mg of AA per gram of protein (reference pattern)}}$$

3.3.8: *In vitro protein digestibility (IVPD)- corrected amino acid score (IVPDCAAS)*

Protein digestion through an *in vitro* protocol was conducted in triplicate, as described by Hsu et al. (1977) with modified enzymatic preparation and regression expression from Tinus et al. (2012) and Franczyk (2018). Pulse flour equal to 10mg of nitrogen was added to 10mL of deionized water, heated to 37°C and allowed to solubilize for one hour before adjusting pH to 8.0 ± 0.05 with 1M NaOH or HCl. A multienzyme solution containing 3.1mg/mL chymotrypsin,

1.6mg/mL trypsin and 1.3mg/mL protease prepared in deionized water was also adjusted to pH 8.0 ± 0.05 at 37°C. The pH-stable multienzyme solution was then transferred to an ice water bath (4°C) for the duration of the assay. Reproducibility was ensured by including casein as a sample standard between batches. The IVPD was determined as the change in pH after exactly 10 minutes from when 1mL of enzyme solution was added to the pulse flour solution. This measure associates the increase of carboxylic acid in solution—as proteins are hydrolyzed to peptides and amino acids—with measures of apparent (IVPD1) and true (IVPD2) protein digestibility found *in vivo*:

$$\mathrm{IVPD1} = 65.66 + 18.10(\Delta \mathrm{pH}_{10\mathrm{minutes}})$$

$$\mathrm{IVPD2} = 76.15 + 15.26(\Delta \mathrm{pH}_{10\mathrm{minutes}})$$

The product from the IVPD (1 or 2) and the AAS are then used to calculate IVPDCAAS:

$$\mathrm{IVPDCAAS}\ (1\ \mathrm{or}\ 2) = \ \mathrm{IVPD}\ (1\ \mathrm{or}\ 2)\ \mathrm{x}\ \mathrm{AAS}$$

3.3.9: *Statistics*

Pre-treatment and flour stream combinations for each pulse produced eighteen unique samples in duplicate (36 total). Crude protein, dispensable AAs, indispensable AAs (including cysteine and tyrosine), IVPD, IVPDCAAS and AAS were subject to ordinary two-way ANOVA with Tukey's multiple comparison test with a single pooled variance. Full effects were compared for moisture and scouring within a pulse class for each stream. Hulls and total by-product were also subject to ordinary two-way ANOVA comparing the effects of scouring or scouring and moisture. Analysis was preformed using GraphPad Prism (V9.5.1., GraphPad Software, Boston, MA, USA) and statistical significance for effects were reported if $P < 0.05$.

3.4: RESULTS & DISCUSSION

3.4.1: *Pre-treated yellow pea and flour streams*

3.4.1.1 Hulls and total by-product

Total by-product, including hulls from the scourer, and hulls and shorts from the mill for yellow peas demonstrated a significant difference between flours produced from scouring and the unscoured for the same high (1%) moisture pre-treatment (Figure 3.1., a), which ranged from 8.1 to 13.8% (starting weight). Hulls removed during roller-milling (Figure 3.1., b) ranged from 4.2 to 8.3% (total product). Unscoured peas with no moisture conditioning resulted in significantly greater hull removal from milling relative to either added moisture condition. This loss is in contrast to hydration favoring separation of hulls and cotyledon in pulses (Vishwakarma et al., 2018). Which may have been expected due to the high porosity of peas, as this can impart enhanced hull separation. Once scoured, high moisture conditioning had a significant decrease in hull collected from the mill. Additionally, scouring at this moisture condition also significantly increased total-by product relative to all other pre-treatments, where otherwise there are no differences between total by-product in seeds which are unscoured or scoured. Flour streams and their pre-treatments in yellow pea and green lentil are evaluated separately, as each pulse differs in physical and mechanical properties, such as seed shape (e.g. length, width, thickness, sphericity) and seed weight which influence milling (Thakur et al., 2019). Evidently a varietal selection can also impact a seeds capacity to hydrate (Ajala et al., 2022), influencing hull separation. Additional factors such as agronomic, climatic variables and machine operating parameters can also influence milling efficiency, which are not extensively evaluated in this study (Kumar et al., 2023).

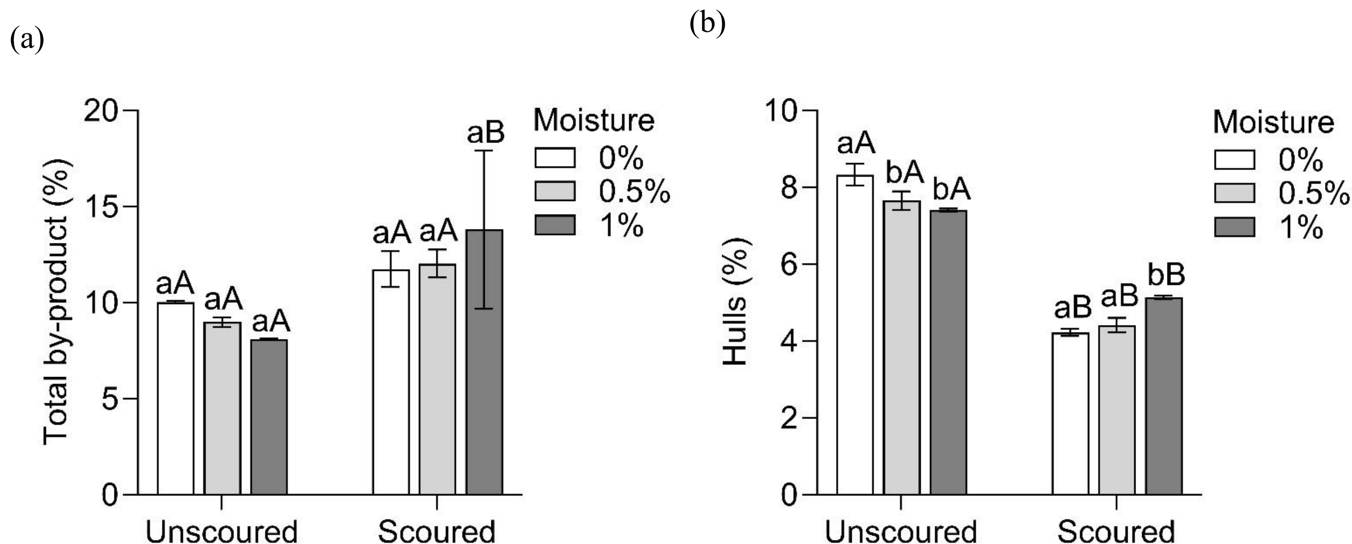

Figure 3.1. Total by-product (starting weight) including hulls removed during scouring and roller milling (a) and (b) hulls removed during roller milling (total product) for yellow peas in unscoured and scoured seeds subject to 0 , 0.5 and 1% moisture pre-treatment. Difference in lowercase letter for mean values indicate significant difference within scouring and uppercase letter for moisture & scouring (P < 0.05).

3.4.1.2 Protein and amino acid composition

Crude protein content ranged from 24.4 to 28.9% (as is) for all pre-treatments and flour streams (Figure 3.2). Protein content was lowest in the BK (24.4 to 24.9%) and highest in the MD (28.1 to 28.6%) flours as previously observed in similar roller-milling activities (Guldiken et al., 2022). Within each stream, protein content was reduced in peas subject to scouring and observed to be significant in BK and SG flours with high moisture conditioning. Scouring in the SG and MD flours were also significantly reduced in protein content with no moisture conditioning. The impact of seed scouring is consistent with total by-product losses, such that scouring and added moisture conditioning increased product loss. The crude protein content is within the range for peas (Nosworthy et al., 2021; Sá et al., 2024), which may be as low as 20.9% and as high as 33.9% (as is). The variety of peas assessed in this study, CDC Spectrum,

was previously reported to be lower in protein content (Warkentin et al., 2017). This may in part be explained by the method of determination—NIR—which can contribute as much as 5% in approximate error in estimating crude protein content of peas (Hacisalihoglu et al., 2020). Notably, most of these protein ranges cover whole peas, where dehulling of pulses increase crude protein content (Thakur et al., 2019). Roller-milling activities in yellow peas, in the absence of pre-treatment—but dehulled—have also yielded a significant range of protein content, which varies based on the selection of rollers and sieves (Motte et al., 2021; Pulivarthi et al., 2021). Nitrogen conversion factors used to estimate protein content in these studies are all 6.25, which is an area of debate (Krul, 2019; Mariotti et al., 2008). Lower nitrogen-protein conversion can alter protein content in pulses, and increase protein quality, when using a proposed factor of 5.4. Protein quality evaluation in this study follows guidelines on protein content claims in Canada and the US, where a factor of 6.25 is recommended. Furthermore, recommendations have yet to be made in adopting specific nitrogen-conversion factors in the area of protein quality (Food and Agriculture Organization of the United Nations, 2013).

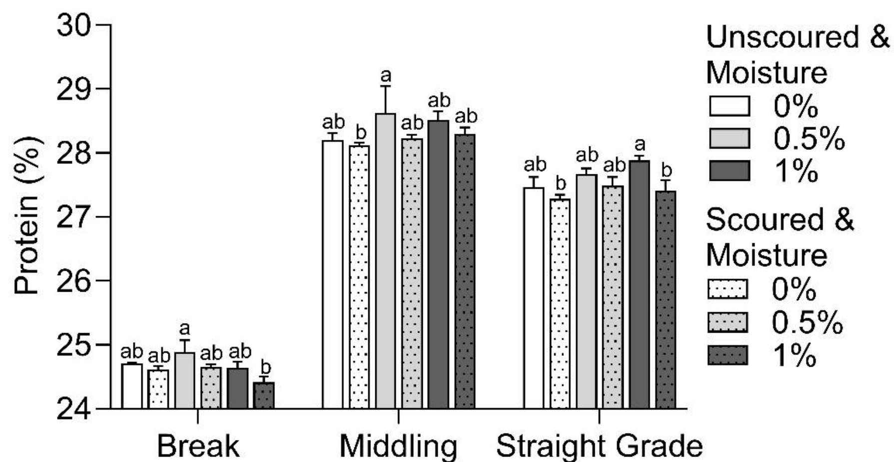

Figure 3.2. Protein content (% as is) of yellow pea flours in unscoured and scoured seeds subject to 0, 0.5 and 1% moisture pre-treatment. Means within the same stream followed by differing lowercase letter are significantly different ($P < 0.05$)

The content of dispensable (Table 3.2) and indispensable (Table 3.3) AAs is provided, along with total IAA content (Figure 3.3), ranging from 8.60 to 10.16% (as is, sample basis). Streams also demonstrated a consistently significant effect for total indispensable AA content as it did for protein content, mainly by differences observed between the BK flours relative to MD or SG flours. Similarly, these streams observe consistently greater aspartic and glutamic acid in MD or SG flours relative to BK flours (Guldiken et al., 2022). The significant effect of the pre-treatments—primarily scouring—on the content of protein is no longer observed on the total content of indispensable AAs. Protein losses may be supported by consistent and, in several instances, significant losses of dispensable AAs, such as glutamic acid in each of the scoured streams, followed by serine and aspartic acid in the MD and SG flours. Seed storage proteins with a higher content of glutamic acid may suggest the losses are primarily from globulins (e.g., vicilin); however, little to no losses are observed from indispensable AAs, which may be expected—based on their content and proportion within the seed (Barac et al., 2010; Rubio et al., 2014). The prevalence of free-form AAs—such as glutamic acid —is more likely, due to their greater content within the endosperm (Melkus et al., 2009). This follows that there is likely a higher content of endosperm lost in flours produced using scouring, as demonstrated by the greater yield of total by-product in scoured flours.

Table 3.2. Dispensable amino acid composition of yellow pea flours (% as is, sample basis)

FS	ML	SL	SER	ARG	GLY	ASP	GLU	ALA	PRO
BK	0	U	1.007 (0.032)	1.630 (0.018)	0.946 (0.007)	2.398 (0.021)	3.628 (0.032)[a]	0.887 (0.006)	0.957 (0.007)
		S	0.993 (0.038)	1.641 (0.009)	0.923 (0.017)	2.377 (0.039)	3.588 (0.047)[ab]	0.876 (0.005)	0.918 (0.008)
	0.5	U	1.029 (0.050)	1.627 (0.082)	0.920 (0.001)	2.394 (0.058)	3.596 (0.117)[ab]	0.877 (0.033)	0.922 (0.045)
		S	0.995 (0.019)	1.603 (0.041)	0.920 (0.034)	2.329 (0.043)	3.521 (0.043)[b]	0.878 (0.028)	0.912 (0.026)
	1	U	1.066 (0.033)	1.664 (0.020)	0.915 (0.006)	2.394 (0.017)	3.616 (0.002)[a]	0.881 (0.001)	0.943 (0.008)
		S	1.020 (0.018)	1.689 (0.022)	0.921 (0.035)	2.405 (0.082)	3.640 (0.121)[a]	0.883 (0.028)	0.941 (0.018)
MD	0	U	1.157 (0.001)[ab]	1.907 (0.018)	1.054 (0.012)	2.690 (0.018)[ab]	4.039 (0.000)[a]	1.000 (0.004)	1.074 (0.004)
		S	1.157 (0.010)[ab]	1.888 (0.031)	1.051 (0.010)	2.714 (0.029)[a]	4.064 (0.042)[a]	1.009 (0.005)	1.048 (0.010)
	0.5	U	1.167 (0.034)[ab]	1.868 (0.128)	1.066 (0.025)	2.732 (0.028)[a]	4.054 (0.008)[a]	1.014 (0.034)	1.053 (0.048)
		S	1.113 (0.020)[b]	1.880 (0.041)	1.021 (0.022)	2.612 (0.061)[b]	3.931 (0.047)[b]	0.982 (0.029)	1.029 (0.028)
	1	U	1.212 (0.043)[a]	1.886 (0.017)	1.082 (0.018)	2.726 (0.072)[a]	4.097 (0.093)[a]	1.021 (0.025)	1.084 (0.007)
		S	1.147 (0.015)[ab]	1.908 (0.066)	1.053 (0.001)	2.703 (0.010)[a]	4.082 (0.024)[a]	1.012 (0.013)	1.067 (0.029)
SG	0	U	1.136 (0.046)[ac]	1.873 (0.032)	1.020 (0.031)	2.606 (0.015)[a]	3.927 (0.078)[a]	0.979 (0.018)	1.045 (0.027)
		S	1.093 (0.036)[a]	1.848 (0.014)	1.003 (0.002)	2.615 (0.014)[a]	3.933 (0.017)[a]	0.968 (0.005)	1.009 (0.003)
	0.5	U	1.250 (0.015)[b]	1.886 (0.053)	1.062 (0.023)	2.711 (0.089)[b]	4.070 (0.121)[b]	1.007 (0.038)	1.066 (0.026)
		S	1.120 (0.002)[a]	1.816 (0.006)	1.015 (0.009)	2.605 (0.042)[a]	3.917 (0.022)[a]	0.970 (0.009)	1.013 (0.003)
	1	U	1.218 (0.000)[bc]	1.898 (0.031)	1.020 (0.006)	2.662 (0.008)[ab]	3.989 (0.000)[ab]	0.984 (0.002)	1.046 (0.002)
		S	1.109 (0.040)[a]	1.865 (0.018)	1.014 (0.021)	2.621 (0.036)[ab]	3.950 (0.054)[a]	0.981 (0.011)	1.045 (0.004)

FS, Flour stream; BK, Break; MD, Middling; SG, Straight grade; SL, Scouring level; U, Unscoured; S, Scoured; ML, Moisture level (%); Ser, Serine; Arg, Arginine; Gly, Glycine; Asp, Aspartic acid; Glu, Glutamic acid; Ala, Alanine; Pro, Proline; Means within a column of the same stream followed by differing lowercase letter are significantly different ($P < 0.05$), SD in brackets

Table 3.3. Indispensable amino acid composition of yellow pea flours (% as is, sample basis)

FS	ML	SL	HIS	THR	CYS	LYS	TYR	MET	VAL	ILE	LEU	PHE	TRP
BK	0	U	0.456 (0.006)	0.792 (0.001)	0.307 (0.001)	1.483 (0.013)	0.709 (0.056)	0.219 (0.003)	1.089 (0.008)	0.951 (0.010)	1.541 (0.007)	1.064 (0.004)	0.192 (0.001)
		S	0.443 (0.006)	0.781 (0.006)	0.312 (0.004)	1.505 (0.012)	0.731 (0.006)	0.201 (0.011)	1.038 (0.003)	0.937 (0.003)	1.537 (0.024)	1.056 (0.001)	0.198 (0.004)
	0.5	U	0.446 (0.015)	0.780 (0.023)	0.295 (0.007)	1.471 (0.081)	0.723 (0.011)	0.232 (0.015)	1.038 (0.057)	0.915 (0.057)	1.529 (0.036)	1.049 (0.040)	0.187 (0.005)
		S	0.406 (0.036)	0.771 (0.018)	0.305 (0.011)	1.477 (0.030)	0.732 (0.010)	0.207 (0.001)	1.033 (0.030)	0.932 (0.013)	1.499 (0.019)	1.039 (0.016)	0.199 (0.001)
	1	U	0.445 (0.008)	0.782 (0.013)	0.298 (0.001)	1.483 (0.017)	0.728 (0.050)	0.229 (0.024)	1.063 (0.007)	0.945 (0.000)	1.519 (0.008)	1.057 (0.001)	0.194 (0.000)
		S	0.449 (0.021)	0.796 (0.036)	0.309 (0.001)	1.504 (0.055)	0.721 (0.008)	0.214 (0.004)	1.069 (0.018)	0.952 (0.034)	1.534 (0.048)	1.059 (0.029)	0.183 (0.010)
MD	0	U	0.519 (0.008)	0.896 (0.002)	0.339 (0.001)	1.699 (0.031)	0.854 (0.012)^{ab}	0.240 (0.002)	1.214 (0.008)	1.058 (0.003)	1.740 (0.002)	1.202 (0.007)	0.221 (0.002)
		S	0.524 (0.010)	0.897 (0.008)	0.345 (0.006)	1.731 (0.020)	0.807 (0.018)^{ab}	0.245 (0.002)	1.196 (0.012)	1.065 (0.018)	1.763 (0.012)	1.211 (0.017)	0.230 (0.003)
	0.5	U	0.521 (0.006)	0.901 (0.028)	0.339 (0.003)	1.703 (0.005)	0.876 (0.056)^{a}	0.259 (0.004)	1.194 (0.055)	1.061 (0.051)	1.723 (0.039)	1.194 (0.024)	0.215 (0.002)
		S	0.518 (0.005)	0.884 (0.022)	0.355 (0.001)	1.693 (0.022)	0.783 (0.087)^{b}	0.249 (0.007)	1.164 (0.017)	1.051 (0.006)	1.714 (0.058)	1.182 (0.023)	0.228 (0.003)
	1	U	0.524 (0.011)	0.918 (0.015)	0.342 (0.006)	1.727 (0.041)	0.830 (0.019)^{ab}	0.288 (0.018)	1.241 (0.032)	1.087 (0.014)	1.765 (0.026)	1.211 (0.002)	0.227 (0.000)
		S	0.503 (0.016)	0.903 (0.014)	0.342 (0.003)	1.717 (0.016)	0.858 (0.061)^{ab}	0.244 (0.004)	1.213 (0.034)	1.057 (0.015)	1.769 (0.019)	1.206 (0.010)	0.228 (0.001)
SG	0	U	0.576 (0.116)	0.871 (0.013)	0.335 (0.006)	1.664 (0.011)	0.815 (0.101)	0.237 (0.004)	1.193 (0.032)	1.041 (0.053)	1.677 (0.003)	1.156 (0.029)	0.215 (0.002)
		S	0.495 (0.004)	0.870 (0.013)	0.344 (0.004)	1.649 (0.001)	0.862 (0.021)	0.234 (0.002)	1.149 (0.004)	1.032 (0.021)	1.683 (0.016)	1.151 (0.003)	0.225 (0.003)
	0.5	U	0.514 (0.008)	0.897 (0.023)	0.318 (0.001)	1.697 (0.085)	0.851 (0.038)	0.245 (0.009)	1.209 (0.033)	1.058 (0.023)	1.743 (0.058)	1.194 (0.037)	0.214 (0.004)
		S	0.510 (0.010)	0.870 (0.007)	0.331 (0.007)	1.657 (0.038)	0.794 (0.002)	0.233 (0.010)	1.155 (0.003)	1.039 (0.031)	1.695 (0.013)	1.167 (0.001)	0.225 (0.001)
	1	U	0.523 (0.013)	0.883 (0.003)	0.330 (0.005)	1.673 (0.013)	0.835 (0.047)	0.281 (0.029)	1.186 (0.002)	1.043 (0.002)	1.719 (0.012)	1.177 (0.003)	0.216 (0.000)
		S	0.490 (0.001)	0.875 (0.004)	0.336 (0.001)	1.665 (0.055)	0.787 (0.023)	0.231 (0.004)	1.191 (0.003)	1.043 (0.022)	1.706 (0.006)	1.169 (0.001)	0.218 (0.001)

FS, Flour stream; BK, Break; MD, Middling; SG, Straight grade; SL, Scouring level; U, Unscoured; S, Scoured; ML, Moisture level (%); His, Histidine; Thr, Threonine; Cys, Cystine; Lys, Lysine; Tyr, Tyrosine; Met, Methionine; Val, Valine; Ile, Isoleucine; Leu, Leucine; Phe, Phenylalanine; Trp, Tryptophan; Means within a column of the same stream followed by differing lowercase letter are significantly different ($P < 0.05$), SD in brackets

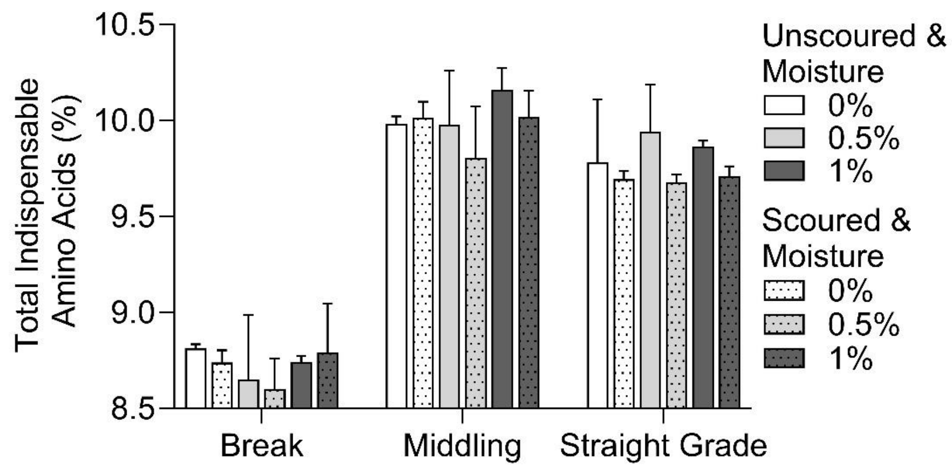

Figure 3.3. Total indispensable amino acid content (% as is, sample basis) content of yellow pea flours in unscoured and scoured seeds subject to 0, 0.5 and 1% moisture pre-treatment. Means within the same stream followed by differing lowercase letter are significantly different ($P < 0.05$).

3.4.1.3 Amino acid score

When calculating the most limiting AA based on the reference pattern for children aged 2-5 (Food and Agriculture Organization of the United Nations & World Health Organization, 1991), yellow peas were consistently limiting in tryptophan (Table 3.4). The AAS in all yellow pea flours ranged from 0.68 to 0.75. Roller-milled yellow peas in the absence of pre-treatments also found similar, but slightly higher AAS (Guldiken et al., 2022), which were also limiting in both tryptophan and sulfur AAs. Flour produced in any stream using scouring had consistently higher AASs' than the unscoured; however, at high moisture conditioning this effect was negated. Several significant differences are observed in these specific conditioned flours, particularly in relation to the unscoured and the moderate (0.5%) moisture conditioned samples which decreased in AAS across all three streams. These effects are not significant when observing the content of tryptophan, whether from streams or pre-treatments. Small differences in the content of tryptophan and larger differences observed on protein content, explain the varied AAS in these flours, while maintaining the same limiting AA. Whether peas are whole, dehulled, or receive thermal treatments, tryptophan or sulfur AAs are consistently limiting (Boye et al., 2012; Nosworthy et al., 2021; Nosworthy, Franczyk, Medina, et al., 2017; Sá et al., 2024). Depending on treatments, specifically through thermal or biological (fermentation) means (Boye et al., 2012; Nosworthy, Franczyk, Medina, et al., 2017), peas increase in AAS (i.e. > 0.75) over raw flours and may no longer have a limiting AA (i.e. AAS ≥ 1.00). Growing location, year, genotype and their interaction (Larmure et al., 2005; Nosworthy et al., 2021; Wang & Daun, 2004) have also been factors effecting the AAS of peas, in part due to changes in protein and indispensable AA content. Analysis of 480 pea samples evaluating the influence of different AAS patterns demonstrated sulfur AAs to be the most limiting, following by tryptophan or

leucine, depending on the scoring pattern (Sá et al., 2024). If scoring patterns are adjusted to these guidelines—which are not yet adopted by Canada or the US—for children aged 6 months to 3 years (Food and Agriculture Organization of the United Nations, 2013), the sulfur AAs would become the most limiting in this study and AAS would increase (0.74 to 0.84; data not shown).

Table 3.4. *In vitro* protein digestibility and quality of yellow pea flours (%)

FS	ML	SL	IVPD1	IVPD2	AAS	LAA	IVPDCAAS1	IVPDCAAS2
BK	0	U	81.86 (0.80)	89.81 (0.68)	0.71 (0.00)[ab]	TRP	57.90 (0.71)[ab]	63.52 (0.69)[ab]
		S	82.79 (0.84)	90.60 (0.71)	0.73 (0.02)[a]	TRP	60.39 (1.19)[a]	66.09 (1.35)[a]
	0.5	U	82.58 (0.62)	90.42 (0.52)	0.68 (0.02)[b]	TRP	56.28 (1.82)[b]	61.62 (1.98)[b]
		S	83.25 (1.46)	90.98 (1.23)	0.73 (0.00)[a]	TRP	61.11 (0.37)[a]	66.78 (0.29)[a]
	1	U	83.19 (1.09)	90.93 (0.92)	0.72 (0.00)[ab]	TRP	59.69 (0.71)[ab]	65.24 (0.68)[ab]
		S	82.89 (0.71)	90.67 (0.60)	0.68 (0.04)[b]	TRP	56.53 (3.05)[b]	61.84 (3.30)[b]
MD	0	U	81.86 (0.65)	89.81 (0.55)	0.71 (0.00)[ab]	TRP	58.42 (0.48)[ab]	64.10 (0.51)[ab]
		S	82.55 (0.59)	90.39 (0.50)	0.74 (0.01)[a]	TRP	61.40 (0.80)[a]	67.23 (0.87)[a]
	0.5	U	82.13 (0.85)	90.04 (0.72)	0.68 (0.02)[b]	TRP	56.17 (1.07)[b]	61.58 (1.26)[b]
		S	82.92 (1.28)	90.70 (1.08)	0.73 (0.01)[a]	TRP	60.88 (0.61)[a]	66.60 (0.73)[a]
	1	U	82.98 (0.74)	90.75 (0.62)	0.72 (0.00)[ab]	TRP	60.12 (0.04)[a]	65.75 (0.05)[a]
		S	82.58 (0.68)	90.42 (0.57)	0.73 (0.00)[a]	TRP	60.46 (0.45)[a]	66.19 (0.38)[a]
SG	0	U	82.28 (0.88)	90.16 (0.74)	0.71 (0.01)[ab]	TRP	58.61 (0.33)[b]	64.23 (0.48)[b]
		S	83.22 (1.48)	90.95 (1.25)	0.75 (0.01)[a]	TRP	62.36 (0.75)[a]	68.15 (0.80)[a]
	0.5	U	81.59 (0.63)	89.58 (0.53)	0.70 (0.02)[b]	TRP	57.49 (1.44)[b]	63.12 (1.54)[b]
		S	83.04 (1.02)	90.80 (0.86)	0.74 (0.01)[ab]	TRP	61.86 (0.26)[a]	67.64 (0.33)[ac]
	1	U	82.79 (0.87)	90.60 (0.74)	0.71 (0.00)[b]	TRP	58.38 (0.31)[b]	63.88 (0.33)[bc]
		S	82.49 (0.47)	90.34 (0.39)	0.72 (0.01)[ab]	TRP	59.57 (0.89)[ab]	65.24 (0.91)[ab]

FS, Flour stream; BK, Break; MD, Middling; SG, Straight grade; SL, Scouring level; U, Unscoured; S, Scoured; ML, Moisture level (%); IVPD(1 or 2), *In vitro* protein digestibility (1 or 2); AAS, Amino acid score; LAA, Limiting amino acid; TRP, Tryptophan; IVPDCAAS(1 or 2), *In vitro* protein digestibility corrected amino acid score (1 or 2); Means within a column of the same stream followed by differing lowercase letter are significantly different ($P < 0.05$). SD given in brackets

3.4.1.4 In vitro protein digestibility and quality

In vitro protein digestibility using the first regression equation (IVPD1) ranged from 81.59 to 83.25% and the second equation (IVPD2) from 89.58 to 90.98% (Table 3.4). These ranges are calculated from two different regressions, using the same change in pH data. However, IVPD1 estimates apparent protein digestibility, whereas IVPD2 estimates true protein digestibility. This important distinction reflects (1) the occurrence of IVPD1 (i.e. using the pH drop method and the same regression equation) used in literature when determining protein digestibility and (2) true protein digestibility (accounting for endogenous losses, the metabolic nitrogen loss in absence of dietary nitrogen) as the recommended basis for calculating the PDCAAS (Food and Agriculture Organization of the United Nations & World Health Organization, 1991). No effects of the pre-treatments in any of the streams for yellow peas were observed in the estimation of IVPD1 or IVPD2. Our previous work on roller-milled yellow peas also reported no significant differences for IVPD1 in the range of 80.89 to 81.41% for untreated BK, MD and SG flours (Guldiken et al., 2022). Roller milling to produce a split yellow pea has also produced greater IVPD1 of 85.63%, whereas a hammer milled flour was lower, at 83.99% (Ma et al., 2017). Following the same method of IVPD, a wide range may be expected in IVPD1, in part due to location, such as a comparison between Canadian (78.4%) and Egyptian (80.1%) peas (Khattab et al., 2009). From 40 randomly selected samples of unprocessed field peas, with distinct genetic origin, growing location and year, IVPD1 produced a mean of 85.4% (Sá et al., 2024), whereas a similar evaluation of six varieties produced a mean of 77.7% (Nosworthy et al., 2021). These IVPD values are in close proximity to rarely assessed *in vivo* values, where an apparent and true protein digestibility coefficient of 81.9% and 82.6% respectively was found in peas (Frias et al., 2011; Urbano et al., 2005). Yellow peas demonstrate a wide range of tannin

content (0.6-10.5g/kg), mainly within the seed coat (i.e., hull), in which a decrease is often associated with an improvement in protein digestibility (Gilani et al., 2012; Patterson et al., 2017; Sá et al., 2019). Tannin content in peas can be an order of magnitude different when varieties have white (low tannin) or purple flowers (high tannin), where monogastric animals (rats, chickens & pigs) observe distinct differences in protein digestibility (Smulikowska et al., 2001). Low tannin content in four varieties of peas with white flowers showed no effect on protein and AA digestibility (Kluth et al., 2005). Although this effect was not measured in this study, significant decreases in tannin content through dehulling and roller milling have been demonstrated in yellow peas (Ma et al., 2017), which observed a slight but not significant difference in protein digestibility (*in vitro*) between split (hull removed; low tannin) and raw (hulls re-added; high tannin) yellow peas. The tannin content of CDC Spectrum may be negligible, given that it is a white flowering variety, which may explain no observed differences in IVPD, even if tannin content is significantly reduced. However, without direct knowledge of the tannin content, in addition to variety, year and environment may also be factors (Nikolopoulou et al., 2007). Another potential explanation to unchanged IVPD in peas may be due to a shift in overall content of antinutritional factors, such that phytate or trypsin inhibitors may increase, as tannins decrease, in dehulled pea flours (Ma et al., 2017; Saldanha Do Carmo et al., 2022). Secondary structure analyses have also been associated with changes with IVPD of some pulses, such that the proportion of specific structures can decrease protein digestibility (Carbonaro et al., 2012). Protein digestibility of thermally treated yellow and green peas identified a relationship following a different digestion method, but the association was not significant with the inclusion of raw peas (Yu et al., 2015). Evaluation of six varieties of navy beans grown in two locations found no relationship between the proportion of secondary

structures and IVPD—where significant differences in IVPD were otherwise observed due to variety or variety and environment (Guldiken et al., 2021).

When subject to correction with the AAS, the IVPDCAAS indicated a strong effect of scouring and moisture. The range of protein quality values in IVPDCAAS1 is 56.17 to 62.36%, and for IVPDCAAS2, from 61.58 to 68.15% (Table 3.4). The differences observed between pre-treatments within a stream is similar to those seen in the AAS, where flour produced using scouring with no moisture or moderate moisture addition, demonstrated the highest protein quality within each stream. At high moisture conditioning, BK flours observe a significant decrease in IVPDCAAS, whereas MD flours do not. Regressions following either IVPDCAAS1 or IVPDCAAS2 identify the same effects of the pre-treatments in BK and MD flours, but notably do not in the SG flours. This is observed in the higher moisture, scoured or unscoured conditions, which is likely due to differences in the regression intercepts of IVPD1 (65.66) and IVPD2 (76.15), narrowing the range of estimated values in IVPDCAAS2. In the absence of pre-treatments, our previous work produced a similar but narrower range—with fewer samples—only calculated for IVPDCAAS1 (Guldiken et al., 2022). Literature coefficients for IVPDCAAS1 in unprocessed peas may be as low as 59% (Boye et al., 2012), but more recent ranges are from 62.7 to 85.9% (Nosworthy et al., 2021; Sá et al., 2024). The breadth of differences has been associated with genotype, location, year, and their interaction, as a function of differences in crude protein and amino acid content and to a lesser degree IVPD. Estimated coefficients of true protein digestibility from IVPD2 follow that IVPDCAAS2 is greater in magnitude than IVPDCAAS1—which have seldom been produced—and may present a better estimate of the PDCAAS. This approach warrants caution, as these regression analyses are not developed solely on raw proteins, in part due to the difficulty of assessment *in vivo* (i.e. animal

welfare). This digestion method and the subsequent regression analyses have demonstrated sensitivity to, and associations with changes in antinutritional contents (Boye et al., 2012; Sá et al., 2019) and protein secondary structure (Carbonaro et al., 2012).

3.4.2: *Pre-treated green lentil and flour streams*

3.4.2.1 Hulls and total by-product

Total by-product yields from green lentils are dramatically greater in flour produced using scouring, from 19.5 to 25.7%, than the unscoured, from 8.7 to 11.2% (Figure 3.4., a). Unscoured seeds in this regard were unaffected by moisture addition, where scouring at both moderate and high moisture conditioning increased total by-product. Overall, the hulls removed during roller milling ranged from 7.5 to 10.7% (Figure 3.4., b). The unscoured seed subject to moisture conditioning significantly increased hull collected from the roller mill, in line with the moisture treatments on improving hull removal efficiency (Vishwakarma et al., 2018), whereas scouring and moisture conditioning had no effect. Scouring with no moisture and moderate moisture conditioning also resulted in significantly less hull collected from the mill relative to any of the unscoured conditions.

(a) (b)

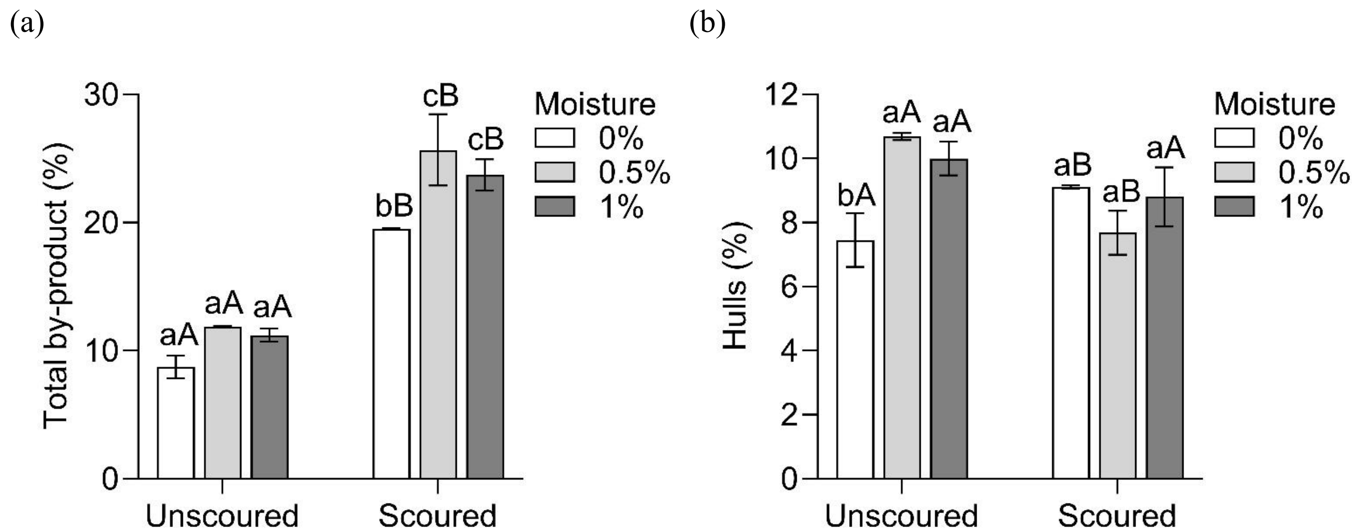

Figure 3.4. Total by-product (starting weight) including hulls removed during scouring and roller milling (a) and (b) hulls removed during roller milling (total product) for green lentils in unscoured and scoured seeds subject to 0 , 0.5 and 1% moisture pre-treatment. Difference in lowercase letter for mean values indicate significant difference within scouring and uppercase letter for moisture & scouring ($P < 0.05$).

3.4.2.2 Protein and amino acid composition

Crude protein content ranged from 26.6 to 29.9% (as is) for all flour streams and pre-treatments (Figure 3.5). Eight varieties of raw and dehulled lentils have observed crude protein ranges of 25.2 to 29.3% and 26.4 to 31.2% (dry matter basis), respectively (Wang et al., 2009). Analyses of 1290 whole lentil samples—with 324 unique varieties grown in two locations in two years—produced a ranged in crude protein of 24.3 to 34.6% (as is) and a mean of 29.7% (Sá et al., 2023). Flour streams are responsible for significant differences between products, where MD flours produced the highest protein (28.9 to 29.9%) products. In contrast, BK flours produced flours have the lowest protein (26.6 to 27.2%) products, reflecting the same trend reported previously in these roller-milled green lentils in the absence of pre-treatment (Guldiken et al., 2022). Flours produced from seeds that are scoured trend towards lower protein content but were

not significant in the BK and MD streams. Seeds subject to scouring with moderate and high moisture for the SG flours became significantly lower in protein content.

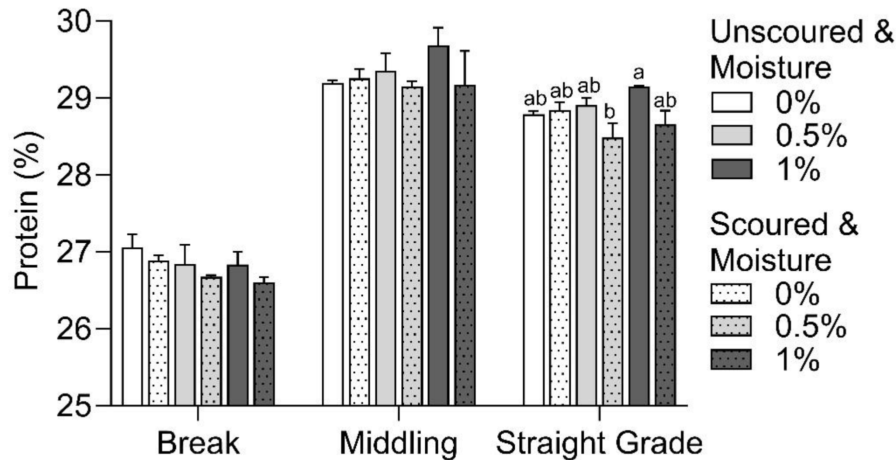

Figure 3.5. Protein content (% as is) of green lentil flours in unscoured and scoured seeds subject to 0, 0.5 and 1% moisture pre-treatment. Means within the same stream followed by differing lowercase letter are significantly different ($P < 0.05$).

The content of indispensable AAs ranged from 8.60 to 9.91% (as is, sample basis), with the lowest content in the BK flours (8.60 to 8.86%) except for the BK flour with no pre-treatment (9.23%), which was significantly greater than its scoured and high moisture counterpart (Figure 3.6). Similarly with yellow pea, the strongest impact on protein or total indispensable AA content is due to the resultant streams, previously established (Guldiken et al., 2022). Additionally, hull and total by-product losses translate to significant changes in protein or total indispensable AA content, mainly due to seed scouring. Green lentils observe a greater change in total indispensable AAs in BK and SG flours, when otherwise only observed in the SG flours for protein content. This specific instance occurs in the unscoured BK and SG flours with no moisture conditioning, which are significantly greater in total indispensable AAs than their scoured high moisture counterpart. Overall dispensable (Table 3.5) and indispensable (Table 3.6)

AA composition of green lentils demonstrate that arginine, aspartic acid, glutamic acid, and histidine are greatly impacted within each pre-treatment and flour stream. The BK flours had the highest number of AAs affected by pre-treatments, with notable differences in histidine and tyrosine. These flours demonstrated a greater loss in dispensable and indispensable AAs, relative to MD and SG flours. Unlike yellow pea, green lentil BK flours observed additional losses in tyrosine and leucine for all but the scoured high moisture and unscoured no moisture pre-treatments. Although not measured in this study, a greater loss of these indispensable AAs in the BK stream may also be due to proportional changes or abundance of seed storage proteins. The AA composition of the albumin protein fraction in lentils contain greater histidine, leucine and slightly greater tyrosine than globulin fractions (Ghumman et al., 2016). These three indispensable AAs begin to decrease in BK flours provided they are scoured, or moisture pre-treated. This suggests that many of the BK flours contained more globulins than albumins, however, these changes do not clearly correspond to all observed changes in AA composition.

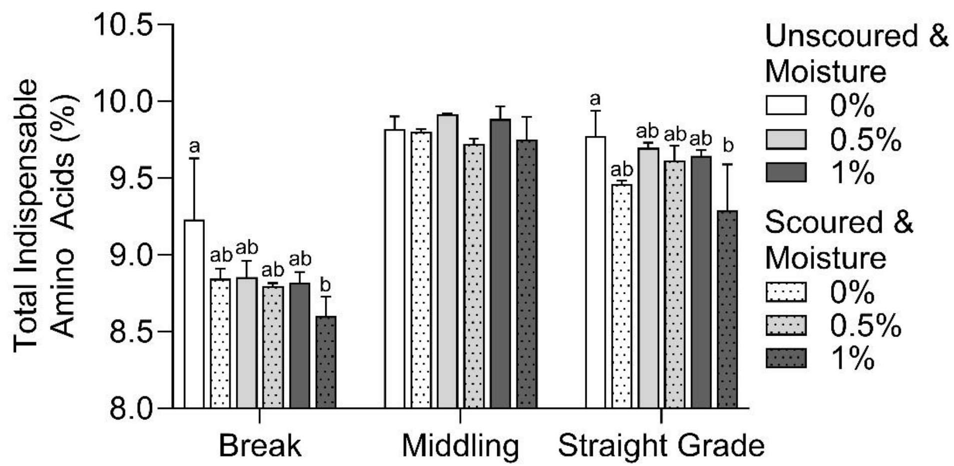

Figure 3.6. Total indispensable amino acid content (% as is, sample basis) content of green lentil flours in unscoured and scoured seeds subject to 0, 0.5 and 1% moisture pre-treatment. Means within the same stream followed by differing lowercase letter are significantly different ($P < 0.05$).

Table 3.5. Dispensable amino acid composition of green lentil flours (% as is, sample basis)

FS	ML	SL	SER	ARG	GLY	ASP	GLU	ALA	PRO
BK	0	U	1.104 (0.005)	1.537 (0.019)^{b}	0.862 (0.014)	2.500 (0.001)^{a}	3.593 (0.033)^{ab}	0.884 (0.008)	0.938 (0.008)
		S	1.147 (0.012)	1.708 (0.016)^{a}	0.842 (0.008)	2.519 (0.003)^{a}	3.625 (0.011)^{a}	0.869 (0.010)	0.931 (0.006)
	0.5	U	1.129 (0.009)	1.714 (0.001)^{a}	0.852 (0.005)	2.504 (0.042)^{a}	3.585 (0.025)^{ab}	0.864 (0.020)	0.926 (0.007)
		S	1.134 (0.008)	1.646 (0.023)^{a}	0.845 (0.016)	2.513 (0.006)^{a}	3.577 (0.023)^{ab}	0.863 (0.004)	0.919 (0.010)
	1	U	1.105 (0.002)	1.651 (0.067)^{a}	0.849 (0.026)	2.482 (0.022)^{a}	3.543 (0.001)^{b}	0.865 (0.006)	0.921 (0.001)
		S	1.066 (0.021)	1.464 (0.068)^{b}	0.835 (0.004)	2.382 (0.062)^{b}	3.461 (0.071)^{c}	0.840 (0.016)	0.905 (0.021)
MD	0	U	1.202 (0.023)^{ab}	1.692 (0.028)^{b}	0.955 (0.039)	2.680 (0.006)^{bc}	3.890 (0.033)^{cb}	0.956 (0.008)	1.022 (0.016)
		S	1.267 (0.004)^{a}	1.906 (0.020)^{a}	0.936 (0.001)	2.759 (0.037)^{a}	3.982 (0.052)^{a}	0.958 (0.005)	1.027 (0.003)
	0.5	U	1.234 (0.006)^{ab}	1.854 (0.084)^{ac}	0.967 (0.029)	2.748 (0.013)^{ac}	3.938 (0.025)^{ab}	0.965 (0.001)	1.031 (0.001)
		S	1.246 (0.010)^{ab}	1.829 (0.077)^{c}	0.932 (0.049)	2.730 (0.023)^{ac}	3.917 (0.025)^{ab}	0.939 (0.013)	1.009 (0.005)
	1	U	1.231 (0.008)^{ab}	1.880 (0.034)^{ac}	0.957 (0.027)	2.734 (0.050)^{ac}	3.900 (0.024)^{bc}	0.970 (0.005)	1.027 (0.004)
		S	1.187 (0.000)^{b}	1.679 (0.018)^{b}	0.916 (0.008)	2.652 (0.040)^{b}	3.832 (0.001)^{c}	0.939 (0.006)	1.009 (0.002)
SG	0	U	1.187 (0.000)	1.647 (0.035)^{a}	0.941 (0.008)	2.645 (0.018)^{a}	3.840 (0.010)^{a}	0.945 (0.002)	1.009 (0.007)
		S	1.221 (0.004)	1.794 (0.105)^{b}	0.916 (0.035)	2.669 (0.040)^{a}	3.811 (0.061)^{a}	0.929 (0.004)	0.989 (0.006)
	0.5	U	1.219 (0.002)	1.770 (0.049)^{b}	0.962 (0.011)	2.708 (0.039)^{a}	3.869 (0.035)^{a}	0.951 (0.015)	1.014 (0.001)
		S	1.235 (0.004)	1.821 (0.052)^{bc}	0.929 (0.030)	2.709 (0.002)^{a}	3.855 (0.120)^{a}	0.937 (0.003)	1.003 (0.000)
	1	U	1.193 (0.008)	1.850 (0.008)^{b}	0.916 (0.004)	2.701 (0.022)^{a}	3.852 (0.040)^{a}	0.943 (0.007)	1.001 (0.006)
		S	1.143 (0.041)	1.570 (0.057)^{ac}	0.895 (0.026)	2.551 (0.068)^{b}	3.718 (0.089)^{b}	0.901 (0.028)	0.968 (0.026)

FS, Flour stream; BK, Break; MD, Middling; SG, Straight grade; SL, Scouring level; U, Unscoured; S, Scoured; ML, Moisture level (%); Ser, Serine; Arg, Arginine; Gly, Glycine; Asp, Aspartic acid; Glu, Glutamic acid; Ala, Alanine; Pro, Proline; Means within a column of the same stream followed by differing lowercase letter are significantly different ($P < 0.05$), SD in brackets

Table 3.6. Indispensable amino acid composition of green lentil flours (% as is, sample basis)

FS	ML	SL	HIS	THR	CYS	LYS	TYR	MET	VAL	ILE	LEU	PHE	TRP
BK	0	U	0.885 (0.355)^{bc}	0.796 (0.004)	0.227 (0.004)	1.482 (0.025)	0.707 (0.025)^{b}	0.177 (0.006)	1.106 (0.007)	0.964 (0.006)	1.600 (0.025)^{a}	1.110 (0.000)	0.176 (0.014)
		S	0.694 (0.001)^{ab}	0.804 (0.002)	0.224 (0.003)	1.480 (0.006)	0.618 (0.019)^{a}	0.187 (0.000)	1.061 (0.014)	0.931 (0.006)	1.558 (0.044)^{ab}	1.087 (0.009)	0.203 (0.001)
	0.5	U	0.722 (0.004)^{a}	0.805 (0.003)	0.222 (0.001)	1.448 (0.089)	0.618 (0.000)^{a}	0.189 (0.011)	1.075 (0.006)	0.949 (0.021)	1.561 (0.005)^{ab}	1.093 (0.011)	0.176 (0.001)
		S	0.675 (0.007)^{ab}	0.790 (0.005)	0.222 (0.004)	1.471 (0.010)	0.618 (0.025)^{a}	0.186 (0.006)	1.056 (0.011)	0.925 (0.023)	1.567 (0.007)^{ab}	1.087 (0.007)	0.202 (0.001)
	1	U	0.690 (0.007)^{ab}	0.790 (0.005)	0.221 (0.001)	1.465 (0.005)	0.596 (0.015)^{a}	0.184 (0.007)	1.068 (0.001)	0.932 (0.011)	1.581 (0.012)^{a}	1.120 (0.049)	0.173 (0.004)
		S	0.610 (0.017)^{c}	0.761 (0.011)	0.221 (0.001)	1.423 (0.011)	0.654 (0.052)^{ab}	0.190 (0.012)	1.054 (0.020)	0.915 (0.006)	1.516 (0.001)^{b}	1.066 (0.003)	0.193 (0.003)
MD	0	U	0.732 (0.016)^{b}	0.874 (0.013)	0.241 (0.005)	1.633 (0.059)	0.745 (0.093)	0.193 (0.008)	1.204 (0.020)	1.062 (0.024)	1.722 (0.018)	1.210 (0.012)	0.204 (0.004)
		S	0.777 (0.011)^{ab}	0.884 (0.001)	0.238 (0.004)	1.603 (0.024)	0.713 (0.013)	0.205 (0.008)	1.177 (0.001)	1.016 (0.005)	1.760 (0.002)	1.206 (0.006)	0.222 (0.001)
	0.5	U	0.829 (0.001)^{a}	0.894 (0.001)	0.243 (0.003)	1.630 (0.008)	0.700 (0.011)	0.200 (0.004)	1.193 (0.004)	1.043 (0.013)	1.771 (0.015)	1.220 (0.005)	0.193 (0.001)
		S	0.765 (0.011)^{ab}	0.875 (0.004)	0.245 (0.001)	1.607 (0.001)	0.701 (0.010)	0.198 (0.001)	1.163 (0.001)	1.022 (0.023)	1.723 (0.012)	1.205 (0.015)	0.220 (0.001)
	1	U	0.806 (0.026)^{a}	0.889 (0.001)	0.242 (0.003)	1.636 (0.029)	0.704 (0.032)	0.201 (0.000)	1.195 (0.001)	1.033 (0.002)	1.764 (0.001)	1.221 (0.002)	0.197 (0.005)
		S	0.797 (0.120)^{ab}	0.854 (0.001)	0.237 (0.001)	1.597 (0.014)	0.711 (0.023)	0.197 (0.004)	1.194 (0.013)	1.032 (0.002)	1.728 (0.010)	1.192 (0.011)	0.213 (0.008)
SG	0	U	0.845 (0.170)^{ab}	0.868 (0.005)	0.237 (0.001)	1.624 (0.025)	0.681 (0.010)	0.189 (0.004)	1.191 (0.010)	1.030 (0.011)	1.727 (0.006)	1.193 (0.007)	0.191 (0.000)
		S	0.743 (0.033)^{ab}	0.856 (0.003)	0.224 (0.007)	1.552 (0.001)	0.690 (0.006)	0.203 (0.018)	1.133 (0.001)	0.995 (0.018)	1.687 (0.019)	1.162 (0.013)	0.221 (0.011)
	0.5	U	0.809 (0.000)^{a}	0.876 (0.005)	0.240 (0.004)	1.582 (0.028)	0.667 (0.020)	0.208 (0.009)	1.176 (0.005)	1.039 (0.018)	1.714 (0.008)	1.196 (0.002)	0.193 (0.002)
		S	0.753 (0.018)^{a}	0.867 (0.006)	0.237 (0.004)	1.576 (0.055)	0.695 (0.001)	0.195 (0.006)	1.152 (0.005)	1.001 (0.006)	1.727 (0.004)	1.198 (0.011)	0.217 (0.008)
	1	U	0.781 (0.004)^{a}	0.867 (0.004)	0.237 (0.001)	1.609 (0.007)	0.690 (0.003)	0.202 (0.001)	1.163 (0.005)	1.007 (0.008)	1.721 (0.011)	1.182 (0.006)	0.188 (0.001)
		S	0.669 (0.033)^{b}	0.813 (0.031)	0.236 (0.002)	1.552 (0.011)	0.706 (0.061)	0.187 (0.007)	1.134 (0.035)	0.993 (0.047)	1.651 (0.039)	1.148 (0.041)	0.200 (0.011)

FS, Flour stream; BK, Break; MD, Middling; SG, Straight grade; SL, Scouring level; U, Unscoured; S, Scoured; ML, Moisture level (%); His, Histidine; Thr, Threonine; Cys, Cystine; Lys, Lysine; Tyr, Tyrosine; Met, Methionine; Val, Valine; Ile, Isoleucine; Leu, Leucine; Phe, Phenylalanine; Trp, Tryptophan; Means within a column of the same stream followed by differing lowercase letter are significantly different ($P < 0.05$), SD in brackets

3.4.2.3 Amino acid score

Green lentils were most frequently limiting in sulfur AAs, with several flours produced from unscoured seeds becoming limiting in tryptophan first (Table 3.7). Analysis of the duplicates in some instances would identify sulfur AAs or tryptophan on the margin of most limiting AA in either. The range of these AASs' across lentil flours was 0.58 to 0.62, with no significant effects observed in the MD or SG flours. Previous analyses conform to the same ranges and identifying the first limiting AA as the sulfurs (Guldiken et al., 2022). Unscoured lentils from the BK flours with no moisture conditioning were significantly lower in AAS than the other pre-treatments in this stream. This same observation only trended in the unscoured lentils with high moisture in the BK flours. Analysis of 40 lentil varieties using the same AA reference pattern find a range of 0.63 to 0.81 for sulfur AAs—being the first limiting AA—followed by 0.66 to 0.74 for tryptophan (Sá et al., 2023). This study contrasted the impact of different reference patterns for calculating AAS, returning a range of 0.58 to 0.72 (6 months to 3 years old) for sulfur AAs, maintaining their limiting acid but also lowering the AAS. If adjusted to the same pattern, roller-milled green lentils subject to these pre-treatments would also decrease in AAS, with a range of 0.54 to 0.59 (data not shown). The AAS—0.66, 0.61 and 0.57—for green lentils are also always limiting in sulfur AAs when processed as an extrudate, cooked (stovetop) or baked (cracker), respectively (Nosworthy et al., 2018).

Table 3.7. *In vitro* protein digestibility and quality of green lentil flours (%)

FS	ML	SL	IVPD1	IVPD2	AAS	LAA	IVPDCAAS1	IVPDCAAS2
BK	0	U	77.70 (0.99)^{b}	86.30 (0.84)	0.58 (0.02)^{b}	M+C & TRP	44.98 (2.37)^{b}	49.95 (2.50)^{b}
		S	80.86 (0.78)^{a}	88.97 (0.66)	0.61 (0.01)^{ab}	M+C	49.47 (0.73)^{a}	54.42 (0.74)^{a}
	0.5	U	78.03 (0.66)^{ab}	86.58 (0.56)	0.60 (0.00)^{ab}	M+C & TRP	46.52 (0.01)^{ab}	51.62 (0.04)^{ab}
		S	79.96 (0.74)^{ab}	88.21 (0.62)	0.61 (0.02)^{ab}	M+C	48.90 (2.35)^{a}	53.94 (2.29)^{a}
	1	U	78.51 (1.07)^{ab}	86.98 (0.90)	0.58 (0.01)^{ab}	TRP	45.92 (0.99)^{ab}	50.87 (1.00)^{ab}
		S	79.02 (0.66)^{ab}	87.42 (0.56)	0.62 (0.02)^{a}	M+C	48.68 (1.50)^{a}	53.85 (1.61)^{a}
MD	0	U	78.00 (0.69)	86.55 (0.58)	0.59 (0.02)	M+C	46.25 (1.50)	51.33 (1.65)
		S	80.53 (0.73)	88.69 (0.62)	0.61 (0.02)	M+C	48.80 (1.71)	53.75 (1.85)
	0.5	U	77.73 (0.52)	86.32 (0.44)	0.60 (0.00)	TRP	46.55 (0.09)	51.70 (0.07)
		S	80.05 (0.65)	88.28 (0.55)	0.61 (0.00)	M+C	48.61 (0.17)	53.61 (0.24)
	1	U	78.21 (0.93)	86.73 (0.78)	0.59 (0.00)	M+C & TRP	46.35 (0.28)	51.40 (0.29)
		S	79.55 (0.22)	88.56 (2.43)	0.60 (0.00)	M+C	47.36 (0.11)	52.72 (0.52)
SG	0	U	77.12 (0.87)^{b}	85.81 (0.74)^{b}	0.59 (0.01)	M+C	45.58 (0.16)	50.72 (0.00)
		S	80.92 (0.62)^{a}	89.02 (0.52)^{a}	0.59 (0.02)	M+C	47.88 (1.18)	52.67 (1.33)
	0.5	U	78.45 (1.11)^{ab}	86.93 (0.93)^{ab}	0.60 (0.00)	M+C & TRP	47.27 (0.31)	52.38 (0.21)
		S	79.60 (1.70)^{ab}	87.90 (1.43)^{ab}	0.61 (0.01)	M+C	48.31 (0.04)	53.35 (0.17)
	1	U	77.61 (0.68)^{b}	86.22 (0.57)^{ab}	0.59 (0.00)	TRP	45.49 (0.68)	50.54 (0.67)
		S	79.08 (1.65)^{ab}	87.47 (1.39)^{ab}	0.59 (0.01)	M+C	46.68 (0.85)	51.63 (0.90)

FS, Flour stream; BK, Break; MD, Middling; SG, Straight grade; SL, Scouring level; U, Unscoured; S, Scoured; ML, Moisture level (%); IVPD(1 or 2), *In vitro* protein digestibility (1 or 2); AAS, Amino acid score; LAA, Limiting amino acid; TRP, Tryptophan; M+C, Methionine and Cysteine; IVPDCAAS(1 or 2), *In vitro* protein digestibility corrected amino acid score (1 or 2); Means within a column of the same stream followed by differing lowercase letter are significantly different ($P < 0.05$). SD given in brackets

3.4.2.4 In vitro protein digestibility and quality

In vitro protein digestibility using the first regression equation (IVPD1) ranged from 77.12 to 80.92% and the second equation (IVPD2) from 85.81 to 89.02% (Table 3.7). Regression using either equation for IVPD demonstrated no differences in the pre-treatments' effect in MD flours. Interestingly, the IVPD2 for BK flours did not reveal the same effect for any of the pre-treatments relative to IVPD1. Whereas IVPD2 in SG flours did not observe a difference in the unscoured high moisture conditioning treatment relative to IVPD1. Unscoured and no moisture

conditioned BK and SG flour demonstrated the lowest IVPD, which may also reflect having a greater content of albumin fractions or otherwise a lower content of globulins (Ghumman et al., 2016). The proportion of secondary structure components in lentils may also identify potential differences in protein digestibility, where a higher proportion of β sheets, or anti-parallel β sheets, are associated with lower protein digestibility (Carbonaro et al., 2012). Alternatively, these unscoured lentils may contained a greater tannin content, with lower total by-product losses relative to the scoured seeds. Dehulling of lentils was previously demonstrated to be an effective means to decrease tannin content across eight varieties (Wang et al., 2009). This decrease is likely responsible for the increases in IVPD of lentil seeds subject to scouring, as hull losses increase, tannin content decreases, and IVPD increases. Decreases in other antinutritional factors including trypsin inhibitors, or an increase in phytate, may also influence changes in IVPD as a result of dehulling in lentils (Patterson et al., 2017; Wang et al., 2009). Notably, phytate has also be shown to decrease in lentils when dehulled, which may be due to varietal selection, the method of dehulling (i.e. by hand) or the method of determining phytate (Pal et al., 2017). The IVPD of BK, MD and SG flours with no pre-treatment are the same as previous work using the same roller-milling procedures (Guldiken et al., 2021). Alternatively, IVPD measured using the same digestion procedures and regression (Barbana & Boye, 2013) found slightly lower protein digestibility (75.9%), likely due measuring whole lentils. Similar digestion protocols and regression equation for estimating true protein digestibility of whole lentils (Carbonaro et al., 1997) was greater in IVPD (82.5%) than the untreated SG (dehulled) IVPD1 (77.1%), but lower than SG IVPD2 (85.8%). Processes such as extrusion, cooking (stovetop) and baking (cracker) demonstrated a modest increase in protein digestibility whether *in vivo* or *in vitro* over that observed for whole raw lentil flour (Nosworthy et al., 2018; Porres et al., 2002). Notably, the *in*

vivo apparent and true protein digestibility of whole raw lentils is 79.0 and 81.3, respectively (Porres et al., 2002). This may indicate a closer estimation of IVPD1 than IVPD2, although a range of *in vivo* coefficients are not available, to which these values may be lower due to the influence of tannins (Woyengo, 2022). Good associations with *in vivo* protein digestibility has been demonstrated in peas and lentils using the IVPD method used in this study (Nosworthy et al., 2018; Nosworthy, Franczyk, Medina, et al., 2017). Standardized approaches employing digestive protocols which more closely follow human digestion have also been proposed (Brodkorb et al., 2019), but have yet to be established for use in estimating protein digestibility or quality with values founds *in vivo*. A criticism of the pH Drop method for estimating protein digestibility relates to the fact that it is a static monocompartmental method not is not necessarily physiologically relevant (FAO, 2013). However, this method has been employed in numerous studies and was positioned by the FAO (1991) as a potential *in vitro* model for evaluating overall protein digestibility. Advances in the INFOGEST 2.0 model (Brodkorb et al., 2019) have now been published to measure protein and amino acid digestibility (Sousa et al., 2023), and the method is undergoing validation. Similarly, the pH Drop method is undergoing a ring trial for method validation (Goldberg and House, 2023) to determine its fit for purpose. For the current application of estimating protein digestibility in similar ingredients subjected to different processing steps, the pH Drop method provides a rapid, inexpensive and relative method for the estimation of the impact of processing on overall protein digestibility. Comparison between a gastro-intestinal digestion model and the pH drop method found similar outcomes in IVPD in a large sample of plant-based proteins, including pulses, relative to true protein digestibility (Franczyk, 2018).

In calculating the IVPDCAAS, the differences in quality measures determined with either regression demonstrated the same effects from the pre-treatments (Table 3.7). These coefficients range for IVPDCAAS1 from 44.98 to 49.47% and IVPDCAAS2 from 49.95 to 54.42%. Overall IVPDCAAS2 values are greater, which is in effect due to the regression used to calculate IVPD2. Unlike yellow pea, only the BK flours in green lentils observed significant differences in protein quality. Lentils subject to scouring within this stream maintained protein quality at every moisture conditioning level, whereas protein quality significantly decreased in relation to the unscoured lentils with no moisture conditioning. Pre-treatments in SG flours demonstrated no differences in either IVPDCAAS1 or IVPDCAAS2, even though differences were observed in their IVPD. The MD flours maintained no significant differences in protein quality, as no differences were observed previously in either IVPD or AAS in each pre-treatment. The greater protein quality from scoured seeds, with the highest in the BK stream, are notably lower than a robust range of IVPDCAAS values for whole lentils, which find a range of 53.1 to 61.0%, following the same reference pattern (Sá et al., 2023). The PDCAAS determined in rodents for raw (71.2%) and autoclaved (66.4%) lentils decrease in quality when thermally treated (Porres et al., 2002), although these analyses did not include tryptophan as a potential limiting AA. When whole lentils are cooked (51.46%), extruded (55.95%) or baked (45.03%), protein quality also remains low (Nosworthy et al., 2018). The AAS remained low in these thermally treated lentils, implying the need to focus on varietal selection to improve the protein quality. Alternatively, it has been proposed that one of the strongest effects observed on protein digestibility in pulses are due to structural phenomena (Carbonaro et al., 1997, 2012), otherwise protein-tannin interactions (Carbonaro et al., 1996). In either case, a combination of milling and milling pre-treatments may be an effective means to improve lentils' protein quality.

3.5: CONCLUSION

The evolving plant-based protein landscape may benefit from front-of-package labelling, offered by protein quality evaluation. Milling quality parameters such as crude protein content may seek to select for flours of higher protein quality for this specific end-use application. Within this novel research, it has been demonstrated that roller-milling can produce a range of flours which can improve in protein quality when yellow peas or green lentils are scoured. This follows due to small losses in crude protein content and AAs, in addition to improvement in protein digestibility. Moisture conditioning alone can also aid hull removal in roller-milling of green lentils. These results highlight the necessity to evaluate effective milling strategies to reduce hull contents in refined pulse flours, as these strategies will differ between pulse types—in part due to their underlying physical differences. *In vitro* methodology can be a valuable tool in the assessment of protein digestibility and quality, however this method (pH-drop) requires validation to facilitate lab-to-lab comparisons of the impact of variables, including pre-treatment, on the protein quality of pulses and other protein foods. The choice of regression equation used to estimate protein digestibility was observed to yield minor differences between methods but impacts the magnitude of estimated protein quality coefficients.

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3.7: AUTHORSHIP

A.F. Conceived and designed the study, carried out the research for each component, analyzed the data and wrote and reviewed each draft of the manuscript. A.S. conceived the milling trial design including pre-treatments. N.B. and J.C. assisted all collections of *in vitro* protein digestibility data and amino acid hydrolysis, which was analyzed by J.N. Sample preparation, including pre-treatments and milling was completed by L.B. M.N. was the nominated principal investigator and J.H., E.S. and J.P. were co-investigators involved in conceiving and designing the study, reviewing data analysis, and approving the final draft of the manuscript.

3.8: CONFLICT OF INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

3.9: References

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3.10: Supplementary Data

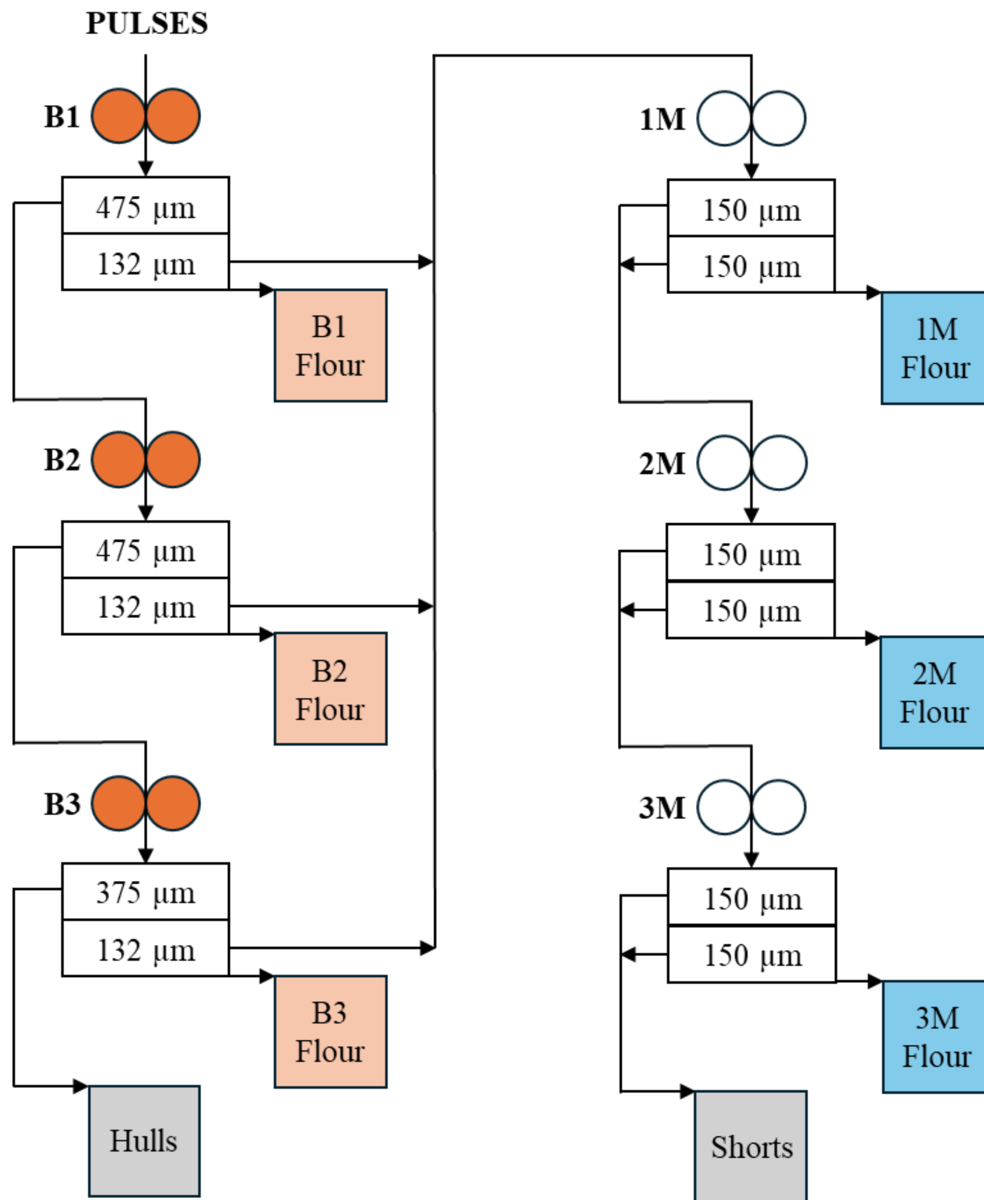

Figure 3.7. Schematic representation adapted from Guldiken et al. (2022) for the roller-milling process utilized on pre-treated yellow peas and green lentils

Table 3.8. Dry matter (%) for yellow peas

Scouring Level		Unscoured			Scoured	
Moisture Level	**0%**	**0.5%**	**1%**	**0%**	**0.5%**	**1%**
Break	92.91 (0.04)	91.66 (0.97)	92.15 (0.20)	92.77 (0.06)	92.66 (0.03)	93.03 (0.11)
Middling	92.83 (0.18)	91.65 (1.04)	92.27 (0.39)	92.67 (0.04)	92.49 (0.11)	92.91 (0.18)
Straight Grade	92.91 (0.01)	92.14 (0.11)	91.86 (0.10)	92.67 (0.03)	92.48 (0.20)	92.84 (0.49)

Means with SD in brackets

Table 3.9. Dry matter (%) for green lentils

Scouring Level	Unscoured			Scoured		
Moisture Level	**0%**	**0.5%**	**1%**	**0%**	**0.5%**	**1%**
Break	91.34 (0.08)	91.24 (0.15)	90.81 (0.04)	90.75 (0.01)	90.65 (0.08)	90.79 (0.02)
Middling	91.65 (0.01)	91.25 (0.01)	91.07 (0.18)	90.82 (0.12)	90.79 (0.11)	90.51 (0.68)
Straight Grade	91.62 (0.15)	91.35 (0.02)	91.07 (0.13)	90.90 (0.30)	90.97 (0.30)	90.77 (0.52)

Means with SD in brackets

3.11: BRIDGE TO CHAPTER 4.

Building on the evaluation of lentils and peas in Chapter 3, the following chapter applies a similar approach to investigate protein quality in chickpeas and navy beans. As with the previous study, moisture conditioning and scouring were used as pre-milling treatments prior to roller milling, and the effects on protein and amino acid composition, IVPD, and IVPDCAAS were assessed. This work complements earlier findings on functional and nutritional characteristics of these pulses (Guldiken et al., 2022), offering comparative insight into how species-specific responses to processing influence protein quality outcomes.

CHAPTER 4. THE ASSESSMENT OF *IN VITRO* PROTEIN DIGESTIBILITY AND QUALITY ON MECHANICALLY SCOURED AND MOISTURE CONDITIONED NAVY BEANS (*PHASEOLUS VULGARIS*) AND CHICKPEAS (*CICER ARIETINUM*) SUBJECT TO ROLLER-MILLING

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4.1: Abstract

Background and objectives: Roller-milling practices for pulses to establish potential end-use applications are required. Scouring and moisture conditioning may improve milling efficiency and hull removal, in addition to influencing nutritional quality. The extent of these conditions on the protein digestibility and quality are evaluated *in vitro* on navy beans and chickpeas in this study.

Findings: Scouring with no additional moisture conditioning increased protein quality in both navy beans and chickpeas, whereas the addition of 1% moisture conditioning in combination with scouring was detrimental to protein quality. Moisture conditioning at 0.5% without scouring also decreased protein quality in navy beans. Changes in protein quality were primarily due to compositional changes in both protein and amino acid content, reflected in amino acid scoring. Sulfur amino acids were the sole limiting amino acids in navy beans, while tryptophan was the sole limiting amino acid in chickpeas, irrespective of the roller-milled flour stream or pre-treatment.

Conclusion: Scouring with no added moisture conditioning effectively improves the protein quality in both navy beans and Kabuli chickpeas due to changes in their amino acid scoring pattern.

Significance and novelty: Balancing milling practices and nutritional quality are important in developing navy bean and Kabuli chickpea products.

4.2: INTRODUCTION

Pulses including beans, peas, lentils and chickpeas serve as an important source of dietary protein worldwide. Following India and China, Canada remains a leader in overall global pulse production, with a smaller share in beans and chickpeas (Bekkering, 2014; Luzardo-Ocampo et al., 2020). This high production is in stark contrast to overall low consumption rates in Canada, where consumption of bean-based dishes and products is highest, followed by lentils and chickpea (Mudryj et al., 2012). Diets with the inclusion of pulses have been associated with improved nutrient intakes and a reduced burden of chronic diseases, including cancer, cardiovascular disease and diabetes (Mudryj et al., 2014).

Guidance for pulse consumption in Canada has recently been updated with messaging encouraging the consumption of plant-based protein, featuring pulses as a key dietary component (Fernandez et al., 2020). An evaluation of adult Canadians consuming a higher proportion of plant-based foods showed lower total protein intake and reduced protein quality—a product of the first limiting indispensable amino acid (AA) and how complete a protein digests (Marinangeli et al., 2021). In the group with the greatest (≥75%) plant-based consumption, cereal-based products accounted for nearly 40% of the diet. Cereal grains are known to be limiting in lysine, an indispensable AA required for normal human metabolism and growth, where a low content would be associated with poorer protein quality. Pulses, on the other hand, are rich in lysine but limited in sulfur-containing AAs (methionine and cysteine). When combined with cereals, pulses can complement the AA profile, improving protein quality compared to either source alone. The regulatory framework for communicating content claims to consumers on front-of-package labelling in Canada and the US requires substantiation of protein quality (Marinangeli & House, 2017). The assessment of these claims involves animal bioassays to determine the protein

digestibility component—a limiting factor in evaluating protein quality due to the cost, time and ethical considerations of these assays. Evaluation of protein digestibility through *in vitro* methods is an important tool in understanding the effects of different processing and treatments on food formulations (Mansilla et al., 2020), which have demonstrated good agreement with *in vivo* evaluations of beans and chickpeas (Nosworthy et al., 2018, 2020).

Nutrient content claims can serve as an effective tool for communicating the benefits of food products to consumers, potentially increasing their selection (Kaur et al., 2017). Despite their nutritional benefits, pulses are underutilized in Western diets, partly due to the inconvenience of their time-intensive preparation, such as soaking and cooking, as well as dietary preferences that favor animal-based proteins, cultural practices that do not traditionally include pulses, and a lack of awareness about their health benefits and versatility (Mitchell et al., 2021; Mudryj et al., 2012; Niva et al., 2017). Given the high consumption of cereal-based foods, such as rice, wheat, and corn products, there is a significant opportunity to enhance the nutritional quality of these foods by incorporating pulses. Products like soups, sauces, bakery goods, bread, pasta, and meat substitutes can be fortified with pulses to improve both total protein content and protein quality (Boye et al., 2010). Moreover, pulses provide a low-cost alternative to animal-based proteins, especially in scenarios of rising food prices, helping to mitigate the effects of increasing commodity costs. (Gazan et al., 2021). Enhanced nutritional benefits have already been demonstrated in pulse-enriched pasta, which has shown similar positive effects on net protein utilization and muscle protein synthesis as animal-based proteins (Berrazaga et al., 2020). By integrating pulses with cereal-based products, it is possible to create more balanced and nutritionally complete foods for consumers.

The type of milling process can play a critical role in pulse flour quality, in part due to the variability of pulse seed sizes and the mill's capabilities to adequately grind the material in addition to sufficient removal of the seed hull (Thakur et al., 2019). Relative to cereal grains, international standards for pulse milling are lacking in accordance with their specified application (Scanlon et al., 2018). Roller-milling allows for the production of various flour streams of desired particle sizes, as seeds pass through a series of rollers, thus allowing flours to be produced with specific characteristics, including streams devoid of the hull (Cappelli et al., 2020). Scouring, or mechanical abrasion, uses specialized machinery with abrasive surfaces or rotating drums to gently grind the outer seed coat, loosening it without harming the inner cotyledon. Whereas increasing the moisture content of pulse seeds can weaken pectin and lignin bonds between the hull and cotyledon, facilitating separation (Vishwakarma et al., 2018). By employing methods like moisture conditioning, scouring, and mechanical abrasion, seed pre-treatments can improve milling efficiency and hull removal (Thakur et al., 2019). Controlling hull removal from pulses is crucial for achieving a desired flour quality, as it directly impacts techno-functional properties. Specifically, managing starch gelatinization and adjusting the proportions of starch, fiber, and protein-rich fractions can significantly influence the final quality of products like cookies and cakes, ultimately affecting consumer acceptance. (Bravo-Núñez & Gómez, 2023; Wang & Toews, 2011). Selective incorporation of the hull fraction can also be an effective strategy to boost dietary fiber, primarily insoluble fiber, in products like high-fiber breads, providing comparable quality and consumer acceptance to whole grain bread while offering a higher fiber content (Zhong et al., 2018).

Within the context of protein quality, pulse hulls can be especially high in tannins, an antinutritional factor that can form tannin-protein complexes, reducing protein digestibility and

quality (Sá et al., 2019). Pulses overall can contain a wide range of tannin content, being greatest in whole ground beans but among the lowest in chickpeas, whereas dehulling has been demonstrated to significantly diminish the overall tannin content (Gilani et al., 2012; Patterson et al., 2017). Relative to whole ground flour, dehulled pulse flours contain a greater proportion of cotyledon material, thus shifting the protein fractions and the AA composition, which can alter protein quality (Patterson et al., 2017). This shift may also concentrate other antinutritional factors, such as trypsin inhibitors and phytic acid, which may be reduced or eliminated as a result of additional processing, including thermal treatments (Sá et al., 2019).

Previous studies have examined the techno-functional properties (e.g., foaming, oil absorption, and emulsion capacity) and protein quality of roller-milled navy beans and Kabuli chickpeas without pre-treatments (Guldiken et al., 2022). Furthermore, key physical and compositional characteristics, including protein content of the Kabuli chickpeas used in this study have been documented (Choo et al., 2022). This study aims to build on previous findings by evaluating the effects of pre-treatments, including scouring and moisture conditioning, followed by roller-milling on navy beans and Kabuli chickpeas, using protein quality as the primary evaluation metric. Protein quality, an often-overlooked factor in traditional milling standards, will be assessed through *in vitro* protein digestibility and AA composition. This approach seeks to provide a more comprehensive understanding of how pre-treatments influence the nutrient quality of roller-milled pulse flours.

4.3: MATERIALS AND METHODS

4.3.1: *Materials*

Navy bean (Nautica) and Kabuli chickpea (CDC Orion) were provided by Hensall Co-op Ltd. (Hensall, ON) and Reisner Farm Ltd. (Limerick, SK), respectively, and harvested from the 2018 crop year. Seeds were stored at ambient room temperature (22 ± 2 °C) and transferred to a freezer (-20°C) once milled. All chemicals were ACS or reagent grade and purchased from Fisher Scientific (Ottawa, ON, Canada). Digestive enzymes, including chymotrypsin (bovine pancreas ≥40 units/mg protein), trypsin (porcine pancreas 13,000-20,000 BAEE units/mg protein) and protease (Streptomyces griseus ≥15 units/mg solid) were acquired from Sigma-Aldrich (Oakville, ON, Canada). High nitrogen casein (80 mesh) was also acquired from Dyets Inc. (Bethlehem, PA, USA).

4.3.2: *Moisture conditioning and scouring pre-treatment*

The protocols for moisture conditioning and scouring reported by Choo et al., (2022) were applied to navy beans in this study, which also used the same Kabuli chickpeas. The initial seed moisture content was measured using an infrared grain analyzer (Inframatic 9500 Grain and Flour Analyzer, Perten Instruments NA, Inc., Winnipeg, MB) prior to moisture conditioning. Whole pulses were treated at three moisture levels: no addition (0% increase), moderate addition (0.5% increase w/w), and high addition (1% increase w/w). The terms 'moderate' and 'high' were assigned arbitrarily to represent the relative differences in moisture levels, with percentages indicating the amount of water added relative to the initial seed moisture and the mass of the sample. Pulse seeds

were then mechanically scoured on a Bühler MHXA 50/70 (Bühler Group, Uzwil, Switzerland) or left unscoured.

4.3.3: *Flour milling and streams*

Prior to milling, seeds underwent an initial pre-break using the first break roll on a Bühler MDDM 1000/250 pilot roller-mill (Bühler Group, Uzwil, Switzerland). Pre-broken seeds were milled on a Bühler MLU 202 (Bühler Group, Uzwil, Switzerland) laboratory scale roller-mill. Navy beans were separated into three streams consisting of either break (BK; B1+B2+B3), middling (MD; 1M+2M+3M) or straight-grade (SG) flours. Chickpea flours had four streams—including BK and SG—where the middling flours were separated into middling 1 (MD1; 1M) and middling 2 streams (MD2; 2M+3M). The SG flours were representative of the total yield of BK and MD flours produced in either pulse. Further details regarding flour streams (Figure 4.7) including, sieve size, feed rate, and clearances, have also been previously described (Choo et al., 2022). Hulls specifically removed during the roller-milling process were collected and weighed, to be calculated separately on a total product basis. Total milled by-products including these hulls from the roller-mill, in addition to shorts from the mill and material collected during scouring, were calculated from a starting sample weight basis. These hulls and by-products were not further analyzed in this study. The combinations of unscoured, scoured, and moisture conditioned levels, as well as their corresponding flour streams, are summarized in Table 4.1.

Table 4.1. Summary of seed pre-treatments and flour streams for both pulses

Scouring Level (SL)	Moisture Level % (ML)	Navy Bean Flour Streams	Chickpea Flour Streams
Unscoured (U)	0, 0.5, 1	Break (BK), Middling (MD), Straight Grade (SG)	Break (BK), Middling 1 (MD1), Middling 2&3 (MD2), Straight Grade (SG)
Scoured (S)	0, 0.5, 1	Break (BK), Middling (MD), Straight Grade (SG)	Break (BK), Middling 1 (MD1), Middling 2&3 (MD2), Straight Grade (SG)

4.3.4: *Protein and amino acid composition*

Nitrogen was determined in duplicate through Dumas combustion on a LECO nitrogen analyzer (FP828, LECO Corporation, St. Joseph, MI, USA) and used to calculate crude protein content (N × 6.25). The nitrogen-to-protein conversion factor of 6.25 is selected in accordance with protein content claim substantiation and nutrition facts tables in Canada and the US (House et al., 2024). Dry matter for navy beans (Table 4.8) and chickpeas (Table 4.9) was determined according to standardized protocols (AOAC 925.10) but were not utilized in this study.

Amino acid (AA) hydrolysis was conducted in singlets and divided into three distinct procedures. The first procedure involved acid hydrolysis for general AAs (AOAC 982.30). The second procedure involved oxidation of cysteine and methionine to cysteic acid and methionine sulfone, respectively, using performic acid before acid hydrolysis (AOAC 994.12). The third procedure employed alkaline hydrolysis to determine tryptophan (ISO 13904). Regular and oxidized samples were derivatized using the AccQ-tag ultra derivatization kit (Waters Corporation, Milford, MA, USA) and analyzed by UHPLC (Nexera X2, Shimadzu Corporation, Kyoto, Japan) with an AccQ-tag Ultra RP 10 cm x 2.1 column. Tryptophan was analyzed on the same UHPLC system but with a 15 cm x 4.6 mm Luna column (Phenomenex Inc., Torrance, CA, USA), following derivatization and procedures previously outlined by Nosworthy et al. (2017). Appropriate reference standards were included in each procedure. The AA composition was categorized into dispensable AA, which can be synthesized by the human body, and indispensable AA, which cannot be synthesized and must be obtained through the diet (Preston et al., 2019). The results were reported as free AAs (% as is, sample basis).

4.3.5: *In vitro protein digestibility and in vitro protein digestibility-corrected amino acid score*

The pH drop method was used to determine *in vitro* protein digestibility (IVPD), as described by Hsu et al. (1977) with modified enzymatic preparation and regression expression (Franczyk, 2018; Tinus et al., 2012). Triplicate pulse flour samples, each containing the equivalent of 10 mg of nitrogen, were hydrated with 10 mL of deionized water, stirred for one hour, heated to 37°C, and adjusted to a pH of 8.0 ± 0.05 using 1M NaOH or HCl.. Separately, a multi-enzyme solution containing 3.1 mg/mL chymotrypsin, 1.6 mg/mL trypsin and 1.3 mg/mL protease was dissolved in deionized water, warmed to 37°C and adjusted to pH 8.0 ± 0.05 before being placed in an ice water bath (4°C). An indirect measure of enzyme activity was accounted for by digesting a casein control, included between batches of IVPD. The addition of 1 mL of cooled multi-enzyme solution was added to the pH-stable sample solution, and the subsequent change in pH was used to calculate the IVPD over a period of 10 min. The decrease in pH of the solution is an indirect measure of the degree of hydrolysis, due to the increase of carboxylic acid in solution from proteins hydrolyzed to peptides and AAs, which are associated (regression) with apparent (IVPD1) and true protein digestibility (IVPD2) found *in vivo*:

$$\text{IVPD1} = 65.66 + 18.10(\Delta\text{pH}_{\text{10minutes}})$$

$$\text{IVPD2} = 76.15 + 15.26(\Delta\text{pH}_{\text{10minutes}})$$

The amino acid score (AAS) is calculated as a ratio, by dividing each indispensable AA by its relative abundance, expressed as milligrams of AA for each gram of protein in the sample, by the relative abundance of each AA from a reference pattern for children aged 2-5 (FAO/WHO, 1991),

as required by the US and accepted by Canadian regulatory bodies. The resulting AA with the lowest ratio—the limiting AA—was selected as the amino acid score (AAS).

$$\text{AAS} = \frac{\text{mg of AA per gram of protein (sample)}}{\text{mg of AA per gram of protein (reference pattern)}}$$

The product from the IVPD (1 or 2) and the AAS were used to calculate the *in vitro* protein digestibility-corrected amino acid score (IVPDCAAS):

$$\text{IVPDCAAS}(1 \text{ or } 2) = \text{IVPD}(1 \text{ or } 2) \text{ x AAS}$$

4.3.6: *Statistics*

Pre-treatment and roller-milled streams for navy beans produced 18 unique combinations in duplicate (36 total) and 24 unique combinations (48) for chickpeas. Milled by-product, hulls, crude protein, dispensable AAs, indispensable AAs, IVPD (1 or 2), IVPDCAAS (1 or 2) and AAS were subject to ordinary two-way ANOVA with Tukey's multiple comparison test with a single pooled variance. Within a pulse class, the full effect was compared for the pre-treatments relative to their flour stream. The effect of flour stream is not a parameter in the analysis of milled by-product or hulls. Statistical significance is indicated when $P < 0.05$.

4.4: RESULTS & DISCUSSION

4.4.1: *Pre-treated navy beans and flour streams*

4.4.1.1 *Milling by-products and hulls*

Hulls removed during the roller milling of navy beans alone (Figure 4.1., a) accounted for 1.8 to 7.3% of the total product. In contrast, milled by-products (Figure 4.1., b), which include mill-collected hulls, scouring hulls, and shorts, ranged from 9.1 to 14.5% of the initial weight. As expected, more hulls were removed during the milling of unscoured seeds compared to scoured seeds, as most of the hulls from scoured seeds were collected during the scouring process. Seed hydration is anticipated to enhance hull separation by forming a bubble between the hull and cotyledon (Vishwakarma et al., 2018), thereby facilitating detachment. Although this separation was not statistically significant, scoured seeds showed a slight linear increase in hull content collected with increasing levels of moisture conditioning. Both moderate (0.5%) and high (1%) moisture conditioning combined with scouring resulted in significant increases in milled by-products compared to unscoured seeds under the same moisture conditions. A slight but non-significant linear decrease in milled by-products was observed in unscoured seeds as moisture levels increased, with no corresponding trend in hulls alone. This suggests that higher moisture levels may reduce the content of shorts in roller-milling. The hull content of navy beans is estimated to range from 7 to 15% (Aguilera et al., 1982; Anton et al., 2008), indicating that the scouring procedure may be more effective in varieties with higher hull content. Overall, for navy beans, combining moisture conditioning with scouring yields greater milled by-product, whereas moisture addition to unscoured seeds may reduce it. Each pulse was evaluated separately due to distinct physical and mechanical properties, such as seed shape (length, width, thickness, sphericity) and seed weight, which influence the milling process (Thakur et al., 2019).

Additionally, varietal differences within genotypes, such as those observed in navy beans and chickpeas, can affect seed hydration capacity (Bassett et al., 2021; Kaur & Prasad, 2023), influencing hull separation. Machine operating parameters also play a role in milling efficiency, though they were not extensively examined in this study (Wood & Malcolmson, 2021).

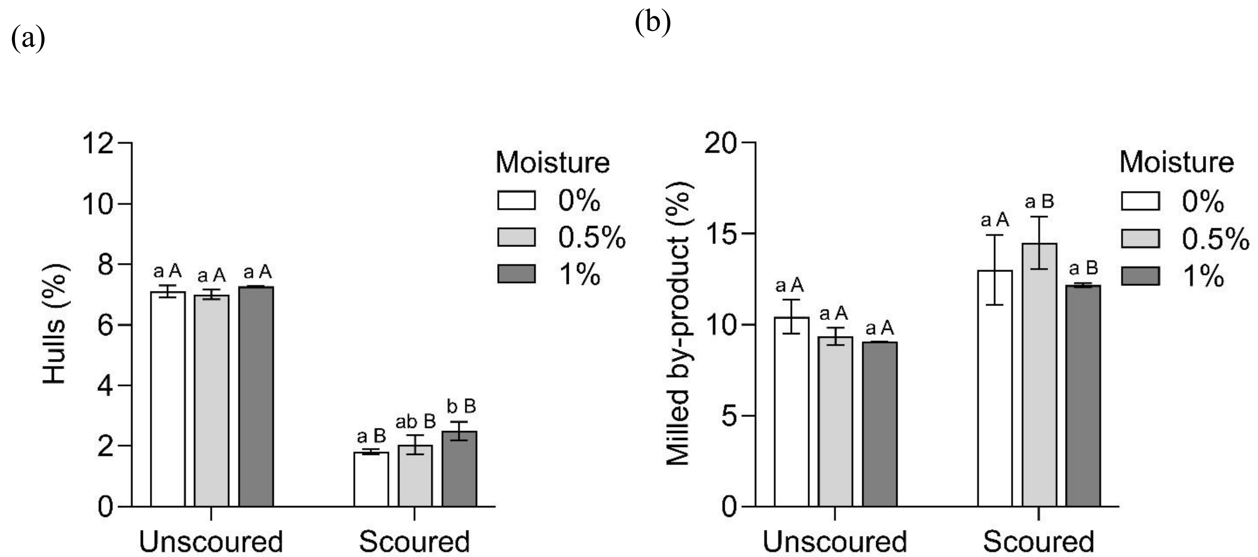


Figure 4.1. Hulls removed (total product) during roller milling (a) and total by-product (starting weight) including hulls removed during scouring and roller milling (b) for navy beans in unscoured and scoured seeds subject to 0, 0.5 and 1% moisture conditioning. Difference in lowercase letter for mean values indicate significant difference within scouring and uppercase letter for moisture and scouring ($P < 0.05$).

4.4.1.2 *Protein and amino acid composition*

The crude protein content ranged from 25.6 to 30.0% (as is; Figure 4.2), and the total indispensable AA content ranged from 9.6 to 11.5% (as is; Figure 4.3), with the lowest content of either in the BK flours, followed by SG and MD flours. Scouring of seeds slightly decreased protein content relative to unscoured seeds within in flour stream. These decreases were not significant but may be expected as there is a tendency for protein to be lost following dehulling, due to proportional changes in starch relative to protein (Thakur et al., 2019). Previous findings

indicated that BK flours (22.5% db) had significantly lower crude protein content compared to MD (27.0–30.5% db) and SG (27.0% db) flours, emphasizing the role of roller-milling in producing higher-protein flours. In contrast, flours produced using an alternative milling method, the Ferkar mill (26.3% db), also exhibited significantly lower protein content (Guldiken et al., 2022) The crude protein content of whole milled navy beans may also be modifiable through selection of variety, growing location and year, which ranged from 24.4 to 26.6% (db) across four cultivars and 20.6 to 24.9% (db) in Nautica (Guldiken et al., 2021; N. Wang et al., 2017). The difference in crude protein content may be partially explained by particle size (volume-weighted mean), as indicated by our prior findings, which showed that BK flour had a lower volume-weighted mean compared to the roller-milled flours (Guldiken et al., 2022). Particle size may also influence the protein content of navy beans during Ferkar milling (Bourré et al., 2019), as shown by a linear increase in crude protein content (25.3 to 26.1% db) with the use of progressively larger screen sizes, from 0.5 mm to 1.27 mm. Similarly, particle size separations of milled navy bean flours into small (<74 µm), medium (74–297 µm), and large (297–500 µm) screenings showed a somewhat linear increase in crude protein content, with values of 16.3%, 22.2%, and 21.2%, respectively. (Byars et al., 2021).

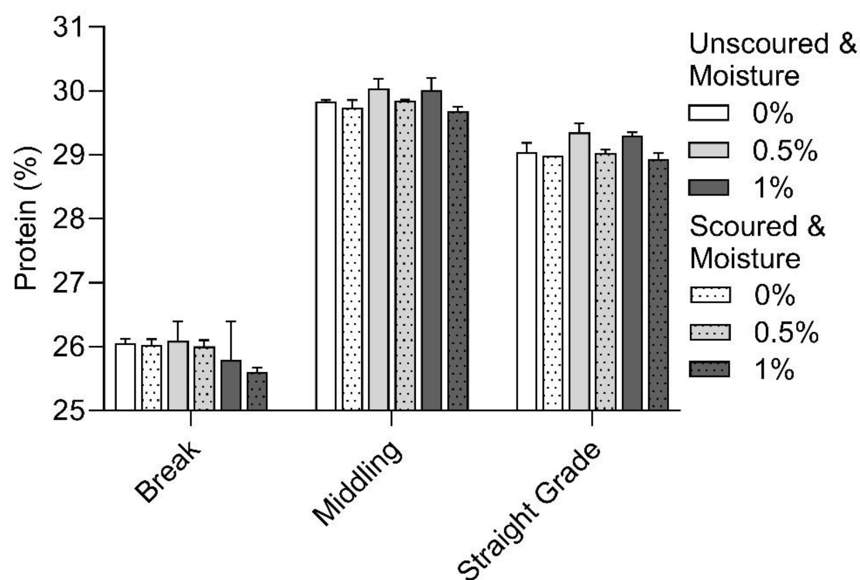


Figure 4.2. Protein content (% as is) of navy bean flours in unscoured and scoured seeds subject to 0, 0.5 and 1% moisture pre-treatment. Means within the same stream followed by differing lowercase letter are significantly different (P < 0.05).

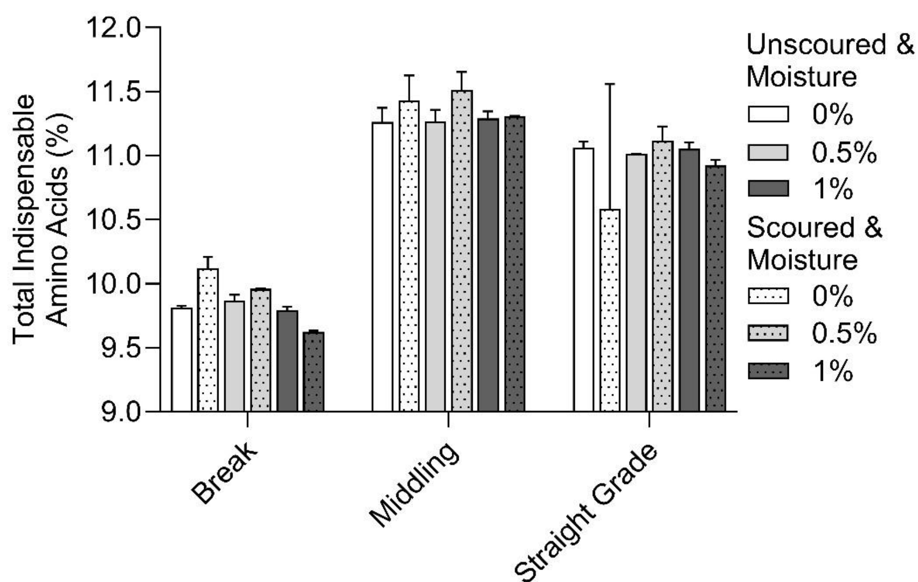


Figure 4.3. Total indispensable amino acid content (% as is) content of navy bean flours in unscoured and scoured seeds subject to 0, 0.5 and 1% moisture pre-treatment. Means within the same stream followed by differing lowercase letter are significantly different (P < 0.05).

Lower crude protein content in BK flours corresponded to lower total indispensable AAs compared to MD and SG flours. Across all flour streams with scouring, no (0%) and moderate moisture conditioning appeared to result in greater total indispensable AA content than high (1%) moisture conditioning; however, these differences were not statistically significant. Individual dispensable and indispensable AAs in BK flours (Tables 4.2 & 4.3) exhibited the greatest number of significant changes ($P < 0.05$) due to scouring and moisture conditioning. Serine, glycine, aspartic acid and proline were consistently lower ($P < 0.05$) in navy bean BK flours, subjected to scouring with high moisture conditioning and, to a lesser extent, in unscoured navy beans under the same condition. Similarly, these two treatments in the BK flours produced a lower content of indispensable AAs, except for histidine, cysteine, and tryptophan. In contrast, fewer differences were observed in the MD flours, where serine content was greater in flours produced from scouring with no or moderate moisture conditioning. For the MD flour, histidine content was highest, and tyrosine content was lowest in unscoured seeds with high moisture conditioning compared to other pre-treatments within this stream. The SG flours showed no differences in indispensable AAs, while dispensable AAs, specifically arginine, aspartic acid, and glutamic acid, were significantly lower in scoured seeds with no moisture conditioning. Greater losses of milled by-product in scoured navy beans with no and moderate moisture conditioning, compared to high moisture conditioning may partially explain shifts in AA composition relative to crude protein content. Although the specific location of these losses were not evaluated in this study, they likely impact the distribution of major storage protein fractions. Phaseolin accounts for the highest proportion of nitrogen (40-50%) in common beans, followed by glutelin (20-30%), free AAs (5-9%), and prolamin (2-4%; Montoya et al., 2010). Some loss of the cotyledon layer (e.g., shorts) is expected in the scoured pre-treatment, as indicated by increased milled by-product, which may also

influence the starch content. Notably, starch-rich fractions in navy beans produced through solvent-free electrostatic separation exhibited significant contributions of both dispensable and indispensable AAs relative to protein-rich fractions, indicating that protein- or AA-bound starch granules contribute significantly to the overall AA pool (Jafari et al., 2016).

Table 4.2. Dispensable amino acid composition of navy bean flours (% as is, sample basis)

FS	ML	SL	SER	ARG	GLY	ASP	GLU	ALA	PRO
BK	0	U	1.314 (0.009)^{b}	1.298 (0.026)	0.870 (0.006)^{ac}	2.795 (0.004)^{a}	3.464 (0.021)	0.927 (0.004)	0.839 (0.007)^{ab}
		S	1.445 (0.001)^{a}	1.280 (0.005)	0.896 (0.009)^{a}	2.760 (0.004)^{ac}	3.402 (0.006)	0.931 (0.005)	0.882 (0.013)^{a}
	0.5	U	1.318 (0.003)^{b}	1.296 (0.037)	0.845 (0.002)^{bc}	2.802 (0.006)^{a}	3.440 (0.017)	0.928 (0.007)	0.836 (0.001)^{ab}
		S	1.406 (0.017)^{a}	1.312 (0.009)	0.867 (0.016)^{ab}	2.690 (0.045)^{b}	3.324 (0.024)	0.900 (0.010)	0.867 (0.009)^{ac}
	1	U	1.330 (0.017)^{b}	1.324 (0.026)	0.834 (0.021)^{bc}	2.788 (0.017)^{ac}	3.421 (0.014)	0.911 (0.017)	0.828 (0.006)^{bc}
		S	1.287 (0.004)^{b}	1.284 (0.053)	0.818 (0.016)^{b}	2.741 (0.016)^{bc}	3.356 (0.008)	0.902 (0.001)	0.807 (0.002)^{b}
MD	0	U	1.491 (0.023)^{b}	1.591 (0.037)	0.965 (0.036)	3.094 (0.027)	3.866 (0.019)	1.057 (0.000)	0.955 (0.013)
		S	1.617 (0.026)^{a}	1.558 (0.046)	1.022 (0.021)	3.119 (0.045)	3.907 (0.054)	1.058 (0.008)	0.993 (0.013)
	0.5	U	1.511 (0.007)^{b}	1.560 (0.035)	0.975 (0.003)	3.145 (0.043)	3.933 (0.053)	1.068 (0.013)	0.957 (0.006)
		S	1.610 (0.030)^{a}	1.543 (0.056)	1.024 (0.024)	3.120 (0.024)	3.891 (0.019)	1.057 (0.010)	0.997 (0.007)
	1	U	1.516 (0.008)^{b}	1.554 (0.050)	0.968 (0.008)	3.138 (0.013)	3.914 (0.008)	1.069 (0.006)	0.961 (0.012)
		S	1.509 (0.022)^{b}	1.530 (0.058)	0.975 (0.007)	3.164 (0.016)	3.920 (0.025)	1.068 (0.007)	0.951 (0.006)
SG	0	U	1.470 (0.008)	1.572 (0.041)^{a}	0.948 (0.024)	3.090 (0.019)^{a}	3.861 (0.038)^{a}	1.043 (0.008)	0.940 (0.011)
		S	1.481 (0.132)	1.399 (0.104)^{b}	0.926 (0.090)	2.877 (0.273)^{b}	3.569 (0.329)^{b}	0.967 (0.097)	0.917 (0.085)
	0.5	U	1.471 (0.003)	1.537 (0.036)^{ab}	0.943 (0.013)	3.094 (0.013)^{a}	3.826 (0.047)^{a}	1.039 (0.003)	0.927 (0.002)
		S	1.570 (0.012)	1.473 (0.033)^{ab}	0.992 (0.018)	3.048 (0.008)^{a}	3.800 (0.000)^{a}	1.036 (0.009)	0.973 (0.003)
	1	U	1.483 (0.001)	1.573 (0.044)^{a}	0.935 (0.004)	3.077 (0.034)^{a}	3.845 (0.011)^{a}	1.045 (0.001)	0.932 (0.001)
		S	1.468 (0.009)	1.444 (0.049)^{ab}	0.960 (0.006)	3.077 (0.019)^{a}	3.818 (0.014)^{a}	1.029 (0.006)	0.921 (0.003)

FS, Flour stream; BK, Break; MD, Middling; SG, Straight grade; SL, Scouring level; U, Unscoured; S, Scoured; ML, Moisture level (%); Ser, Serine; Arg, Arginine; Gly, Glycine; Asp, Aspartic acid; Glu, Glutamic acid; Ala, Alanine; Pro, Proline; Means within a column of the same stream followed by differing lowercase letter are significantly different ($P < 0.05$), SD in brackets

Table 4.3. Indispensable amino acid composition of navy bean flours (% as is, sample basis)

FS	ML	SL	HIS	THR	CYS	LYS	TYR	MET	VAL	ILE	LEU	PHE	TRP
BK	0	U	0.558 (0.009)	0.979 (0.001)[ab]	0.214 (0.002)	1.442 (0.021)[ab]	0.707 (0.039)[bc]	0.301 (0.000)[b]	1.207 (0.009)[ab]	1.029 (0.003)[ab]	1.818 (0.015)[ab]	1.305 (0.004)[ab]	0.254 (0.014)
		S	0.563 (0.010)	1.027 (0.008)[a]	0.209 (0.003)	1.457 (0.012)[a]	0.774 (0.028)[a]	0.329 (0.000)[ab]	1.237 (0.009)[a]	1.056 (0.014)[a]	1.853 (0.048)[a]	1.341 (0.019)[a]	0.274 (0.001)
	0.5	U	0.551 (0.004)	0.982 (0.003)[ab]	0.212 (0.005)	1.406 (0.012)[b]	0.773 (0.031)[a]	0.308 (0.019)[ab]	1.204 (0.009)[ab]	1.021 (0.001)[ab]	1.824 (0.020)[ab]	1.318 (0.005)[ab]	0.271 (0.003)
		S	0.549 (0.010)	1.006 (0.014)[ab]	0.225 (0.027)	1.418 (0.024)[ab]	0.761 (0.016)[a]	0.356 (0.033)[a]	1.214 (0.021)[ab]	1.034 (0.004)[ab]	1.818 (0.042)[ab]	1.316 (0.028)[ab]	0.263 (0.006)
	1	U	0.544 (0.008)	0.983 (0.008)[ab]	0.222 (0.002)	1.423 (0.020)[ab]	0.744 (0.029)[ab]	0.293 (0.010)[b]	1.188 (0.002)[ab]	1.013 (0.000)[ab]	1.804 (0.027)[b]	1.298 (0.008)[ab]	0.271 (0.003)
		S	0.558 (0.007)	0.959 (0.002)[b]	0.202 (0.000)	1.401 (0.018)[b]	0.690 (0.043)[c]	0.281 (0.002)[b]	1.184 (0.003)[b]	1.004 (0.003)[b]	1.791 (0.011)[b]	1.285 (0.010)[b]	0.270 (0.002)
MD	0	U	0.628 (0.024)[b]	1.116 (0.011)	0.253 (0.012)	1.646 (0.015)	0.838 (0.012)[ab]	0.342 (0.010)	1.389 (0.017)	1.170 (0.006)	2.083 (0.035)	1.490 (0.021)	0.309 (0.003)
		S	0.606 (0.057)[b]	1.167 (0.011)	0.231 (0.001)	1.685 (0.002)	0.852 (0.074)[a]	0.368 (0.009)	1.404 (0.015)	1.199 (0.039)	2.091 (0.017)	1.515 (0.019)	0.316 (0.004)
	0.5	U	0.638 (0.009)[ab]	1.128 (0.008)	0.242 (0.007)	1.627 (0.024)	0.861 (0.003)[a]	0.351 (0.008)	1.383 (0.009)	1.176 (0.005)	2.071 (0.041)	1.483 (0.006)	0.306 (0.002)
		S	0.633 (0.017)[b]	1.158 (0.025)	0.238 (0.018)	1.673 (0.027)	0.876 (0.017)[a]	0.377 (0.014)	1.411 (0.019)	1.210 (0.021)	2.104 (0.024)	1.524 (0.019)	0.310 (0.005)
	1	U	0.707 (0.109)[a]	1.124 (0.004)	0.259 (0.012)	1.678 (0.023)	0.774 (0.032)[b]	0.334 (0.017)	1.374 (0.005)	1.178 (0.003)	2.061 (0.011)	1.490 (0.004)	0.312 (0.009)
		S	0.645 (0.006)[ab]	1.124 (0.008)	0.235 (0.005)	1.667 (0.016)	0.823 (0.028)[ab]	0.338 (0.008)	1.386 (0.007)	1.176 (0.009)	2.102 (0.017)	1.498 (0.008)	0.311 (0.002)
SG	0	U	0.618 (0.002)	1.096 (0.002)	0.241 (0.005)	1.625 (0.041)	0.844 (0.044)	0.343 (0.009)	1.354 (0.000)	1.144 (0.002)	2.052 (0.013)	1.457 (0.005)	0.287 (0.017)
		S	0.584 (0.052)	1.073 (0.102)	0.243 (0.021)	1.559 (0.139)	0.737 (0.078)	0.393 (0.034)	1.294 (0.133)	1.100 (0.101)	1.916 (0.175)	1.386 (0.123)	0.307 (0.004)
	0.5	U	0.613 (0.012)	1.100 (0.001)	0.230 (0.004)	1.576 (0.002)	0.877 (0.010)	0.343 (0.020)	1.340 (0.003)	1.150 (0.022)	2.035 (0.010)	1.451 (0.002)	0.299 (0.000)
		S	0.576 (0.089)	1.133 (0.005)	0.227 (0.012)	1.650 (0.005)	0.812 (0.011)	0.358 (0.008)	1.363 (0.005)	1.150 (0.001)	2.063 (0.018)	1.478 (0.009)	0.310 (0.003)
	1	U	0.627 (0.034)	1.100 (0.001)	0.253 (0.009)	1.612 (0.027)	0.844 (0.035)	0.342 (0.002)	1.344 (0.008)	1.154 (0.000)	2.021 (0.011)	1.460 (0.009)	0.297 (0.027)
		S	0.623 (0.009)	1.095 (0.008)	0.227 (0.000)	1.601 (0.018)	0.783 (0.013)	0.340 (0.008)	1.348 (0.004)	1.137 (0.025)	2.018 (0.006)	1.449 (0.009)	0.303 (0.000)

FS, Flour stream; BK, Break; MD, Middling; SG, Straight grade; SL, Scouring level; U, Unscoured; S, Scoured; ML, Moisture level (%); His, Histidine; Thr, Threonine; Cys, Cystine; Lys, Lysine; Tyr, Tyrosine; Met, Methionine; Val, Valine; Ile, Isoleucine; Leu, Leucine; Phe, Phenylalanine; Trp, Tryptophan; Means within a column of the same stream followed by differing lowercase letter are significantly different ($P < 0.05$), SD in brackets

4.4.1.3 *Amino acid score*

The AAS for all navy bean pre-treatments ranged from 0.76 to 0.88 (Table 4.4), with the total sulfur AAs methionine and cysteine being the sole limiting AAs. Scoured navy beans with no moisture pre-treatment achieved the greatest AAS in the SG flour, with the lowest present in the scoured, high moisture BK flour. Within the SG stream, the scoured seed with no moisture pre-treatment produced flour which had significantly greater AAS than its high moisture counterpart, in addition to unscoured flour at moderate moisture. No differences were observed in the MD flours, and BK flours had significantly lower AAS for the scoured high moisture condition relative to scoured treatment with no moisture pre-treatment. Reported AAS values for raw whole navy beans range from 0.79 and 1.03 (Deushi et al., 2023; Guldiken et al., 2021; Guldiken, Franczyk, et al., 2022). These ranges were narrower due to roller-milling or sulfur fertilization and were largely impacted by geographical location. Notably, the Nautica variety of navy beans was observed to have a 12% difference between the two geographical locations (Guldiken et al., 2021). However, in each study, the sulfur AAs remained as the limiting AAs, which also persisted across five common beans varieties following thermal treatments, including stove-top cooking, simulated cracker baking and extrusion (Nosworthy et al., 2018).

Table 4.4. *In vitro* protein digestibility and quality of navy bean flours (%)

FS	ML	SL	IVPD1	IVPD2	AAS	LAA	IVPDCAAS1	IVPDCAAS2
BK	0	U	73.65 (0.53)	82.89 (0.45)	0.79 (0.00)[ab]	M+C	58.25 (0.53)[ab]	65.55 (0.45)[ab]
		S	73.99 (0.97)	83.17 (0.82)	0.83 (0.00)[ab]	M+C	61.20 (0.27)[ab]	68.80 (0.20)[ab]
	0.5	U	73.29 (0.98)	82.58 (0.83)	0.80 (0.03)[ab]	M+C	58.32 (1.94)[ab]	65.71 (2.20)[ab]
		S	73.11 (0.43)	82.43 (0.36)	0.87 (0.05)[a]	M+C	63.32 (3.62)[a]	71.40 (4.11)[a]
	1	U	73.32 (0.86)	82.61 (0.73)	0.80 (0.01)[ab]	M+C	58.60 (0.23)[ab]	66.02 (0.32)[ab]
		S	72.81 (0.23)	82.18 (0.20)	0.76 (0.00)[b]	M+C	55.04 (0.67)[b]	62.12 (0.58)[b]
MD	0	U	73.26 (0.53)	82.56 (0.45)	0.80 (0.00)	M+C	58.40 (0.50)	65.81 (0.46)
		S	73.53 (0.64)	82.79 (0.54)	0.80 (0.01)	M+C	59.14 (0.82)	66.59 (0.87)
	0.5	U	72.69 (0.82)	82.08 (0.69)	0.79 (0.00)	M+C	57.39 (0.46)	64.80 (0.45)
		S	73.05 (0.66)	82.38 (0.56)	0.82 (0.04)	M+C	60.12 (2.80)	67.80 (3.27)
	1	U	73.35 (0.69)	82.64 (0.58)	0.79 (0.03)	M+C	58.01 (2.61)	65.35 (2.89)
		S	73.65 (0.74)	82.89 (0.62)	0.77 (0.02)	M+C	56.91 (1.91)	64.05 (2.01)
SG	0	U	73.29 (0.62)	82.58 (0.52)	0.80 (0.02)[ab]	M+C	58.98 (2.22)[ab]	66.46 (2.35)[ab]
		S	73.87 (0.21)	83.07 (0.18)	0.88 (0.08)[a]	M+C	64.76 (5.44)[a]	72.83 (6.17)[a]
	0.5	U	73.11 (0.28)	82.43 (0.23)	0.78 (0.03)[b]	M+C	57.04 (1.92)[b]	64.31 (2.16)[b]
		S	73.44 (0.86)	82.71 (0.73)	0.81 (0.03)[ab]	M+C	59.20 (2.02)[ab]	66.67 (2.24)[ab]
	1	U	73.41 (0.21)	82.69 (0.18)	0.81 (0.01)[ab]	M+C	59.61 (1.04)[ab]	67.14 (1.17)[ab]
		S	72.99 (0.53)	82.33 (0.45)	0.78 (0.01)[b]	M+C	57.27 (1.18)[b]	64.59 (1.29)[b]

FS, Flour stream; BK, Break; MD, Middling; SG, Straight grade; SL, Scouring level; U, Unscoured; S, Scoured; ML, Moisture level (%); IVPD(1 or 2), *In vitro* protein digestibility (1 or 2); AAS, Amino acid score; LAA, Limiting amino acid; M+C, Methionine and Cysteine; IVPDCAAS(1 or 2), *In vitro* protein digestibility corrected amino acid score (1 or 2); Means within a column of the same stream followed by differing lowercase letter are significantly different ($P < 0.05$). SD given in bracket

4.4.1.4 *In vitro protein digestibility*

Protein digestibility determined through IVPD1 ranged from 72.7 to 74.0% and 82.1 to 83.2 for IVPD2 (Table 4.4). The pH drop method employed to evaluate protein digestibility in this study has demonstrated good association with *in vivo* rat assays, previously reported as IVPD1—an estimation of apparent protein digestibility (Nosworthy et al., 2018). Estimates of true protein digestibility are also included, which account for endogenous losses, as represented by IVPD2. This regression provides a better protein digestibility coefficient for PDCAAS, where

otherwise true protein digestibility determined *in vivo* is used. Establishing *in vivo* values to determine true protein digestibility for raw navy beans to enable a direct comparison to *in vitro* values is challenging from an animal welfare perspective since feed intake is negligible (Kakade & Evans, 1965) due to the presumed presence of thermolabile antinutritive factors. Early reports for apparent digestibility were very low (43.5%) but may be expected to increase if corrected for endogenous losses (Sathe et al., 1984). Previously published reports on the *in vitro* protein digestibility of raw navy beans, either hulled or dehulled, have not been determined using IVPD2, although white beans following a similar method and regression have demonstrated a lower (73.5%) IVPD2 (Carbonaro et al., 2012). Previously, IVPD1 ranged from 73.29 to 73.87% using the same navy bean variety, indicating no differences observed between milling types (roller vs. knife) or stream. Two varieties of navy beans subjected to different sulfur fertilization conditions also yielded a narrow range of IVPD1, from 73.9 to 74.4% (Deushi et al., 2023). Six different varieties of navy bean, including Nautica, grown in two locations, showed that IVPD1 ranged from 70.1 to 73.1%, where both genotype and genotype-by-environment interactions were factors influencing *in vitro* protein digestibility (Guldiken et al., 2021). One study, including a composite of navy beans from different growing locations demonstrated a higher IVPD1, with untreated navy beans at 78.15% (Sánchez-Velázquez et al., 2021). Notably, in the previous three studies, dehulling of navy beans was not utilized or not indicated. Dehulling has been shown to significantly reduce the tannin content of kidney beans (by 93%), another common bean (*Phaseolus vulgaris*), and significantly increase IVPD1 from 68.1 to 71.6%. The seed coat colour of kidney beans has been a distinct factor in protein digestibility, where white and red varieties were reported to have an IVPD1 of 78.0 and 70.5%, respectively (Khattab et al., 2009, p. 1). This difference in protein digestibility may be partly due to growing location (Egypt and Canada), as red kidney beans

contained 2.5 times more tannin than white kidney beans (Khattab & Arntfield, 2009, p. 2), a component known to suppress protein digestibility. Elsewhere, following a pepsin-only protein digestion protocol, despite differences in tannin content between raw white and red kidney beans, protein digestibility was similar, at 34.0 and 33.8%, respectively (Rehman & Shah, 2005), indicating that protocols for digestion may also influence protein digestibility outcomes as trypsin hydrolysis would be expected to influence *in vitro* protein digestibility of beans (Romero & Ryan, 1978). Although not measured in this study, tannins may have been effectively decreased between all pre-treatments, as hull losses were equal from the mill. Additionally, as a measure of milled by-product, losses significantly reflect seed coat removal and thus a decrease in tannin content. Alternatively, as IVPD1 falls within the range of reported values for navy bean, dehulled or whole, it may be that these select varieties' tannin content does not significantly impact protein digestibility. Tannins or other antinutritional factors have been demonstrated to decrease protein digestibility in pulses (Gilani et al., 2012; Sá et al., 2019), but other factors such as the type of storage protein or protein secondary structure, may play a larger role (Carbonaro et al., 1996, 2000, 2012). The storage protein, globulin G1, was shown to be more digestible in navy beans (89.5%) and other common beans (92.5%) (Liener & Thompson, 1980; Marquez & Lajolo, 1990) than their other storage proteins, namely albumins and glutelins (79.1 and 73.2%, respectively). This may be partly due to methionine liberation during digestion (Coelho & Sgarbieri, 1994) but also potentially due to polyphenol-protein reactions (Ozdal et al., 2013). While some early works are unclear in their association between the ratio of globulins to albumins on protein digestibility (Sgarbieri et al., 1979), the relative proportion of legumin to vicilin was associated with a modest negative ($r = -0.50$, $P < 0.05$) correlation with IVPD1 in navy beans (Guldiken et al., 2021). Due

to the narrow range of either IVPD1 or IVPD2 within each flour and pre-treatment, the results may indicate minimal differences in the proportion of storage proteins.

Recently, the pH-drop method was officially accepted by AOCS (Ka 1b-2024: *In vitro* Assay for Protein Digestibility Using the pH-Drop Method) for assessing TPD and IVPDCAAS. Meanwhile, the INFOGEST 2.0 model (Brodkorb et al., 2019), which incorporates physiologically relevant procedures, has also shown significant advances in measuring protein and amino acid digestibility (Sousa et al., 2023) and is currently undergoing validation. In contrast, the pH-drop method has been specifically developed within the context of TPD and PDCAAS, which may have more direct implications for content claims in Canada and the U.S. (House et al., 2024), offering a rapid, inexpensive, and relatively simple approach for estimating the effects of processing on overall protein digestibility.

4.4.1.5 *In vitro protein quality*

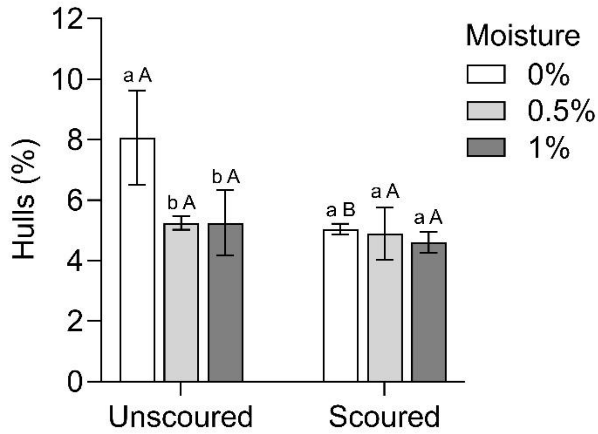
Protein quality determined via IVPDCAAS1 ranged from 55.0 to 64.8%, and IVPDCAAS2 from 62.2 to 72.8% (Table 4.4). The same AAS was used to correct both protein quality values, with only a change in IVPD1 or IVPD2. The greater values observed in IVPD2, as discussed in the previous section, are due to the estimation of true protein digestibility, which is a better fit for PDCAAS estimation. Given the methods used to estimate both IVPDCAAS1 and IVPDCAAS2, the factor that most influenced protein quality was the difference observed in the AAS. In both the BK and SG flours, those previously subjected to scouring and either no or moderate moisture treatment showed greater protein quality, as defined by IVPDCAAS, whereas high moisture conditioning with scouring led to decreased protein quality. The range of protein quality found across all flours aligns with effects observed due to differences in growing location for six varieties of navy beans, which ranged from 60.1 to 73.3%; however, in the latter case, both IVPD and AAS influenced final protein quality (Guldiken et al., 2021). Two varieties grown under different levels of sulfur fertilization demonstrated a narrow range of protein quality, from 60 to 63% (Deushi et al., 2023). When dehulling was not explicitly used, in a composite sample of raw navy beans, IVPDCAAS1 was 49%, and was similarly low in other common beans analyzed (Sánchez-Velázquez et al., 2021).

4.4.2: *Pre-treated chickpeas and flour streams*

4.4.2.1 *Milling by-products and hulls*

Moisture addition combined with scouring in chickpeas resulted in a significantly greater amount of milled by-products compared to their unscoured counterparts under moderate and high moisture conditions, with total milled by-product losses ranging from 10.1 to 23.2% of the starting weight (Figure 4.4., b). However, within either the unscoured or scoured chickpeas, varying moisture conditions did not significantly affect milled by-product losses. Hulls removed during milling accounted for 4.6 to 8.1% of the total product (Figure 4.4., a). Unscoured chickpeas without moisture pre-treatment yielded significantly more hulls at the roller-mill compared to those subjected to moderate and high moisture conditioning. In contrast, moisture conditioning in scoured chickpeas did not alter the amount of hulls collected, resulting in similar hull content across all moisture levels. These findings suggest that while moisture conditioning is typically expected to enhance hull removal by improving detachment through seed hydration (Vishwakarma et al., 2018), this effect was diminished when combined with roller-milling for chickpeas. Notably, the hull content of Kabuli chickpeas is reported to range from 4.5 to 9.5%, significantly lower than the 10.1 to 22.0% range observed for Desi chickpeas (Zhong et al., 2018). Based on the observed ranges in this study, it is likely that the scouring procedure combined with roller-milling significantly increased the loss of shorts in Kabuli chickpeas, while unscoured seeds fell within the expected hull content range.

(a) (b)



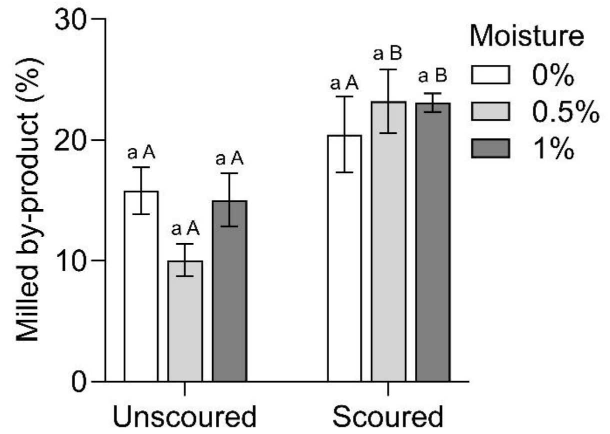


Figure 4.4. Hulls removed (total product) during roller milling (a) and total by-product (starting weight) including hulls removed during scouring and roller milling (b) for chickpeas in unscoured and scoured seeds subject to 0 , 0.5 and 1% moisture conditioning. Difference in lowercase letter for mean values indicate significant difference within scouring and uppercase letter for moisture and scouring ($P < 0.05$).

4.4.2.2 *Protein and amino acid composition*

The effect of moisture conditioning and scouring on chickpea crude protein content (Figure 4.5) has been previously reported by Choo et al. (2022), ranging from 20.3 to 23.7% (as is). The BK flours maintained a lower protein content amongst all streams, whereas the MD2 flours were highest. These trends in flour streams match those previously published (Guldiken et al., 2022) in absence of pre-treatment, in the range of 18.9 - 22.9% (db). Scouring consistently led to decreased protein content trends for MD1, MD2 and SG flours across all moisture conditioning levels. This observation is only significant in both unscoured MD1 and MD2 at moderate moisture conditioning, where protein content is greater relative scoured and no moisture conditioning within the same streams. Unscoured MD2 at moderate moisture conditioning also had significantly greater protein content than its scoured counterpart. Unscoured BK flours demonstrated a significant effect of moisture conditioning, where both moderate and high moisture conditioning were significantly lower in protein than the no moisture pre-treatment. Protein contents of both Kabuli and Desi chickpea varieties have been previously reported (Saha et al., 2023) within the range of 10.9 to 29.2% (as is), with an average of 18.6 ± 3.5%. Notably, the CDC Orion variety in the aforementioned analysis was lower, at 14.9% (as is), potentially influenced by the reconditioning of moisture used in the study. Higher protein was also reported at 23.6% (db) for CDC Orion in whole milled seed (He et al., 2019). Environmental factors, such as heat stress, have been demonstrated to influence the protein content of chickpeas and may also be one of the factors observed to affect the protein content of CDC Orion across studies (Samineni et al., 2022). Notably, the seed coat of chickpeas contains a high proportion of nonprotein nitrogen relative to the embryo, cotyledon or overall whole seed. Thus, changes in AA composition may not entirely

coincide with protein content, as nonprotein nitrogen contributes to total nitrogen content but does not provide indispensable AAs required for protein synthesis (Singh & Jambunathan, 1982).

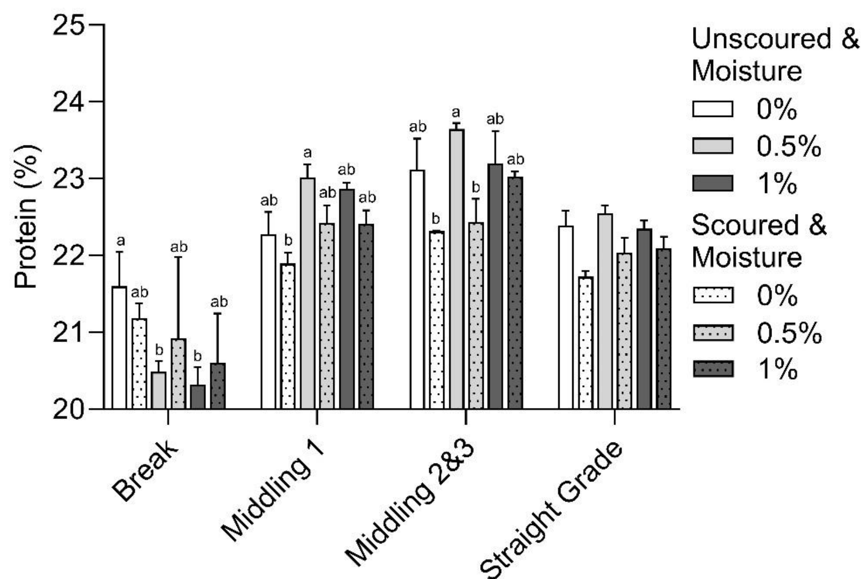


Figure 4.5. Protein content (% as is) of chickpea flours in unscoured and scoured seeds subject to 0, 0.5 and 1% moisture pre-treatment. Means within the same stream followed by differing lowercase letter are significantly different ($P < 0.05$).

The content of indispensable AA followed the same trend as protein, being lower in BK and higher in MD2, with values ranging from 7.2 to 8.5% (Figure 4.6). Changes in protein content did not translate to differences in total indispensable AA content in most pre-treatments and flour streams, except for the BK flours. In the BK flours, chickpeas with moderate and high moisture conditioning without scouring had lower total indispensable AA than their scoured counterparts. The highest total indispensable AA content was observed in the scoured high moisture BK flour, which was significantly different from the moderate moisture unscoured BK flour. The only decrease in indispensable AAs was observed in MD2 flours, where both lysine and tyrosine content decreased from scouring, and consistently with the application of moderate moisture levels (Table

4.6). All other changes occurred in dispensable AA (Table 4.5), particularly, arginine and glutamic acid, which decreased across all flours. This reduction may be attributed to overall hull losses, as the seed coat of Desi chickpeas has been reported to contain a higher proportion of glutelin (33.2%), followed by residue (30.5%), globulin (22.8%), albumin (3.5%), and prolamin (3.4%; Singh & Jambunathan, 1982). Although a lower proportion may be expected in Kabuli chickpeas used in this study—given the seed coat contributes a lower proportion of the total seed (4.5–9.5%)—it still provides a significant contribution (Zhong et al., 2018). Both glutelin and globulins contain a high amount of arginine but a lower amount of glutamic acid relative to other protein fractions, which may partially explain this decrease . Additional reductions in aspartic acid were observed in both MD1 and MD2 streams, possibly due to changes in the proportion of storage proteins, where albumins and globulins contain the highest levels of aspartic acid (Singh & Jambunathan, 1982).

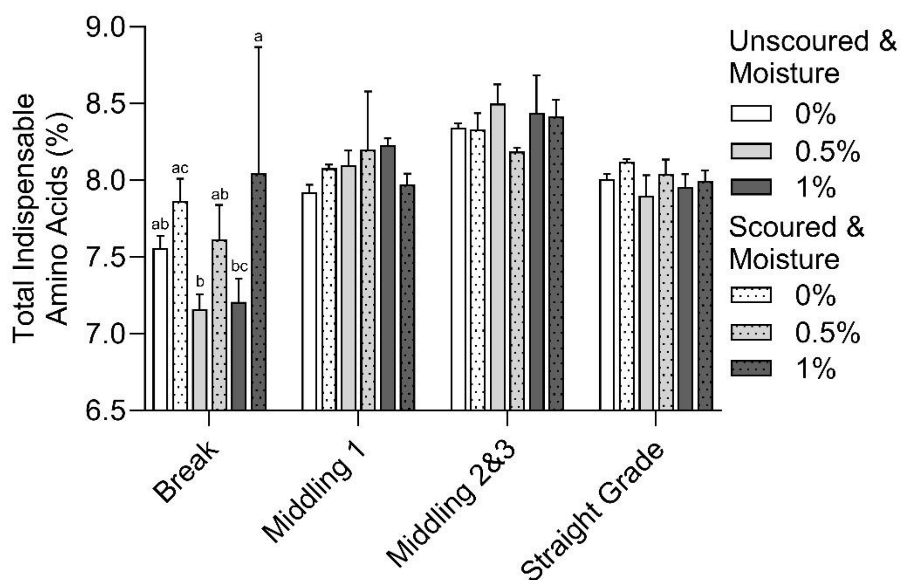


Figure 4.6. Total indispensable amino acid content (% as is) content of chickpea flours in unscoured and scoured seeds subject to 0, 0.5 and 1% moisture pre-treatment. Means within the same stream followed by differing lowercase letter are significantly different (P < 0.05).

Table 4.5. Dispensable amino acid composition of chickpea flours (% as is, sample basis)

FS	ML	SL	SER	ARG	GLY	ASP	GLU	ALA	PRO
BK	0	U	0.918 (0.011)	1.655 (0.067)^{ab}	0.681 (0.001)	2.107 (0.037)	3.083 (0.029)^{ab}	0.745 (0.014)	0.782 (0.011)
		S	0.939 (0.031)	1.631 (0.054)^{ab}	0.701 (0.036)	2.177 (0.032)	3.199 (0.074)^{ac}	0.766 (0.010)	0.794 (0.021)
	0.5	U	0.874 (0.007)	1.621 (0.050)^{ab}	0.632 (0.001)	1.993 (0.042)	2.939 (0.048)^{b}	0.707 (0.014)	0.743 (0.006)
		S	0.923 (0.016)	1.667 (0.000)^{ab}	0.681 (0.030)	2.097 (0.069)	3.081 (0.074)^{ab}	0.747 (0.020)	0.771 (0.014)
	1	U	0.882 (0.009)	1.581 (0.014)^{b}	0.654 (0.012)	2.000 (0.036)	2.993 (0.006)^{bc}	0.705 (0.019)	0.747 (0.018)
		S	0.988 (0.083)	1.802 (0.222)^{a}	0.732 (0.037)	2.183 (0.329)	3.277 (0.471)^{a}	0.779 (0.111)	0.825 (0.098)
MD1	0	U	0.964 (0.002)	1.711 (0.002)^{bc}	0.721 (0.001)	2.184 (0.016)^{b}	3.194 (0.020)^{b}	0.778 (0.007)	0.812 (0.005)
		S	0.973 (0.013)	1.774 (0.030)^{ab}	0.721 (0.001)	2.205 (0.017)^{ab}	3.270 (0.008)^{ab}	0.789 (0.003)	0.826 (0.010)
	0.5	U	0.988 (0.010)	1.771 (0.040)^{ab}	0.730 (0.001)	2.246 (0.012)^{ac}	3.279 (0.037)^{a}	0.795 (0.000)	0.832 (0.004)
		S	1.006 (0.053)	1.780 (0.001)^{ac}	0.747 (0.063)	2.293 (0.101)^{a}	3.328 (0.167)^{a}	0.813 (0.039)	0.826 (0.039)
	1	U	0.998 (0.006)	1.796 (0.040)^{a}	0.737 (0.027)	2.275 (0.013)^{ac}	3.303 (0.031)^{ab}	0.807 (0.007)	0.844 (0.006)
		S	0.954 (0.005)	1.693 (0.011)^{b}	0.725 (0.018)	2.217 (0.023)^{bc}	3.223 (0.032)^{b}	0.792 (0.008)	0.812 (0.009)
MD2	0	U	1.003 (0.008)	1.812 (0.029)^{a}	0.750 (0.021)	2.279 (0.030)^{ab}	3.311 (0.022)^{bcd}	0.823 (0.014)	0.848 (0.014)
		S	0.998 (0.008)	1.777 (0.005)^{a}	0.758 (0.013)	2.281 (0.020)^{ab}	3.371 (0.081)^{ad}	0.818 (0.005)	0.848 (0.028)
	0.5	U	1.022 (0.002)	1.897 (0.018)^{b}	0.747 (0.001)	2.286 (0.005)^{ab}	3.337 (0.020)^{ab}	0.824 (0.015)	0.861 (0.003)
		S	0.984 (0.009)	1.796 (0.055)^{a}	0.738 (0.013)	2.241 (0.002)^{b}	3.276 (0.026)^{bc}	0.806 (0.004)	0.829 (0.008)
	1	U	1.017 (0.010)	1.833 (0.024)^{ab}	0.757 (0.002)	2.285 (0.048)^{ab}	3.334 (0.053)^{ac}	0.827 (0.020)	0.866 (0.015)
		S	1.017 (0.012)	1.820 (0.003)^{a}	0.773 (0.014)	2.317 (0.037)^{a}	3.381 (0.055)^{a}	0.830 (0.019)	0.859 (0.005)
SG	0	U	0.961 (0.010)	1.723 (0.125)^{ab}	0.704 (0.005)	2.212 (0.013)	3.212 (0.001)^{ab}	0.788 (0.001)	0.826 (0.004)
		S	0.979 (0.016)	1.765 (0.039)^{a}	0.725 (0.026)	2.226 (0.012)	3.289 (0.047)^{a}	0.795 (0.004)	0.823 (0.023)

0.5	U	0.953 (0.020)	1.613 (0.258)^{b}	0.746 (0.066)	2.143 (0.114)	3.145 (0.122)^{b}	0.772 (0.019)	0.802 (0.020)
	S	0.967 (0.020)	1.756 (0.036)^{a}	0.723 (0.009)	2.224 (0.050)	3.233 (0.045)^{ab}	0.793 (0.015)	0.812 (0.009)
1	U	0.968 (0.002)	1.754 (0.059)^{a}	0.716 (0.012)	2.202 (0.015)	3.220 (0.023)^{ab}	0.784 (0.005)	0.821 (0.010)
	S	0.970 (0.001)	1.776 (0.012)^{a}	0.706 (0.008)	2.224 (0.034)	3.207 (0.052)^{ab}	0.786 (0.005)	0.819 (0.008)

FS, Flour stream; BK, Break; MD1, Middling 1; MD2, Middling 2&3; SG, Straight grade; SL, Scouring level; U, Unscoured; S, Scoured; ML, Moisture level (%); Ser, Serine; Arg, Arginine; Gly, Glycine; Asp, Aspartic acid; Glu, Glutamic acid; Ala, Alanine; Pro, Proline; Means within a column of the same stream followed by differing lowercase letter are significantly different ($P < 0.05$), SD in brackets

Table 4.6. Indispensable amino acid composition of chickpea flours (% as is, sample basis)

FS	ML	SL	HIS	THR	CYS	LYS	TYR	MET	VAL	ILE	LEU	PHE	TRP
BK	0	U	0.389 (0.017)	0.647 (0.010)	0.259 (0.009)	1.151 (0.014)	0.540 (0.017)	0.292 (0.010)	0.875 (0.019)	0.806 (0.006)	1.360 (0.010)	1.064 (0.003)	0.174 (0.001)
		S	0.403 (0.002)	0.669 (0.007)	0.259 (0.004)	1.221 (0.017)	0.540 (0.007)	0.355 (0.051)	0.894 (0.015)	0.837 (0.011)	1.408 (0.019)	1.104 (0.017)	0.177 (0.004)
	0.5	U	0.362 (0.004)	0.603 (0.015)	0.238 (0.004)	1.102 (0.043)	0.507 (0.034)	0.273 (0.004)	0.830 (0.017)	0.764 (0.025)	1.305 (0.004)	1.018 (0.015)	0.161 (0.002)
		S	0.393 (0.001)	0.650 (0.019)	0.245 (0.011)	1.153 (0.052)	0.572 (0.010)	0.293 (0.020)	0.870 (0.022)	0.821 (0.018)	1.375 (0.037)	1.086 (0.024)	0.160 (0.009)
	1	U	0.366 (0.010)	0.614 (0.015)	0.231 (0.008)	1.102 (0.047)	0.521 (0.021)	0.267 (0.001)	0.834 (0.022)	0.763 (0.016)	1.314 (0.028)	1.030 (0.021)	0.168 (0.005)
		S	0.433 (0.021)	0.693 (0.053)	0.243 (0.009)	1.189 (0.221)	0.584 (0.122)	0.284 (0.010)	0.938 (0.098)	0.871 (0.111)	1.475 (0.130)	1.178 (0.100)	0.159 (0.014)
MD1	0	U	0.389 (0.007)	0.675 (0.002)	0.264 (0.009)	1.208 (0.024)	0.602 (0.027)	0.307 (0.011)	0.911 (0.001)	0.844 (0.007)	1.427 (0.001)	1.118 (0.015)	0.178 (0.004)
		S	0.422 (0.002)	0.691 (0.014)	0.269 (0.008)	1.248 (0.009)	0.535 (0.011)	0.329 (0.010)	0.923 (0.005)	0.874 (0.008)	1.465 (0.015)	1.143 (0.008)	0.181 (0.002)
	0.5	U	0.409 (0.009)	0.693 (0.007)	0.270 (0.001)	1.227 (0.007)	0.611 (0.021)	0.306 (0.006)	0.941 (0.012)	0.857 (0.002)	1.463 (0.025)	1.142 (0.004)	0.179 (0.003)
		S	0.403 (0.063)	0.706 (0.032)	0.261 (0.003)	1.267 (0.067)	0.587 (0.003)	0.326 (0.004)	0.946 (0.036)	0.882 (0.048)	1.494 (0.065)	1.161 (0.064)	0.170 (0.006)
	1	U	0.444 (0.036)	0.698 (0.007)	0.266 (0.000)	1.282 (0.006)	0.568 (0.039)	0.313 (0.004)	0.956 (0.005)	0.867 (0.020)	1.489 (0.002)	1.159 (0.015)	0.188 (0.002)
		S	0.418 (0.016)	0.676 (0.010)	0.272 (0.002)	1.234 (0.043)	0.540 (0.024)	0.322 (0.011)	0.921 (0.013)	0.860 (0.018)	1.437 (0.011)	1.130 (0.010)	0.162 (0.006)
MD2	0	U	0.433 (0.037)	0.715 (0.008)	0.286 (0.004)	1.288 (0.039)^{ab}	0.610 (0.051)^{ab}	0.339 (0.030)	0.961 (0.011)	0.874 (0.012)	1.501 (0.001)	1.151 (0.005)	0.186 (0.002)
		S	0.422 (0.030)	0.709 (0.005)	0.279 (0.009)	1.286 (0.024)^{ab}	0.589 (0.032)^{b}	0.333 (0.007)	0.953 (0.013)	0.904 (0.009)	1.502 (0.027)	1.168 (0.009)	0.188 (0.002)
	0.5	U	0.477 (0.073)	0.729 (0.005)	0.285 (0.002)	1.304 (0.031)^{ab}	0.632 (0.003)^{ab}	0.322 (0.002)	0.976 (0.003)	0.901 (0.005)	1.515 (0.005)	1.170 (0.005)	0.188 (0.001)
		S	0.417 (0.021)	0.703 (0.000)	0.271 (0.002)	1.244 (0.004)^{b}	0.587 (0.025)^{b}	0.332 (0.004)	0.938 (0.006)	0.882 (0.012)	1.482 (0.004)	1.159 (0.006)	0.174 (0.011)
	1	U	0.424 (0.008)	0.730 (0.024)	0.275 (0.005)	1.288 (0.047)^{ab}	0.657 (0.055)^{a}	0.311 (0.007)	0.974 (0.024)	0.876 (0.012)	1.538 (0.037)	1.176 (0.021)	0.192 (0.002)
		S	0.423 (0.002)	0.727 (0.007)	0.278 (0.002)	1.318 (0.017)^{a}	0.592 (0.024)^{ab}	0.325 (0.001)	0.980 (0.011)	0.899 (0.014)	1.524 (0.033)	1.188 (0.023)	0.171 (0.021)
SG	0	U	0.389 (0.003)	0.684 (0.001)	0.273 (0.003)	1.213 (0.004)	0.604 (0.016)	0.311 (0.005)	0.926 (0.001)	0.849 (0.008)	1.446 (0.008)	1.134 (0.007)	0.180 (0.001)
		S	0.451 (0.055)	0.691 (0.008)	0.266 (0.001)	1.254 (0.009)	0.558 (0.027)	0.326 (0.009)	0.917 (0.000)	0.860 (0.004)	1.470 (0.002)	1.155 (0.034)	0.177 (0.004)
	0.5	U	0.449 (0.056)	0.668 (0.013)	0.265 (0.010)	1.231 (0.020)	0.552 (0.046)	0.298 (0.007)	0.903 (0.026)	0.839 (0.019)	1.414 (0.046)	1.105 (0.036)	0.178 (0.000)

	S	0.414 (0.017)	0.683 (0.011)	0.265 (0.004)	1.229 (0.017)	0.604 (0.002)	0.324 (0.021)	0.922 (0.011)	0.862 (0.017)	1.438 (0.032)	1.127 (0.018)	0.176 (0.004)
1	U	0.405 (0.012)	0.685 (0.001)	0.258 (0.001)	1.237 (0.028)	0.560 (0.069)	0.300 (0.013)	0.919 (0.003)	0.843 (0.002)	1.443 (0.004)	1.126 (0.007)	0.181 (0.000)
	S	0.437 (0.074)	0.679 (0.003)	0.271 (0.005)	1.214 (0.003)	0.577 (0.006)	0.314 (0.002)	0.923 (0.007)	0.853 (0.002)	1.445 (0.008)	1.126 (0.001)	0.159 (0.001)

FS, Flour stream; BK, Break; MD1, Middling 1; MD2, Middling 2&3; SG, Straight grade; SL, Scouring level; U, Unscoured; S, Scoured; ML, Moisture level (%); His, Histidine; Thr, Threonine; Cys, Cystine; Lys, Lysine; Tyr, Tyrosine; Met, Methionine; Val, Valine; Ile, Isoleucine; Leu, Leucine; Phe, Phenylalanine; Trp, Tryptophan; Means within a column of the same stream followed by differing lowercase letter are significantly different ($P < 0.05$), SD in brackets

4.4.2.3 *Amino acid score*

Minor changes in both indispensable AAs and protein content were sufficient to impact the AAS in the pre-treated chickpeas, which were all limiting in tryptophan, ranging from 0.66 to 0.77 (Table 4.7). The MD1, MD2 and the SG flours showed the same effects of moisture conditioning and scouring, such that high moisture conditioning with scouring resulted in the lowest AAS in these flours, significantly different from the highest AAS observed for the no moisture conditioning with scouring pre-treatment. Previously, differences between streams from roller-milled chickpeas were insufficient to show significant differences in AAS, which were also limiting in tryptophan (Guldiken et al., 2022). Alternatively, both untreated Desi and Kabuli chickpeas have been previously reported to be limiting in threonine, with higher AAS of 0.90 and 0.87, respectively (Bai et al., 2018; S. Wang et al., 2020), with the next limiting AAs being methionine and cysteine or tryptophan. The second limiting AA in our study would have also been threonine, ranging from 0.85 to 1.07 (data not shown). No major changes in AAS were observed when thermal tempering was imparted to the Desi chickpeas in the aforementioned study (Bai et al., 2018). However, extrusion processing in Kabuli chickpeas resulted in valine being the first limiting amino acid , followed by tryptophan (S. Wang et al., 2020). Other work, including raw and extruded Kabuli chickpeas, identified tryptophan as the only limiting amino acid (Bekele et al., 2021). Composite chickpeas subjected to cooking, baking or extrusion processing were only limiting in tryptophan (Nosworthy et al., 2020). This study also evaluated three varieties of Kabuli chickpeas grown in the same three locations, indicating the potential for environment and/or genotype effects on whether the first limiting AA is valine or tryptophan. Whether whole or thermally treated, there may not be a common limiting AAs in chickpeas. Although the reported use of dehulling in the literature, as in these analyses, is not always explicitly stated, making it

difficult to determine its contribution to the observed differences in AAS, alongside potential varietal and environmental factors.

4.4.2.4 *In vitro protein digestibility*

The effect of pre-treatments did not alter the *in vitro* protein digestibility of chickpeas, which ranged from 76.1 to 77.3% for IVPD1 and 84.8 to 86.0% for IVPD2 (Table 4.7). More recent reports utilizing IVPD1 are in agreement with the range of protein digestibility found in the current study, from 76.5 to 78.9% (Bai et al., 2018; Sánchez-Velázquez et al., 2021; S. Wang et al., 2020), whereas earlier reports were lower, at 71.8% (Clemente et al., 1998). Dehulling is not always indicated in these evaluations, and varieties may differ, as studies have utilized Desi, Kabuli, and composites. Earlier works on the protein digestibility of seven Kabuli and eight Desi cultivars, albeit using a different *in vitro* digestibility method, demonstrated varietal effects on IVPD, as 70.2 to 77.6% and 54.4 to 69.0%, respectively (Singh & Jambunathan, 1981). When the Kabuli chickpeas were dehulled, a slight increase in IVPD was observed, from 72.7 to 79.1%, whereas Desi chickpeas demonstrated a greater increase, from 63.7 to 76.0%. Protein digestibility in swine using the mobile nylon bag technique also demonstrated a higher range in eight varieties of Kabuli chickpeas (83.7%) relative to eight Desi chickpeas (78.3%), although this effect was not significant (Thacker et al., 2002). Reductions in tannin content, if measured, are generally expected following hull removal of chickpeas (Patterson et al., 2017). Tannins, as a class of polyphenolics, may be expected to directly impact IVPD, where a significant negative correlation (R= -0.87) was observed (Singh & Jambunathan, 1981). While significant differences in milled by-product loss were observed between pre-treatments, reductions in tannin content would likely be similarly high; otherwise, additional increases in IVPD may have been expected. Furthermore, Kabuli chickpeas

have a thinner seed coat relative to Desi chickpeas, which results in slight improvement in IVPD from milling procedures due to increase hull removal, as roller-milling is observed to remove hulls effectively across pre-treatments. The regression for IVPD2 more closely resembles the estimation of true protein digestibility, where a limited number of studies have shown differences in apparent (73.6 ± 7.9) relative to true (78.4 ± 7.9%) protein digestibility (rat fecal) in unheated (decorticated, soaked, defatted) chickpea (Tavano et al., 2008). In the absence of true protein digestibility values, apparent digestibility (rat fecal) values for chickpeas have also been reported as 82.8 ± 1.2% and 78.7 ± 1.6% (Nestares et al., 1996; Rubio et al., 1998). From this, it can be difficult to ascertain if IVPD2 accurately reflects true protein digestibility due to differences in chickpea variety and the lack of exact evaluation of whole or dehulled flours until further studies reflecting true protein digestibility regressions or *in vivo* data are conducted. Changes in the distribution of globulins and albumins are unlikely to have occurred in these chickpea flours, as previous works have demonstrated that IVPD1 can show a significant difference in the protein digestibility of these protein subunits, but *in vivo*, values may show no differences (Clemente et al., 1998; Rubio et al., 1998; Tavano et al., 2016).

Table 4.7. *In vitro* protein digestibility and quality of chickpea flours (%)

FS	ML	SL	IVPD1	IVPD2	AAS	LAA	IVPDCAAS1	IVPDCAAS2
BK	0	U	76.73 (1.51)	85.48 (1.27)	0.73 (0.02)	TRP	56.35 (1.77)	62.78 (1.91)
		S	77.15 (0.74)	85.84 (0.62)	0.76 (0.01)	TRP	58.62 (0.43)	65.22 (0.54)
	0.5	U	76.73 (0.33)	85.48 (0.28)	0.71 (0.00)	TRP	54.70 (0.24)	60.94 (0.27)
		S	77.24 (0.59)	85.92 (0.50)	0.69 (0.01)	TRP	53.54 (0.36)	59.55 (0.42)
	1	U	76.31 (1.03)	84.77 (1.44)	0.75 (0.01)	TRP	57.32 (0.80)	63.67 (0.55)
		S	76.82 (1.07)	85.56 (0.90)	0.70 (0.04)	TRP	53.69 (2.98)	59.80 (3.36)
MD1	0	U	77.33 (1.37)	85.99 (1.16)	0.73 (0.01)[ab]	TRP	56.11 (0.92)[ab]	62.39 (0.93)[ab]
		S	77.00 (0.78)	85.71 (0.66)	0.75 (0.01)[a]	TRP	57.87 (0.76)[a]	64.42 (0.90)[a]
	0.5	U	76.60 (1.07)	85.18 (1.05)	0.71 (0.01)[ab]	TRP	54.12 (0.45)[ab]	60.18 (0.70)[ab]
		S	76.79 (0.64)	85.53 (0.54)	0.69 (0.02)[ab]	TRP	52.82 (1.47)[ab]	58.83 (1.60)[ab]
	1	U	76.79 (0.60)	85.53 (0.51)	0.75 (0.01)[a]	TRP	57.39 (0.33)[a]	63.93 (0.39)[a]
		S	76.70 (0.63)	85.48 (0.54)	0.66 (0.03)[b]	TRP	50.56 (2.19)[b]	56.35 (2.51)[b]
MD2	0	U	76.85 (1.06)	85.59 (0.89)	0.73 (0.01)[ab]	TRP	56.21 (0.31)[ab]	62.60 (0.39)[ab]
		S	77.03 (0.28)	85.74 (0.23)	0.77 (0.01)[a]	TRP	59.07 (0.71)[a]	65.75 (0.78)[a]
	0.5	U	76.34 (0.59)	85.15 (0.50)	0.72 (0.00)[ab]	TRP	55.20 (0.50)[ab]	61.58 (0.47)[ab]
		S	76.73 (0.86)	85.48 (0.73)	0.71 (0.05)[ab]	TRP	54.25 (4.21)[ab]	60.43 (4.66)[ab]
	1	U	76.52 (0.64)	85.31 (0.54)	0.75 (0.01)[a]	TRP	57.61 (0.72)[a]	64.22 (0.74)[a]
		S	76.67 (0.82)	85.43 (0.69)	0.68 (0.08)[b]	TRP	51.80 (5.76)[b]	57.73 (6.55)[b]
SG	0	U	76.81 (0.54)	85.59 (0.41)	0.73 (0.00)[ab]	TRP	56.15 (0.53)[ab]	62.57 (0.50)[ab]
		S	76.79 (0.67)	85.53 (0.56)	0.74 (0.02)[a]	TRP	56.89 (1.35)[a]	63.37 (1.56)[a]
	0.5	U	76.55 (0.80)	85.33 (0.67)	0.72 (0.00)[ab]	TRP	54.92 (0.02)[ab]	61.22 (0.07)[ab]
		S	76.97 (0.68)	85.69 (0.57)	0.73 (0.02)[ab]	TRP	56.03 (1.88)[ab]	62.38 (2.07)[ab]
	1	U	76.94 (0.75)	85.48 (0.83)	0.74 (0.00)[a]	TRP	56.75 (0.25)[a]	63.05 (0.50)[a]
		S	77.21 (1.63)	85.89 (1.37)	0.66 (0.01)[b]	TRP	50.58 (1.17)[b]	56.27 (1.15)[b]

FS, Flour stream; BK, Break; MD1, Middling 1; MD2, Middling 2&3; SG, Straight grade; SL, Scouring level; U, Unscoured; S, Scoured; ML, Moisture level (%); IVPD(1 or 2), *In vitro* protein digestibility (1 or 2); AAS, Amino acid score; LAA, Limiting amino acid; TRP, Tryptophan; IVPDCAAS(1 or 2), *In vitro* protein digestibility corrected amino acid score (1 or 2); Means within a column of the same stream followed by differing lowercase letter are significantly different ($P < 0.05$). SD given in bracket

4.4.2.5 *In vitro protein quality*

The protein quality of chickpea flours, derived from IVPD1 as IVPDCAAS1, ranged from 50.6–59.1%, while IVPD2 for IVPDCAAS2 ranged from 56.3–65.8% (Table 4.7). The method of calculating IVPDCAAS differed only in the magnitude of protein quality estimates, as both methods identified the same significant differences in treatments. The differences in protein quality of chickpea flours from pre-treatments closely reflected the effects observed in the AAS, as no differences were observed in IVPD. Chickpeas subjected to scouring with no moisture conditioning demonstrated improved protein quality, similar to those pre-treated with high moisture conditioning without scouring. However, the addition of scouring at high moisture levels was significantly detrimental to the protein quality of MD1, MD2, and SG flour. Values for IVPDCAAS2 in the literature have not yet been established; however, IVPDCAAS1 has yielded higher protein quality values in raw chickpeas. Factors such as variety, environment, dehulling, and differences in limiting amino acids (e.g., threonine) may contribute to these variations. Notably, since protein content factors into the calculation of AAS, higher-protein (25.4% db) chickpeas coincide with lower IVPDCAAS1 (65.0%), compared to lower-protein (20.9%, as is) chickpeas, which exhibited higher IVPDCAAS1 (69.4%), despite having similar IVPD1 values (76.5% and 76.9%, respectively; Bai et al., 2018; S. Wang et al., 2020). In a previous study, composite unprocessed chickpeas (raw, no dehulling) showed similar IVPDCAAS1 values; however, protein content for the flour was not disclosed (Sánchez-Velázquez et al., 2021).

4.5: CONCLUSION

This study demonstrates that scouring without additional moisture conditioning effectively enhances protein quality in navy beans and Kabuli chickpeas by improving their AAS patterns. However, the combination of scouring with high moisture conditioning (1%) negatively impacted protein quality, highlighting the importance of optimizing pre-treatment conditions. Moderate moisture conditioning without scouring also reduced protein quality in navy beans. These findings emphasize that changes in protein quality were primarily driven by shifts in protein and AA composition, with sulfur AAs and tryptophan remaining the limiting factors in navy beans and chickpeas, respectively, across all flour streams and pre-treatments. Building upon the findings of Franczyk et al. (2025), this study contributes to the growing body of literature supporting the development of nutrient-rich pulse-based products by refining processing techniques and enhancing the functional properties of pulse flours. These results suggest that roller-milling practices can be a target for adjusting the nutritional quality of pulses. Furthermore, the application of *in vitro* digestibility methods provides valuable insights into protein quality assessment, offering a practical alternative to *in vivo* evaluations.

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4.7: AUTHORSHIP

A.F. Conceived and designed the study, carried out the research for each component, analyzed the data and wrote and reviewed each draft of the manuscript. A.S. conceived the milling trial design, including pre-treatments. N.B. and J.C. assisted in all collections of *in vitro* protein digestibility data and amino acid hydrolysis, which was analyzed by J.N. Sample preparation, including pre-treatments and milling, was completed by L.B. M.N. was the nominated principal investigator and J.H., E.S. and J.P. were co-investigators involved in conceiving and designing the study, reviewing data analysis, and approving the final draft of the manuscript.

4.8: CONFLICT OF INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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4.10: SUPPLEMENTARY DATA

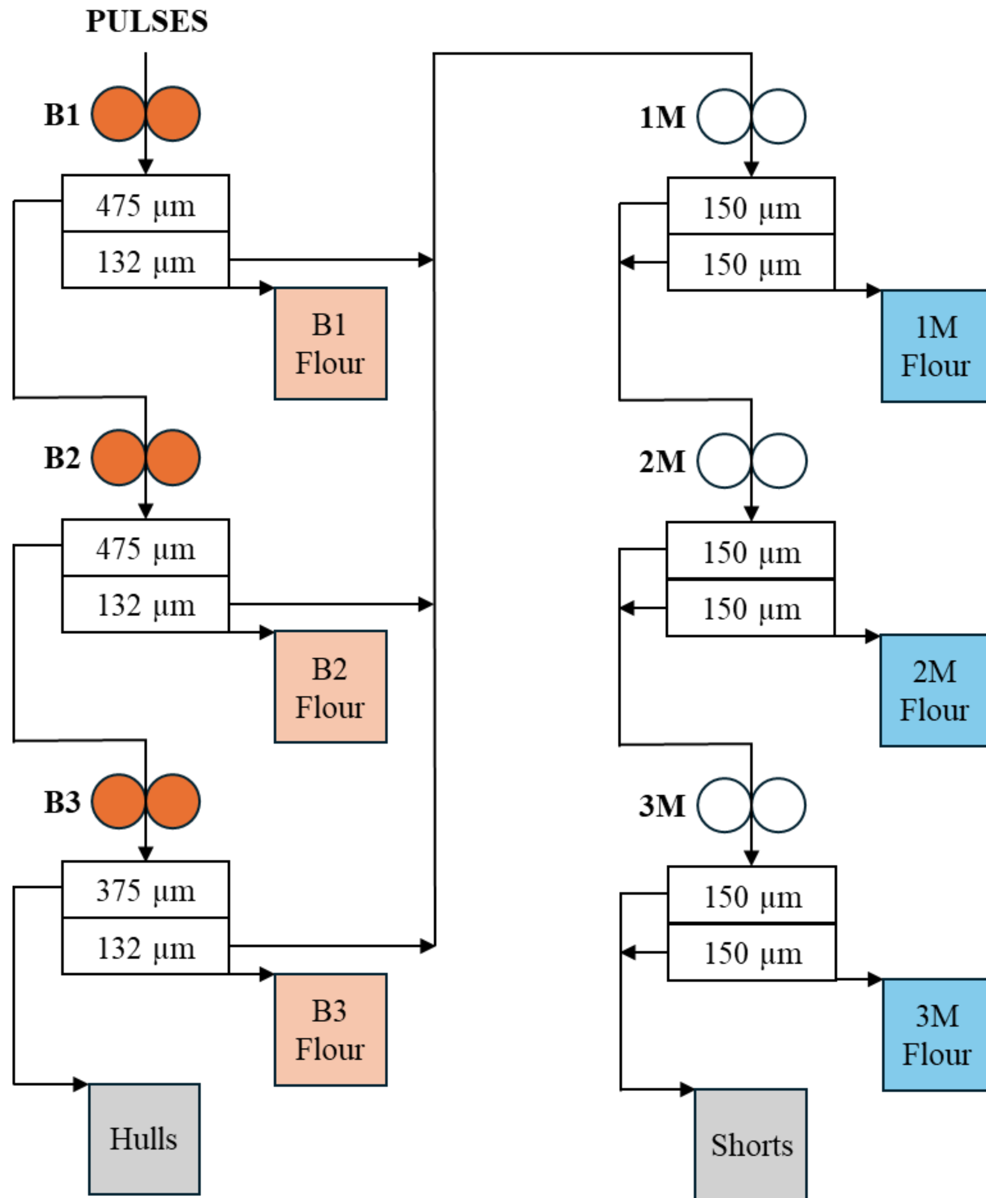

Figure 4.7. Schematic representation adapted from Guldiken et al. (2022) for the roller-milling process utilized on pre-treated navy beans and chickpeas

Table 4.8. Dry matter (%) for navy beans

Scouring Level	Unscoured			Scoured		
Moisture Level	**0%**	**0.5%**	**1%**	**0%**	**0.5%**	**1%**
Break	93.37 (0.25)	92.96 (0.30)	93.72 (1.64)	93.02 (0.09)	92.67 (0.13)	93.34 (0.24)
Middling	93.55 (0.37)	92.75 (0.18)	93.17 (0.39)	93.11 (0.13)	92.60 (0.04)	93.27 (0.15)
Straight Grade	93.54 (0.57)	92.59 (0.33)	92.81 (0.21)	93.06 (0.21)	92.55 (0.08)	92.96 (0.52)

Means with SD in brackets

Table 4.9. Dry matter (%) for chickpeas

Scouring Level	Unscoured			Scoured		
Moisture Level	**0%**	**0.5%**	**1%**	**0%**	**0.5%**	**1%**
Break	92.12 (0.06)	91.50 (0.25)	91.76 (0.01)	93.70 (0.52)	93.00 (1.65)	91.73 (0.11)
Middling 1	92.23 (0.24)	91.69 (0.08)	92.04 (0.01)	94.08 (0.04)	92.52 (0.64)	91.91 (0.09)
Middling 2&3	92.77 (0.08)	91.98 (0.01)	92.47 (0.16)	94.09 (0.02)	92.95 (0.96)	92.29 (0.04)
Straight Grade	92.46 (0.18)	91.79 (0.20)	92.17 (0.43)	93.89 (0.33)	92.79 (1.12)	92.11 (0.31)

Means with SD in brackets

4.11: Bridge to Chapter 5

Building upon the investigation of pre-milling treatments and their influence on the protein quality of navy beans and chickpeas, the next chapter transitions focus from raw flour streams to consumer-relevant products. Chapter 5 explores how both intrinsic factors (antinutritional compounds) and extrinsic factors (protein secondary structure and Maillard reaction products) influence protein quality in chickpea-fortified foods. By evaluating a range of product types—bread, pasta, and extruded snacks—this chapter addresses how formulation and processing translate into changes in *in vitro* protein digestibility, quality and content claims. This applied perspective complements the foundational ingredient-level insights from Chapters 3 and 4, providing a more complete understanding of how protein quality is modulated across the pulse value chain, from raw materials to finished foods.

CHAPTER 5. ROLE OF INTRINSIC AND EXTRINSIC FACTORS IN THE PROTEIN QUALITY OF CHICKPEA-FORTIFIED FOODS: A COMPARATIVE *IN VITRO EVALUATION*

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[x]James House passed away during the course of this work

5.1: ABSTRACT

Chickpeas can enhance the protein quality of cereal-based foods by complementing the lysine-limited amino acid profiles of wheat and corn. Their inclusion also introduces antinutritional factors (ANFs) that may impact protein digestibility and quality. This study evaluated the in vitro protein digestibility and quality of bread, pasta, and extrudates fortified with untreated, germinated, roasted, and micronized chickpeas. Two in vitro protein digestion (IVPD) methods—a pH drop assay and a simulated gastro-intestinal model—were used to assess protein digestibility and calculate the in vitro protein digestibility-corrected amino acid score (IVPDCAAS). Intrinsic (ANFs, polyphenols) and extrinsic (protein structural changes, Maillard reaction products) factors were examined for their influence on IVPD and IVPDCAAS. Chickpea addition improved protein content and amino acid scores across all products. Pre-treatments reduced ANFs, particularly trypsin inhibitors and phytate, with roasting and micronization showing the most consistent effects. Both IVPD and IVPDCAAS varied by product and digestibility method, with roasted chickpeas generally supporting higher digestibility. Polyphenols negatively affected either measure of IVPD, while intermolecular β-sheets, β-sheets, random coil, side chains, and aggregates were correlated only with the gastro-intestinal. These findings demonstrate that chickpea pre-treatments influence both composition and protein secondary structure, affecting protein quality in chickpea-fortified foods. The study supports optimizing formulation and processing strategies to improve the nutritional quality of plant-based products.

Keywords: Protein quality, Protein digestibility correct amino acid score, *in vitro* digestion, protein digestibility, chickpea, bread, pasta, extrudate, content claim

5.2: INTRODUCTION

The shift toward plant-based protein consumption has gained momentum, particularly in light of government dietary recommendations in Canada and the U.S. advocating for increased plant-protein intake (Fernandez et al., 2020). This trend is driven by concerns over the environmental impact of animal agriculture, health implications, and the increasing demand for sustainable and affordable protein sources amid a growing global population. Pulses—such as chickpeas, peas, beans, and lentils—are emerging as a key solution due to their lower carbon footprint, reduced water requirements, and cost-effectiveness (Boye et al., 2010; Gazan et al., 2021). Nutritionally, pulse consumption has been linked to a lower risk of non-communicable diseases, including cardiovascular disease, type II diabetes, and certain cancers, owing to their high fiber, protein, micronutrient, and bioactive compound content (Mudryj et al., 2014).

Pulses are of particular interest in cereal-based food formulations due to their ability to enhance protein content and quality (Mitchell et al., 2021). Cereals are typically low in lysine but rich in sulfur-containing amino acids, while pulses are abundant in lysine but limiting in sulfur-containing amino acids. This complementary amino acid profile improves overall protein quality. The nutritional rationale for combining pulses and cereals in food products is to overcome the amino acid limitations of each source. This is especially important in predominantly plant-based diets, where cereal-based products often make up a large proportion of intake. In one Canadian study, cereals accounted for nearly 40% of intake among adults consuming ≥75% plant-based diets, contributing to lower protein quality due to amino acid limitations (Marinangeli et al., 2021). In both Canada and the United States, protein quality is assessed based on protein digestibility and how well a protein's indispensable amino acid profile aligns with human metabolic requirements (House et al., 2024)—the product of which is identified as the protein

digestibility-corrected amino acid score (PDCAAS). These assessments are challenging, as traditional methods rely on animal bioassays, which raise ethical concerns, contributing significantly to the cost and resource requirements of PDCAAS (Mansilla et al., 2020; Marinangeli & House, 2017). Recent advances in *in vitro* digestion methods, such as the pH drop and gastro-intestinal models, have shown strong correlations with *in vivo* results (Franczyk, 2018; Nosworthy et al., 2020). These methods offer promising alternatives by addressing ethical and resource-related constraints. While the pH drop and pH stat approaches have recently been adopted as standardized methods for estimating PDCAAS (Goldberg et al., 2025), they have not yet been recognized for substantiating protein content claims under North American regulations (Krul et al., 2024)—a requirement for front-of-package labeling. Such claims can help consumers make more informed choices about what constitutes a protein food (A. Kaur et al., 2017), while also supporting manufacturers in meeting the growing demand for innovative plant-based protein products. Aligning regulatory recognition of *in vitro* methods with national dietary goals in both Canada and the United States may ultimately help facilitate greater adoption of plant-based proteins in everyday diets.

Pulses are increasingly incorporated into commercial food products—particularly cereal-based items such as bread, pasta, and extrudates—due to their nutritional, health, economic, and environmental benefits (Boye et al., 2010; Herrera A. & Gonzalez De Mejia, 2021). Integrating pulses into culturally familiar foods also helps reduce barriers to consumption in Western diets, such as unfamiliarity and long preparation times (Niva et al., 2017). Among pulses, chickpeas are of particular interest because of their favorable sensory and functional attributes compared to peas, beans, and lentils (Bravo-Núñez & Gómez, 2023; Wood, 2009). However, pulses contain antinutritional factors (ANFs)—including trypsin inhibitors, phytates, and polyphenols—that can

impair protein digestibility and quality (Gilani et al., 2012; Samtiya et al., 2020). While chickpeas generally have lower ANF levels than other pulses, their content still exceeds that of cereals, necessitating additional processing to improve protein digestibility (Sá et al., 2019).

Processing techniques such as milling are commonly used to produce flours with suitable particle size and functional properties for specific applications (Thakur et al., 2019). A secondary benefit of milling is the reduction of ANF content, particularly through hull removal, as the hull contains tannins—polyphenolic compounds that can interfere with protein digestion by forming enzyme–tannin or protein–tannin complexes (Patterson et al., 2017). Beyond milling, treatments such as germination, roasting, and micronization can significantly influence ANF content (Bai et al., 2018; Xu et al., 2016). Germination activates endogenous enzymes that break down phytates and oligosaccharides, while roasting and micronization—using dry heat and infrared radiation, respectively—further reduce ANF levels (Avilés-Gaxiola et al., 2018; Bai et al., 2018). Historically, intrinsic factors such as ANFs were considered significant impediments to protein digestibility and, by extension, protein quality in pulses. However, recent research using Fourier-transform infrared spectroscopy (FTIR) has highlighted the importance of extrinsic factors such as protein conformation and structure, particularly as they are altered by processing (Carbonaro et al., 2012). In addition, Maillard reaction products (MRPs) such as melanoidins can form during thermal treatments and reduce protein accessibility by creating cross-links that hinder enzymatic hydrolysis (Deng et al., 2017; El Hosry et al., 2025). These reactions also contribute to colour development, making browning intensity a useful indirect indicator of MRP formation.

Conventional milling and product formulation practices often prioritize crude protein content over protein quality. However, emphasizing content alone may fail to communicate which foods—could—serve as meaningful sources of protein in the diet. To address this gap, the

present study evaluated protein digestibility and amino acid composition in Kabuli chickpeas across a range of product types—bread, pasta, and extruded snacks—prepared using pre-milling treatments including germination, roasting, and micronization. Protein quality was assessed using two *in vitro* digestion methods, allowing for comparison of methodological influence on outcomes. In addition to protein quality, the study examined the effects of processing on intrinsic and extrinsic factors—such as ANFs, MRPs and protein secondary structures—that may impact protein digestibility. These findings offer insight into how processing strategies can be optimized to enhance protein quality in chickpea-based food products. The study also evaluated the potential of these products to meet protein content and quality claim requirements in Canada and the United States, contributing to both regulatory and commercial applications.

5.3: Materials and Methods

5.3.1: *Sample pre-treatment and milling*

Kabuli chickpea (CDC Orion) was acquired from McDougall Acres Ltd., Moose Jaw, SK., harvested during the 2020 growing season. Germination was scaled following procedures previously described (Setia et al., 2019). Chickpeas were initially rinsed with tap water, soaked in 0.07% sodium hypochlorite for 30 minutes, rinsed again, and then soaked for 24 hours. The water from the seeds was then strained and placed on screens with a paper towel over the top. Seed racks were placed into dark rooms at ambient room temperature to germinate for 48 hours. Moisture was maintained by spraying with water every 24 hours. The germination rate was 100%, based on a selection of 100 randomly chosen seeds. Immediately following germination,

seeds were dried at 75°C for 15 minutes in a smokehouse, then for an additional 8-12 hours at 55°C until moisture was < 10%.

Prior to roasting, whole chickpeas were tempered to a moisture level of 30% moisture. Tempering was achieved through water addition in 30-60 minute intervals, for a duration of 16-18 hours in a sealed container. Roasting proceeded in a Picard reel oven at 160°C for 30 minutes on perforated trays filled 2-3 seeds high. Moisture was determined prior to tempering and after roasting following the two-stage AACCI method (44-15.02).

Seeds subjected to micronization were also tempered to 20% moisture, which was achieved using the official AACCI method (26-95.01). Seeds were heated to 140°C using a micronizer (Model A 156379- B0, FMC Syntron® Bulk Handling Equipment, Homer City, PA, USA) and processed at InfraReady Products Ltd. (Saskatoon, SK, Canada). Seed surface temperature was confirmed using a hand-held IR thermometer (Oakton, Vernon Hills, IL, USA). Whole chickpeas were passed down through a Syntron feeder (Model F010, Riley Automatic Ltd., Derby, England) to control the speed and volume of passing seeds to another Syntron magnetic feeder (Mode BF2 A, FMC Corporation, Homer City, PA, USA) which conveyed seeds along the heating passageway containing the micronizing burners (Model type R 1603-2 pat, Rinnai, Japan). Chickpeas passing through the burners were situated 19cm below along a 152cm long conveyor.

Whole untreated or pre-treated pulse seeds were stored in sealed plastic bags at ambient room temperature (22 ± 2°C) prior to milling. Seeds were first pre-broken on a Bühler pilot roller mill (MDDM 1000/250, Bühler, Uzwil, Switzerland), followed by a Bühler laboratory roller mill (MLU 202, Bühler Group, Switzerland), using procedures previously described (Choo et al., 2022). Flours were produced from six streams (three break and three middling) and combined to

form a straight grade flour. Canada Western Red Spring (CWRS; G3 Canada Ltd., Winnipeg, MB, Canada) and Canada Western Amber Durum (CWAD; South West Terminal Ltd., Gull Lake, SK, Canada) wheat were acquired from the 2020 crop year. Straight grade flour was produced from CWRS using the pilot roller mill for use in bread applications and semolina was produced from CWAD using the laboratory roller mill for spaghetti applications. Hulls and shorts collected from the pilot and laboratory mills were not included in the straight grade flours or analyzed in this study. Their proportions, along with the straight grade flour yields from pre-treated chickpeas, were measured on a total product basis (Table 5.9).

5.3.2: *Product development*

Production of all products was carried out at Cereals Canada (Winnipeg, MB, Canada). The ratio of pulse inclusion to cereal for each product is summarized on Table 5.1. Bread products were produced using a no-time fermentation baking process. Chickpea breads were formulated with a 20% (replacement of wheat; w/w basis) level of inclusion of untreated or pre-treated flours (germinated, roasted, micronized) based on previous formulation work (Sopiwnyk et al., 2020), in addition to the following (expressed in baker's %): water (variable); 2% gluten (based on preliminary trials), 4% fresh yeast, 1.3% salt, 4% sugar, 4% vegetable shortening, 2% milk powder, and 2% dough conditioner (Puratos, Mississauga, ON, Canada). Wheat bread (100%) was also included as a control, formulated using the same proportions but without the addition of gluten. All ingredients were placed in a spiral mixer (model S-35, Erka Mashinenfabrik, Winnenden, Germany) and mixed on slow speed for 4 minutes (2 minutes for wheat bread) and then fast speed until the dough was fully developed. The initial water addition was determined by Farinograph absorption as a starting point and then assessed by an

experienced baker at the mixer to ensure a soft dough was achieved. The dough was rested on the bench for 10 minutes, then weighed into 640g pieces, rounded by hand, and rested again for an additional 10 minutes. The dough pieces were then molded using a B&B molder (Oliver Packaging & Equipment Co., Walker, MI, USA), placed into baking pans (25.5 cm L × 12.7 cm W × 8.5 cm D), and proofed (85% RH, 37°C) to a target height of 120 mm (measurement taken at the center of the pan using a proof height gauge; average 71 ± 1 min for chickpea breads, 64 ± 0.5 minutes for control). Breads were baked (200°C, 25 min) in a Picard reel oven (model 6-2356, Nicholson Equipment, Vancouver, Canada). Bread products were allowed to cool before being packaged into plastic bags and frozen at -20°C. Samples for analysis were freeze-dried from ~400g of bread, obtained from two loaves including the crumb and an end piece from each loaf.

Table 5.1. Ratio of chickpea inclusion into cereal based products

	Untreated	Germinated	Roasted	Micronized	Control
Bread	20:80	20:80	20:80	20:80	0:100
Pasta	50:50	50:50	50:50	50:50	0:100
Extrudate	60:40	60:40	60:40	60:40	0:100

Chickpea:cereal

Spaghetti products were also produced at Cereals Canada using a Namad Extruder (Rome, Italy). Pasta was formulated from a blend of 50% untreated or pre-treated chickpea flour and 50% semolina, in addition to a control made from 100% semolina. Following the manufacturer's instructions, pasta was produced in 3kg batches. The water bath temperature was set to 20.5°C, and the mixing time was set for 12 minutes. After extrusion, the spaghetti was dried in a Bühler batch dryer (Uzwil, Switzerland). Pre-trial cooking of spaghetti was completed to determine the optimal cooking time, defined as the time when the center core of the spaghetti

just disappears when pressed between two Plexiglas plates. Following this, approximately 200g of dried pasta was boiled in 800g of water using the optimal cooking time for each treatment. Each spaghetti treatment was produced, then cooked, in duplicate. Cooked spaghetti was placed in tin containers and cooled to room temperature before being frozen (-20°C) overnight and freeze-dried.

An extruded puffed snack product was produced using a Clextral EV25 twin screw extruder (Firminy, France). Extrudate was formulated from a blend of 60% untreated or pre-treated chickpea flour and 40% degermed yellow corn meal (Bunge, St. Louis, MI, USA), in addition to a control made entirely from the corn meal. No additional ingredients were included in the formulation. Extrusion processing parameters for the chickpea blends were as follows: water rate 1.6-2.0 L/h, feed rate 20-26 kg/h (feed moisture content: 14.3-15%), screw speed 750 RPM, knife speed 500 RPM and exit barrel temperature 160°C. The corn meal control parameters were: water rate 1.4 L/h, feed rate 25 kg/h (feed moisture content: 13.3%), screw speed 400 RPM, knife speed 500 RPM and exit barrel temperature 130°C. The specific mechanical energy for the chickpea and control extrudates was 298-390 kJ/kg and 74.3 kJ/kg, respectively. Freeze-dried bread and spaghetti, in addition to extruded chickpea products were milled to ≤ 0.75mm (ZM 200 ultra centrifugal mill, Retsch; Haan, Germany) and placed in a freezer (-20°C) prior to further analysis.

5.3.3: *Chemicals*

Digestive enzymes were purchased from Sigma-Aldrich (Oakville, ON, Canada), including chymotrypsin (bovine pancreas ≥40 units/mg protein), trypsin (porcine pancreas 13,000-20,000 BAEE units/mg protein), protease (*Streptomyces griseus* ≥15 units/mg solid), pepsin (Pepsin from porcine gastric mucosa lyophilized powder, 3,200-4,500 units/mg protein)

and pancreatin (Pancreatin from porcine pancreas 8 × USP specifications). Spectrophotometric standards, including phytic acid sodium salt hydrate (93%), gallic acid (95.3%), and L-leucine (≥ 99.5%), were also acquired. High-nitrogen casein (80 mesh), soy protein isolate, and gelatin were obtained from Dyets Inc. (Bethlehem, PA, USA) All other reagents were ACS grade or better, and acquired from Fisher Scientific (Ottawa, ON, Canada).

5.3.4: *Composition*

Dumas combustion on a LECO nitrogen analyzer (LECO Corp., St. Joseph, MI) was used to determine the nitrogen content of samples. Protein was calculated using the same nitrogen conversion factor of 6.25 for all samples, to be consistent with FDA regulations on protein quality assessment for protein content claim substantiation and nutrition facts tables. Samples were not defatted as crude oil content was presumed to be ≤ 7% in whole Khabuli chickpeas, including the variety used in this study (He et al., 2019; Xu et al., 2014). Dry matter (DM%) was determined using a standardized protocol (AOAC 925.10) but was not applied in calculations unless otherwise specified (Table 5.10).

5.3.5: *Amino acids*

Three distinct procedures were employed to produce hydrolysates for amino acid (AA) analysis. Alkaline hydrolysis for the determination of tryptophan (ISO 13904), performic acid oxidation (AOAC 994.12) followed by sodium metabisulfite and acid hydrolysis for methionine and cysteine, and regular acid hydrolysis (AOAC 982.30) for the remaining AAs. Precolumn derivatization utilizing the AccQ-Tag system (Waters Corporation, Milford, MA, USA) was employed for all AAs except tryptophan (Nosworthy et al., 2017). Reverse-phase HPLC was

used to quantify AAs through UV detection or fluorescent emission. Reference standards were included in each batch of analysis to ensure accuracy as tests were completed in singlet.

5.3.6: *In vitro protein digestibility-pH Drop*

Samples were digested by *in vitro* (Hsu et al., 1977) specific to protein with updates to enzymes and regression (Franczyk, 2018). Sample equal to 6.25 mg of protein was suspended in 10 mL of deionized water and mixed on a multi-stir plate for 1 hour at room temperature. A multienzyme solution containing 3.1 mg/mL chymotrypsin, 1.6 mg/mL trypsin and 1.3 mg/mL protease was prepared in deionized water and adjusted to pH 8.0 ± 0.05 using 1 N NaOH or HCl at 37°C. The pH-stable multienzyme solution was then transferred to an ice water bath (4°C) for the duration of the assay. Samples in triplicate were warmed to 37°C and adjusted to a stable pH of 8.0, followed by the addition of 1 mL of multienzyme solution. To establish the consistency of proteolysis of the multienzyme solution, casein was used as a standard. The initial and final pH over a 10-minute period were recorded and used to determine the IVPD by regression:

$$\mathrm{pIVPD} = 76.15 + 15.26(\Delta \mathrm{pH}_{10\mathrm{minutes}})$$

5.3.7: *In vitro gastro-intestinal protein digestion*

Simulated gastro-intestinal digestion was carried out in a two-phase procedure (Franczyk, 2018). The initial gastric phase combined 150 mg of protein with 18.75 mL of 0.1 M potassium phosphate buffer (pH 6.0) and 7.5 mL 0.2 M HCl in a 50 mL screwcap flask. The gastric solution was adjusted to a pH of 2.00 ± 0.05 with 1 M HCl at 39ºC while being magnetically stirred (300

RPM). Flasks were then transferred to a water bath at 39ºC, to which 750 µL of freshly prepared pepsin solution (6.67 mg/mL in 0.1 M HCl) and 375 µL of 0.5% chloramphenicol (w/v; prepared in ethanol) were added to the solutions. The gastric digestates were sealed and shaken (75 RPM) for six hours, then enzymatic action was terminated with 7.5 mL of 0.2 M potassium phosphate buffer (pH 6.8) and 3.75 mL of 0.6 M NaOH. The pH was subsequently adjusted to 7.00 ± 0.05 at 39ºC and transferred to the water bath. The intestinal phase of digestion proceeded after the addition of 750 µL fresh pancreatin solution (66.7 mg/mL in deionized water), followed by sealing and shaking for an additional 18 hours. Casein was included as a digest control, in addition to a digest water blank with and without enzyme.

Intestinal digestion was terminated following transfer of 1.4 mL digestate—under active stirring—to preloaded amber Eppendorf tubes containing 200 µL of 60% trichloroacetic acid. The digestate was vortexed for 6 seconds then rested in a refrigerator (4°C) for 1 hour, before centrifugation at 17,000 × g for 15 minutes. The supernatant was carefully transferred to avoid lipid contamination at the interface and stored at -20°C in cryogenic vials.

Thawed samples were briefly vortexed and 200 µL digestate was transferred to tubes for acid hydrolysis in 6 N HCl at 110°C for 24 hours (without the addition of phenol). Samples were briefly rested at room temperature then neutralized with 25% NaOH (w/v) and rested again. The hydrolysate was transferred to a beaker after three subsequent rinses with deionized water in the same beaker and then mixed. An aliquot of the sample was syringe filtered (0.22 µm) and analyzed the same day using an Evolution One spectrophotometer (Thermo Fisher Scientific Inc., Waltham, USA) for total α-amino acid content, as determined by o-phthaldialdehyde (OPA) at 340 nm (Church et al., 1983). The OPA solution was prepared fresh in an amber volumetric flask, which was composed of 50 mL 100mM sodium tetraborate, 5 mL of 20% (w/w) sodium

dodecyl sulfate, 200 μL β-mercaptoethanol, and 2.0 mL phthaldialdehyde (40 mg/mL in methanol) and brought to a final volume of 100 mL with deionized water and stirred for 1 hour. The response factor was determined from a 10-point standard curve of 1 mM L-leucine (reported purity of 100%) prepared in 100 mM sodium tetraborate. Preloaded 1.5 mL methacrylate cuvettes containing 200 μL of blank (100 mM sodium tetraborate), hydrolysate or L-leucine in duplicate were carefully mixed with 1 mL of OPA reagent. The subsequent absorbance was measured after two minutes—including mixing time—at room temperature.

In vitro protein digestibility using simulated gastro-intestinal digestion (gIVPD) was determined from the initial nitrogen content of the sample (S) and the remaining α-amino acid nitrogen in the digestate (D). Both the water blank (WB) and enzyme blank (EB) from the digestion procedure were used to correct for potential contributing sources of nitrogen. No correction for food matrix effects was included.

$$\text{Digest} = \left(\frac{(\text{D} - \text{WB}) - (\text{EB} - \text{WB})}{\text{S}}\right) \text{x}100$$

A previously established regression was used to estimate true protein digestibility from the former calculation (Franczyk, 2018):

$$\text{gIVPD} = 37.58 + 0.7095(\text{Digest})$$

5.3.8: *Amino acid score & in vitro protein quality*

The AA scoring is determined from indispensable and conditionally indispensable amino acids (IAA), which is determined from the relative abundance of that IAA in milligrams for each gram of protein in the sample, by the relative abundance of each IAA provided by a reference pattern, for ages 2-5 (FAO/WHO, 1991). This pattern is selected based on the currently accepted

practice for protein quality claims. This amino acid score (AAS) is selected from the ratio, which is the lowest (limiting IAA) relative to all other scored IAAs.

$$\text{AAS} = \frac{\text{mg of AA per gram of protein (test protein)}}{\text{mg of AA per gram of protein (reference pattern)}}$$

The product of IVPD and the AAS were used to calculate IVPDCAAS, from both methods of digestion, as follows, resulting in two corrected protein digestibility coefficients:

$$\text{pIVPDCAAS} = \text{pIVPD} \times \text{AAS}$$

$$\text{gIVPDCAAS} = \text{gIVPD} \times \text{AAS}$$

5.3.9: *Antinutritional factors*

Trypsin inhibitor activity (TIA) was evaluated (Liu et al., 2021) indirectly by measuring the inhibitory action of the sample on the synthetic substrate DL-arginine para-nitroanilide, followed by the addition of a known quantity of trypsin. The hydrolysis of the substrate yields a yellow-coloured end-product, which is determined spectrophotometrically at 410 nm. One trypsin unit inhibited (TUI) is defined as 0.02 A410 and calculated on a TUI/g sample (on an as-is basis).

Phytate was determined by extracting samples in an acidic solution with a known iron content (Haug & Lantzsch, 1983). The accessibility of iron in solution decreases from phytate exposure, which is reflected spectrophotometrically by a decrease in a red colour from the addition of a bipyridine solution, measured at 519 nm, 30-60 seconds after addition. The content was determined from a standard curve of phytate from 40 to 4 µg/mL (R^2 =0.996) and reported on a mg phytate/g sample (on an as-is basis).

The total phenolic content was determined using a combined approach from two previous studies, as described in the Folin-Ciocalteu assay (Heimler et al., 2005; Konieczny et al., 2020). A 2 g sample was extracted in 5 mL of 1% concentrated HCl (12 N) in methanol. The solution was initially vortexed for 15 seconds at 3000 RPM then placed on a rotator for 2 hours at 40 RPM. Extracts were then centrifugation at 1050 × g for 10 minutes and supernatant transferred to a separate sealed tube. Extraction procedures were repeated twice, each lasting 20 minutes. The supernatant was pooled, and a 1.5 mL aliquot was centrifuged at 12,700RPM for 10 minutes. From this, 125 µL of supernatant was added to tubes containing 1.5 mL of deionized water and briefly vortexed, followed by 125 µL of Folin–Ciocalteu reagent and additional mixing. The sample mixture was rested for 5 minutes followed by the addition of 1.25 mL of 7 % (w/v) Na_2CO_3 and incubated for 100 minutes. Absorbance was measured at 550 nm against a blank (sample preparation with just 1% in methanol) using a spectrophotometer. Total phenolic contents are expressed as mg GAE (gallic acid equivalent)/g sample, from a calibration curve in the range of 100 to 500 µg/mL (R^2 = 0.992). Standard curves were produced for phytate and polyphenols, both adjusted to reflect the purity reported for their contents. Extraction procedures for all ANFs were completed in duplicate-on-duplicate samples, with analyses performed in a single run. Soy protein isolate was included as a control across all measures of ANFs to ensure experimental replication across multiple days.

5.3.10: *Protein secondary structure*

Characterization of protein secondary structure (Carbonaro et al., 2012) was completed using attenuated total reflectance-Fourier transformed infrared spectroscopy (ATR-FTIR) on the Nicolett 6700 (Thermo Electron Inc., Madison, WI, USA). Infrared spectra were generated from

a range of 400–4000 cm^{-1} at a 2 cm^{-1} resolution from 40 scans. Sample flours were placed in a desiccator with the addition of phosphorus pentoxide for one week prior to analysis. Background spectrum of air was measured prior to and following every 10 samples. Data analysis was performed in Origin 2024 software (OriginLab Corporation, Northampton, MA, USA). Full range of the ATR-FTIR data was imported and normalized, with a quick peaks function in the amide I region (1600-1700 cm^{-1}). To improve the evenness of the data, the region was fit to a straight line, and the baseline was subtracted. Deconvolution of the region was generated through the second derivative and smoothed using the Savitzky-Golay algorithm, and then each selected peak was fit using the Gaussian function. The protein secondary structure reported is corrected based on the proportion of each peak area divided by the sum area for all determined peaks. An overall peak fit was considered acceptable when the chi-square tolerance was $\leq 1\times10^{-6}$ and the R^2 value was ≥ 0.80.

5.3.11: *Colour*

Colour readings were measured using a Minolta CM-3500d spectrophotometer (Toyko, Japan), calibrated against the D65 CIE Standard Illuminant. Results are expressed as L*, defined as darkness (0) to brightness (+100), a* as greenness (−60) to redness (+60) and b* as blueness (−60) to yellowness (+60).

5.3.12: *Statistics*

Duplicate products were produced for 14 of the items (5 bread, 5 pasta, and 4 extrudates), where only a single replicate for the extruded corn control was procured (29 total). Straight-

grade flour mixtures used to produce the products, in addition to whole untreated, germinated, roasted, and micronized flour, were also procured in singlets (19 total). Products were subject to ordinary two-way ANOVA with Tukey's multiple comparison test with a single pooled variance on measures of colour, ANFs, protein secondary structure, IVPD, IVPDCAAS, and AAS. Simple effects within columns were compared for each row of a specific product, in addition to a separate analysis across all products. A Pearson correlation was calculated from a matrix of all variables measured in the products, flour mixtures, and a combination of products and flour mixtures was produced. Analyses were conducted using GraphPad Prism 9 (GraphPad Software, Boston, MA, USA) and significant differences were reported if $P < 0.05$.

5.4: RESULTS & DISCUSSION

5.4.1: *Trypsin inhibitor activity*

The content of trypsin inhibitors across all products was low (Table 5.2), ranging from 140.79 to 867.53 TUI/g sample. The lowest levels were observed in the bread product in part due to its lowest inclusion of chickpeas, where pre-treatments had no significant effect on TIA. However, all pre-treated breads contained significantly greater ($P < 0.05$) TIA than the control. A similar pattern was observed in the pasta products, where most chickpea pastas showed no significant differences in TIA among pre-treatments, but all exhibited higher TIA content ($P < 0.05$) compared to the control pasta. Pasta made from micronized chickpea was the exception, containing significantly lower TIA (641.10 TUI/g sample) than the other pre-treatments (811.10 to 867.53 TUI/g sample). Among the extrudates, untreated chickpeas contained the highest ($P < 0.05$) TIA content. No production duplicates were available for the corn control, but it exhibited

relatively lower TIA (by 74-80%) than the chickpea extrudates, consistent with previous reports on corn extrudates (Brugger et al., 2015).

The most significant reductions in TIA were observed in products made from the untreated and germinated pre-treatments, with decreases ranging from 80.72% to 91.67% relative to their raw flour mixtures. Roasting and micronization resulted in more modest reductions, with decreases of 13.63% to 58.87% across both the pasta and extrudate products, and small increases were observed in the bread products. These smaller changes likely reflect the already substantial reductions in TIA achieved from chickpea pre-treatments for roasting (83.57%) and micronization (84.24%) compared to untreated chickpeas. Trypsin inhibitors are regarded as heat labile, including those in chickpeas (Avilés-Gaxiola et al., 2018), where significant reductions have been previously observed following micronization in chickpeas (Bai et al., 2018) and roasting in peas and beans (Khattab & Arntfield, 2009). In contrast, flour made from germinated chickpeas exhibited a smaller initial decrease (12.95%) compared to untreated chickpeas, in agreement with the literature, which demonstrates that while germination reduces TIA, thermal treatments achieve greater reductions (Alves Diniz et al., 2025; El-Adawy, 2002; Pérez-Ramírez et al., 2023).

Previous work demonstrated that bread made from wheat or a rye mix exhibited increased TIA, while whole wheat bread contained no detectable TIA and mixed cereals maintained their TIA (Kostekli & Karakaya, 2017). These author noted that TIA decreased during dough preparation relative to the starting flour, suggesting that trypsin inhibitors may lose activity during fermentation but become reactivated or more extractable after baking. Interestingly, TIA was reduced more in chickpea protein concentrates treated with L-cysteine alone than in those subjected to heat alone. This suggests that thermal processing may interfere

with the disulfide-reducing action of L-cysteine—possibly by altering protein conformation or promoting aggregation that shields trypsin inhibitor activity from reduction (Avilés-Gaxiola et al., 2018). The effect of fermentation by baker's yeast (*Saccharomyces cerevisiae*) on TIA in chickpeas or bread products remains poorly understood. One study reported that a chickpea-based yogurt fermented with baker's yeast and likely lactic acid bacteria exhibited reduced TIA. However, inhibition was more pronounced when extruded (thermally treated) chickpeas were used (Yağcı et al., 2020). Moreover, lactic acid bacteria alone have been shown to reduce TIA in chickpeas, with the extent of reduction dependent on fermentation time (Sáez et al., 2022).

Pasta made from 15% inclusion of black chickpeas also reported similarly low levels of TIA (De Pasquale et al., 2021). Pre-roasting pulse seeds may reduce TIA in a pulse-type–dependent manner in pulse-fortified pastas, showing only marginal effects in navy and pinto beans but greater reductions in lentils (Bahnassey et al., 1986). The reduction in TIA from roasting becomes more pronounced as inclusion of pulse in pasta formulation increases from 10% to 15%, even though the overall TIA also increases. In contrast, cooking pulse pastas is more effective at reducing TIA, with pastas made entirely from faba bean, lentil, or black gram showing 68 to 81% lower TIA than their corresponding raw flours (Laleg et al., 2016). Corn-chickpea fortified extrudates do not appear to have been previously evaluated for TIA. Available work on whole chickpea extrudates shows substantial reductions in TIA, with reductions of 85.8 to 91.8% when chickpeas were pre-conditioned at 70, 90, or 100°C before extrusion, with chickpeas showing greater reductions than faba bean or field pea (Adamidou et al., 2011). Under different extrusion conditions, whole extruded chickpeas had no detectable TIA, whether or not the initial seeds were soaked, and similarly undetectable values were observed in extruded faba

beans, peas, and kidney beans, suggesting near-complete elimination relative to their raw (Abd El-Hady & Habiba, 2003).

Notably, when trypsin inhibitor content was low (≤ 200 TUI/g sample), it was sometimes necessary to use a larger sample amount to detect TIA reliably. Reaching the detection threshold produced viscous samples, which may impact reliable extraction of trypsin inhibitors. This may partially explain marginal increases in TIA, when those detected in the flours produced from thermal pre-treatments are already very small, relative to the end products (i.e. additional thermal treatment), as error in extraction, or why previous reports have no detectable trypsin inhibitors.

Table 5.2. Trypsin inhibitor activity (TUI/g sample), phytate (mg phytate/g sample) and polyphenol (mg GAE/g sample) content of pre-treated chickpea products

	TIA	%D	Phytate	%D	Polyphenol	%D
Bread						
Control	233.51 (40.17)[b]	-37.2	0.28 (0.22)[d]	-81.1	0.78 (0.00)[a]	-7.3
Untreated	514.63 (105.31)[a]	-80.7	0.71 (0.06)[b]	-87.0	1.20 (0.00)[b]	12.0
Germinated	416.10 (13.90)[a]	-83.7	0.86 (0.01)[a]	-84.4	1.33 (0.04)[c]	14.9
Roasted	505.21 (76.22)[a]	31.5	0.83 (0.07)[ab]	-83.8	1.12 (0.04)[b]	12.0
Micronized	461.78 (81.75)[a]	5.7	1.06 (0.09)[c]	-79.6	1.18 (0.04)[b]	9.6
Pasta						
Control	140.79 (2.93)[b]	-61.8	1.70 (0.06)[c]	4.7	0.39 (0.16)[a]	-39.1
Untreated	811.10 (38.42)[a]	-89.0	5.81 (0.06)[a]	-30.2	0.71 (0.03)[b]	-27.8
Germinated	867.53 (125.62)[a]	-86.8	6.30 (0.09)[b]	-32.5	0.91 (0.10)[c]	-19.9
Roasted	817.26 (49.07)[a]	-13.6	5.86 (0.40)[a]	-21.9	0.93 (0.00)[c]	6.9
Micronized	641.10 (50.54)[c]	-46.9	6.13 (0.19)[b]	-23.8	1.11 (0.06)[d]	30.7
Extrudate						
Control	164.61 N/A	-77.4	1.59 N/A	22.3	0.19 N/A	-68.9
Untreated	842.57 (133.84)[b]	-89.6	6.81 (0.10)	-22.5	0.67 (0.06)[a]	-31.5
Germinated	636.62 (8.94)[a]	-91.7	6.49 (0.09)	-24.2	0.74 (0.06)[a]	-47.9
Roasted	688.57 (16.49)[a]	-48.8	6.95 (0.64)	-13.3	0.59 (0.04)[b]	-37.9
Micronized	625.97 (8.13)[a]	-58.9	6.89 (0.41)	-1.5	0.73 (0.03)[a]	-22.6

TIA, Trypsin inhibitor activity; %D, Difference from raw ingredient or mixture; Significant differences in means ($P < 0.05$) are indicated by different letters within each column for a given product; Standard deviation in brackets

5.4.2: *Polyphenols*

In conjunction with other anti-nutritional factors, polyphenol contents were lowest ($P < 0.05$) in the respective product controls (Table 5.2), while pulse inclusion contributed significant quantities of phenolic compounds. Among flours, germinated chickpeas exhibited a substantial increase (41.86%) in phenolics relative to untreated chickpeas. In contrast, roasting and micronization resulted in decreases of 20.84% and 7.57%, respectively (Table 5.11), with the highest polyphenol content observed in germinated chickpeas and the lowest in roasted samples. These findings are consistent with prior observations of increased phenolics following germination (Alves Diniz et al., 2025). Although roasting at elevated temperatures (e.g., 250–350°C) has been associated with both marginal decreases and subsequent increases in total phenolics (Jogihalli et al., 2017), other studies using lower roasting temperatures (e.g., 150–180°C) have reported smaller and more variable changes (Godrich et al., 2023; Hithamani & Srinivasan, 2014). These inconsistencies suggest that factors such as roasting duration, tempering moisture, and dehulling—as applied in the present study—may significantly influence polyphenol outcomes during roasting.

Despite a lower chickpea inclusion, all chickpea-bread products exhibited significantly greater polyphenolic content, ranging from 1.12 to 1.33 mg GAE/g sample. Breads made with germinated chickpeas had the highest ($P < 0.05$) phenolic content among all pre-treatments. Some of this increase may also be attributed to the wheat flour used, which contained higher phenolic content than the other cereal controls. Notably, chickpea breads exhibited a higher polyphenolic content relative to their raw flour mixtures, with increases ranging from 9.6% to 14.9%. While pulse-enriched breads have previously demonstrated similar increases (Boukid et al., 2019), the mechanisms may be influenced by the detection method and the potential impact

of fermentation. Fermentation may enhance phenolic release, as demonstrated in *L. plantarum*-fermented pea protein concentrate over 1 to 11 hours (Çabuk et al., 2018) and in fermented chickpeas after 24 hours (Sáez et al., 2022). In contrast, fermentation of soybean meal showed no effect with *S. cerevisiae*, and even a decrease in total phenolics with *L. plantarum* after 36 hours (Chi & Cho, 2016). The influence of substrate composition, preparation, and fermentation time may therefore be just as critical as microbial species in determining phenolic outcomes. Interestingly, a study of gluten-free breads made with rice flour and roasted chickpea flour found greater total phenolic content than that of whole or dehulled chickpeas (Kahraman et al., 2022). This study, which assessed crumb and crust separately, observed that the crumb was darker and more yellow—indicating higher MRP formation—while the crust appeared less dark and less red than the untreated control. These colour changes align with increases in phenolic-like compounds, which may partly reflect MRPs rather than native phenolics. The Folin–Ciocalteu reagent used in this study is known to react with reducing sugars and amines, potentially inflating apparent phenolic content due to MRP formation (Kitchen & Williamson, 2024). Other baked products, such as crackers made with increasing proportions of chickpea flour—without any yeast or microbial fermentation—also demonstrated rising phenolic content between doughs and crackers with greater chickpea inclusion (Chatziharalambous et al., 2023). This finding suggests that fermentation may not be required for the increase or retention of polyphenols in baked products, and instead, thermal reactions or matrix effects could be responsible.

In pasta products, the total phenolic content ranged from 0.71 to 1.11 mg GAE/g sample, with untreated chickpea pasta containing the lowest levels ($P < 0.05$) relative to all pre-treatments. Pasta made from micronized chickpeas contained significantly more phenolics than that made from roasted chickpeas. Notably, roasting and micronization increased the phenolic

content relative to their raw flour mixtures by 6.9% and 30.7%, respectively, while pastas from untreated and germinated chickpeas showed reductions of 27.8% and 19.9%, respectively. This trend aligns with findings in lentil pasta, which demonstrated higher phenolic content than semolina-only pasta and retained more phenolics after cooking (Di Stefano et al., 2020). The inclusion of carboxymethyl cellulose in this study was also found to enhance phenolic retention in lentil pasta by limiting leaching and providing protective hydrogen bonding. This may infer a secondary effect of dietary fiber introduced through pulse flours, which may help retain phenolics during cooking. Unlike bread, colour changes in pasta did not suggest extensive MRP formation. Pasta prepared from roasted and micronized chickpeas was brighter, less yellow, and similarly red compared to pasta made from untreated or germinated chickpeas. These observations support the interpretation that apparent phenolic increases in bread may partially reflect non-phenolic MRP detection by the Folin–Ciocalteu method, appear to be less influential in pasta matrices.

Extruded products had the lowest phenolic content overall, ranging from 0.59 to 0.74 mg GAE/g sample, with the lowest ($P < 0.05$) levels observed in extrudates made from roasted chickpeas. Regardless of pre-treatment, extrusion consistently reduced the phenolic content, with losses ranging from 22.63% to 47.89%. This is consistent with widespread observations of phenolic degradation during extrusion processing of both pulses and cereals (Nikmaram et al., 2017; Pasqualone et al., 2020). In contrast to other thermal treatments—such as cooking and baking of chickpeas—which did not significantly reduce total phenolics, extrusion led to notable phenolic losses (Sánchez-Velázquez et al., 2021). Additional factors such as soaking and elevated extrusion temperature further exacerbate phenolic losses (Abd El-Hady & Habiba, 2003). These losses in total phenolics are partly explained by the polymerization reactions that

occur during extrusion, which may convert monomeric phenolics into larger, bound structures, thereby reducing their extractability and apparent concentration (Mironeasa et al., 2023). In a similar formulation, germinated and dehulled chickpeas blended with corn grits exhibited an increased phenolic content compared to the corn-only control (Hegazy et al., 2017). Incremental chickpea inclusion at 10%, 20%, and 30% resulted in stepwise increases in phenolics, surpassing levels measured in their respective raw blends. These findings suggest that, despite the typical losses associated with extrusion, strategic formulation—particularly using high-phenolic, germinated seeds—may help mitigate or even counteract these effects. Notably, the study also included additional ingredients, which may have contributed to phenolic retention where losses might otherwise be expected.

5.4.3: *Phytate*

Among the pre-treatments, germinated chickpea flour had the highest phytate content, followed by untreated, roasted, and micronized samples (Table 5.11). Compared to the untreated chickpea, the phytate content showed a slight relative increase of 1.92% in the germinated sample, while roasted and micronized pre-treatments resulted in reductions of 8.69% and 19.84%, respectively. While germination has been shown to reduce phytate in chickpeas (Marengo et al., 2017), some studies report slight increases (7.41%) when germination is combined with dehulling and boiling (Malunga et al., 2012). In this study, dehulling likely explains the lack of phytate reduction in the germinated sample, as removal of hull and shorts may increase the proportion of phytate-rich seed content. A linear relationship (data not shown) was observed between phytate content and the yield of straight-grade flour collected from the mill. Additional reductions in phytate content have been observed when roasting or germination

pre-treatments are combined with protein isolation procedures, with outcomes influenced by germination duration and chickpea variety (Mesfin et al., 2021).

The phytate content of chickpea-based products was significantly greater ($P < 0.05$) than that of their respective cereal-based controls, where all products ranged from 0.28 to 6.95 mg phytate/g sample (Table 2). Within the bread products, phytate levels ranged from 0.71 to 1.06 mg/g, with bread made from untreated chickpea flour containing the lowest amount, followed by bread made from roasted, germinated, and micronized chickpea pre-treatments. Phytate content was significantly higher ($P < 0.05$) in breads made from micronized chickpea compared to all other pre-treatments, and significantly higher in germinated chickpea bread compared to the untreated version. Across all bread products, phytate levels were reduced by 79.62% to 86.98% compared to the original flour. This substantial reduction may be partially attributed to intrinsic wheat phytase activity (Mayer et al., 2023), which likely acts on the phytate present in the chickpea portion. Endogenous wheat enzymes may be more effective than fermentation under optimal conditions (e.g., pH, time, temperature) (Fretzdorff & Brummer, 1992), although yeast extracts may also contribute to phytate degradation (Vilanculos et al., 2024). In isolation, *Saccharomyces cerevisiae* can exhibit greater phytase activity than wheat (Fekri et al., 2020), but this activity may be suppressed when free phosphorus is present, as phytase expression is downregulated. If micronization increased phosphorus availability initially from pre-treatment, this could explain the higher phytate content in the corresponding bread due to reduced phytase induction. Additionally, dough pH—adjusted by the addition of chickpea and pre-treatment—may influence both wheat and yeast-derived phytase activity.

Chickpea pasta retained considerable phytate, ranging from 5.81 to 6.30 mg/g. Similar to the bread results, germinated and micronized chickpea pre-treatments yielded higher phytate

levels ($P < 0.05$) than untreated or roasted chickpea pastas. All pasta samples exhibited moderate reductions—ranging from 23.77% to 32.48%—in phytate content relative to their original flours. These reductions were greater than those reported for 100% legume pastas made from faba bean, lentil, or black gram, which ranged from 3% to 18% (Laleg et al., 2016). These small or moderate reductions align with phytate's poor water solubility (Abd El-Hady & Habiba, 2003) and the brief cooking time used in pasta preparation. While longer soaking with extended cooking periods can substantially reduce phytate (Sánchez-Velázquez et al., 2021).

Chickpea extrudates also contained high phytate levels, ranging from 6.49 to 6.95 mg/g, with no significant differences observed among pre-treatments. Phytate reductions from the original flours ranged from 1.54% to 24.16%. Although extrusion is generally associated with phytate degradation in pulses (Nikmaram et al., 2017) and corn (Pontoppidan et al., 2007), reported reductions vary widely (11–45%) depending on extrusion parameters such as temperature, moisture content, and screw speed (Abd El-Hady & Habiba, 2003; Adamidou et al., 2011; Pasqualone et al., 2020). In contrast to the bread products, the pasta (semolina) and extrudates (corn) provided little to no endogenous phytase. Semolina, derived from the endosperm of durum wheat, lacks the bran and aleurone layers where phytase is concentrated (Mayer et al., 2023). Corn is naturally low in phytase activity (Ingelmann et al., 2019), and the use of degermed corn in the present study further reduced the potential for endogenous phytate degradation. In contrast, germinated dehulled chickpea–corn extrudates with inclusion rates of 10%, 20%, and 30% showed greater phytate reductions, ranging from 43.30% to 46.51% (Hegazy et al., 2017). Notably, the corn grits used in that study contained nearly twice the phytate content of the degermed corn used here, which likely contributed to the greater reductions observed. Furthermore, the phytate extraction method employed in the present study

may have limitations in detecting low phytate concentrations, which could contribute to variability between studies.

5.4.4: *Protein secondary structure*

The relative spectral weights of secondary structure components in all products and chickpea pre-treatments are summarized in Table 5.3, with structural assignments in the amide I band based on literature values (Shevkani, Singh, Chen, et al., 2019). Among the secondary structures measured in bread, α-helices represented the largest proportion, followed by sidechains in the 1690–1695 cm^{-1} region and β-turns in the 1660–1670 cm^{-1} region. Breads made with untreated chickpeas exhibited the greatest number of differences in structural elements ($P < 0.05$) relative to all other pre-treatments and the control. In contrast, only the control bread and the bread made with micronized chickpea showed a significantly lower proportion of intermolecular β-sheet structures. Previous findings in yellow pea-fortified breads provide partial confirmation of our observations, based on crumb analyses from breads containing 10% untreated or roasted yellow pea flour and 20% roasted yellow pea flour (Kotsiou et al., 2021). While direct comparisons between formulations were not the focus of their FTIR analysis, the data suggest that 20% roasted yellow pea inclusion may increase β-sheet structures, reduce random coil and α-helix structures, and elevate β-turns—potentially due to the dry roasting treatment. Despite differences in spectral assignments, the α-helix and random coil ranges are comparable, with reported proportions nearly double in their control breads. In contrast, the reported β-sheet and β-turn contents—if adjusted to align with broader spectral regions—appear considerably lower than those reported in our samples. Interestingly, the authors also examined the effects of storage and reported that, after four days, control breads showed an increase in

random coil structures, while both untreated and roasted pea formulations exhibited decreases in α-helices and increases in β-turns.

Pasta products primarily contained a large proportion of random coil structures, followed by sidechains and aggregates (1690–1695 cm^{-1}), and β-sheets. Pasta made from micronized chickpeas exhibited a significantly ($P < 0.05$) greater proportion of sidechains and aggregates at 1610–1615 cm^{-1} compared to all other treatments. In contrast, intermolecular β-sheets were more abundant in the roasted chickpea pre-treatment relative to the control. The β-turn content (1660–1670 cm^{-1}) in the control pasta was also notably higher ($P < 0.05$) than in most chickpea pre-treatments, except for pasta made from untreated chickpeas. An increase in the β-sheet to β-turn ratio was observed in semolina pasta formulations with the inclusion of chickpea flour, suggesting a shift toward a more ordered and aggregation-prone protein structure (Garcia-Valle et al., 2021). The absolute proportions of these structures reported remain higher than those observed in our analysis. Notably, our results show a high proportion of random coil and side-chain structures, which may suggest that—despite differing spectral assignments—the overall extent of protein aggregation or structural organization is comparable between the semolina control and high chickpea inclusion (e.g., 50%) for any pre-treatment. Fibre content was not measured in our study; however, the substantially higher dietary fibre values reported by Garcia-Valle et al. (2021) suggest that matrix effects arising from fibre–protein interactions may have contributed to the greater structural ordering observed in their samples. In contrast, the use of dehulled chickpeas in our study likely reduced fibre content substantially, potentially minimizing such interactions and influencing the observed secondary structure profile.

Chickpea extrudates were characterized by a high proportion of β-sheet structures as the dominant secondary structure, followed by random coil and α-helical structures. Extrudates

prepared from micronized chickpeas showed a higher ($P < 0.05$) proportion of α-helices relative to those made from germinated and roasted chickpeas. Additionally, the micronized extrudates exhibited a lower ($P < 0.05$) content of β-turn structures (1660–1670 cm^{-1}) compared to those made from untreated chickpeas. The proportion of β-sheets was also lower ($P < 0.05$) in micronized chickpea extrudates than in those made from roasted chickpeas. Qualitative FTIR analysis of maize grits extrudates fortified with 10% and 40% germinated chickpea suggests that the 10% formulation exhibited greater signal intensity for α-helices, β-sheets, and β-turns compared to both the 40% blend and the maize-only control (Serrano-Sandoval et al., 2022). These differences may align with our own findings at lower inclusion levels, though the presence of intact corn grits in their study—absent from ours—may have influenced the structural outcomes.

Table 5.3. Protein secondary structure components of pre-treated chickpea products

	SC+AGG1 (1610–1615)	I-β Sheet (1620–1630)	β-Sheet (1630–1638)	RC (1640–1648)	α-Helices (1650–1660)	β-T1 (1660–1670)	β-T2 (1670–1680)	AP-β Sheet (1680–1688)	SC+AGG2 (1690–1695)
Bread									
Ctrl	8.43 (0.02)^{a}	9.92 (0.08)^{ab}	11.93 (0.03)^{a}	7.69 (0.00)	15.37 (0.04)^{a}	13.47 (0.03)^{a}	6.07 (0.02)	12.69 (0.01)^{a}	14.42 (0.46)^{a}
Untr	6.70 (0.47)^{b}	14.15 (1.09)^{c}	11.06 (0.27)^{b}	8.04 (0.16)	16.29 (0.23)^{b}	14.61 (0.33)^{b}	5.78 (0.10)	11.34 (0.37)^{b}	12.03 (5.03)^{b}
Grm	8.30 (0.38)^{a}	10.53 (0.91)^{ab}	11.88 (0.13)^{ab}	7.65 (0.06)	15.27 (0.17)^{a}	13.48 (0.29)^{a}	6.04 (0.07)	12.56 (0.26)^{a}	14.30 (4.07)^{a}
Rst	8.25 (0.04)^{a}	10.69 (0.00)^{a}	11.83 (0.06)^{ab}	7.75 (0.04)	15.47 (0.03)^{ab}	13.48 (0.06)^{a}	6.01 (0.02)	12.48 (0.05)^{a}	14.04 (0.84)^{a}
Mcr	8.67 (0.07)^{a}	9.72 (0.15)^{b}	11.97 (0.06)^{a}	7.61 (0.03)	15.15 (0.00)^{a}	13.27 (0.04)^{a}	6.11 (0.03)	12.76 (0.02)^{a}	14.74 (0.28)^{a}
Pasta									
Ctrl	6.43 (1.46)^{ab}	6.82 (0.22)^{a}	13.56 (0.78)	21.00 (1.04)	11.91 (0.15)	12.48 (1.32)^{b}	4.88 (0.16)	6.76 (0.67)	16.16 (4.92)
Untr	5.60 (0.17)^{a}	8.08 (0.34)^{ab}	14.19 (0.01)	20.66 (0.20)	11.95 (0.00)	11.39 (0.06)^{ab}	4.69 (0.01)	7.05 (0.05)	16.39 (0.75)
Grm	6.20 (1.29)^{a}	7.97 (0.48)^{ab}	13.87 (0.92)	20.21 (0.67)	12.11 (0.36)	10.82 (0.53)^{a}	4.53 (0.20)	7.37 (0.57)	16.93 (3.43)
Rst	5.99 (0.64)^{a}	9.18 (0.44)^{b}	13.95 (0.44)	20.01 (0.33)	11.99 (0.10)	10.78 (0.25)^{a}	4.52 (0.12)	7.15 (0.37)	16.43 (2.89)
Mcr	7.97 (0.48)^{b}	8.35 (0.03)^{ab}	12.68 (0.23)	19.57 (0.20)	12.52 (0.30)	10.19 (0.16)^{a}	4.49 (0.16)	7.84 (0.35)	16.39 (4.54)
Extrudate									
Ctrl	7.94 N/A	6.09 N/A	11.57 N/A	21.56 N/A	13.54 N/A	8.75 N/A	5.36 N/A	8.75 N/A	16.45 N/A
Untr	1.46 (0.08)	0.00 (0.00)	22.78 (2.81)^{ab}	19.99 (1.17)	17.02 (3.53)^{ab}	17.89 (2.32)^{a}	5.23 (0.06)	10.59 (0.57)	5.04 (0.12)
Grm	3.13 (0.72)	5.47 (4.03)	21.08 (5.43)^{ab}	14.97 (7.04)	16.09 (11.59)^{a}	14.97 (2.84)^{ab}	8.05 (3.05)	11.89 (3.67)	4.36 (3.01)
Rst	2.39 (1.13)	2.03 (2.87)	26.63 (3.66)^{a}	17.52 (1.04)	15.61 (0.77)^{a}	11.58 (3.48)^{ab}	8.84 (2.56)	10.73 (1.55)	4.68 (1.12)
Mcr	3.39 (0.87)	3.37 (1.46)	16.29 (2.10)^{b}	18.12 (1.53)	25.17 (1.91)^{b}	8.67 (1.42)^{b}	5.60 (0.36)	14.87 (4.53)	4.50 (3.10)

Ctrl, control; Untr, untreated; Rst, roasted; Mcr, micronized; SC+AGG, Sidechains and aggregates (1 or 2); I-β sheet, Intermolecular β sheet; RC, Random coil; β-T, β turn; AP-β, Anti-parallel β sheet; Significant differences in means ($P < 0.05$) are indicated by different letters within each column for a given product; Standard deviation in brackets

5.4.5: *Colour*

Breads produced dark products, with brightness (L*) values ranging from 74.65 to 79.39 (Table 5.4). Compared to their flour mixtures, L* decreased by 0.64–17.07%, the greatest decrease in L* observed across the products. Breads made from untreated, germinated, and micronized chickpeas were significantly darker than the wheat control, with germinated samples being the darkest ($P < 0.05$). Roasted chickpeas produced the lightest breads among pre-treatments. Redness (a*) values ranged from 3.97 to 5.33, with no significant differences between treatments or the control, though the intensity of a* increased (514.9–1255.0%) relative to their raw flours. Yellowness (b*) increased by 20.1–68.4%, ranging from 17.31 to 19.69, with only the roasted chickpea bread significantly more yellow than the control. Previous evaluation of crumb and crust colour in gluten-free breads containing 30% whole chickpea flour—whether derived from modern (Kabuli/Desi), white, or red varieties—showed that chickpea inclusion generally produced darker, redder, and more yellow breads. Notably, red chickpea varieties led to the darkest crumbs and crusts, with crumbs also appearing redder and less yellow than those made with white or modern chickpeas (Parenti et al., 2024). Similarly, a 15% inclusion of whole or germinated chickpea flour decreased L* and increased a* of the breads crust relative to a refined hard wheat control (Guardado-Félix et al., 2020). Progressive addition of germinated chickpea flour (5–20%) into wheat bread has also demonstrated a linear decrease in L*, and linear increase in a* and b* whether in crumb or crust (Atudorei et al., 2022). When breads are made entirely from raw, germinated, toasted (dry-roasted), or cooked chickpeas, germination persists over other treatments, yielding darker and redder breads (Ouazib et al., 2016). These authors did not identify differences in L* between toasted and untreated flours in this study, which may suggest that the moisture conditioning used during roasting influenced brightness.

This aligns with findings by Kaewtapee et al. (2017), where wet-heated full-fat soybeans retained higher L*, a*, and reactive lysine values, while dry heating led to progressive darkening, increased redness, and reduced lysine availability.

Pastas produced light products, with L* ranging from 80.54 to 88.07, although all chickpea treatments were significantly darker than the control. Untreated and germinated chickpeas produced darker pastas than roasted and micronized ones. The control pasta was the only product that became relatively lighter than its flour mixture (+3.85%). Values for a* ranged from 0.94 to 5.40, with untreated and germinated samples being significantly redder than the others, and the control being significantly less red than all chickpea treatments. Increases in a* were greatest in untreated (112.3%) and germinated (277.4%) pastas. All chickpea pastas were more yellow (b* = 22.11 to 25.82) than the control, with roasted chickpeas producing the most yellow product, followed by untreated, germinated, and micronized treatments. Similarly, pasta produced using whole chickpea flour was reported to be darker, redder, and more yellow than the semolina control (Handayani et al., 2019). Upon cooking, the authors observed that the chickpea pasta became lighter than the control, but it lost its redness and yellowness. The use of whole chickpeas may influence these shifts, as seed coat pigments and associated compounds could behave differently during heating compared to dehulled chickpea flour. Differences in the semolina itself—such as variation among durum wheat cultivars—may also contribute to baseline colour differences between studies (Troccoli et al., 2025). Additional work with pulses—including green peas, yellow peas, lentils, and chickpeas—demonstrated that incremental addition of pulse flour (5%, 10%, 15%, 20%, 30%) to semolina resulted in progressively darker and redder uncooked pasta compared to a semolina control (Zhao et al., 2005). For chickpea pasta specifically, yellowness decreased only at the highest inclusion level.

The inclusion of chickpea at 10%, 20%, and 30% into a gluten-free formulation with rice flour resulted in darker and redder pasta with increasing inclusion levels (Bouasla et al., 2017). However, due to the rice-based formulation, control pasta was initially darker, greener, and more yellow. Notably, colour measurements were taken on hydrated, rather than traditionally cooked, pasta. In this state, each chickpea formulation, along with the control, was equally bright and green, but became increasingly yellow as the chickpea content increased.

Extrudate L* ranged from 78.31 to 83.43, with untreated chickpeas yielding the lightest product. Germinated extrudates were the darkest and differed significantly from roasted samples. Redness (a* = 2.26–4.37) was lowest in untreated samples and highest in germinated extrudates, with the latter increasing by 143.65% from its flour base. Yellowness (b* = 26.58–30.73) increased in all extrudates by 3.80–32.26%, but unlike breads and pastas, extrudates from untreated chickpeas were significantly less yellow than those made from germinated, micronized, or roasted flours. Previous research on extruded dry-milled corn and chickpea grit under similar processing conditions reported lighter products with increasing chickpea inclusion levels (25%, 50%, 75%), but a darker appearance at 100% chickpea grit (Shevkani, Singh, Rattan, et al., 2019). Redness (a*) and yellowness (b*) appeared primarily influenced by corn grit, though both values were lowest in the 50:50 blend. While the corn extrudate in this research was produced under modified extrusion conditions, a similar reduction in redness (a*) and yellowness (b*) was observed relative to the untreated chickpea extrudate, suggesting that the chickpea pre-treatments impart distinct effects on colour attributes. Despite slight differences in processing parameters, findings from extrudates composed primarily of sorghum with a smaller proportion of Kabuli chickpea—specifically, 80% and 20%, respectively—indicate that incremental adjustments to feed moisture (14%, 16%, 18%) and barrel temperature (130°C,

145°C, 170°C) can lead to clear, linear decreases in L*, a*, and b* values, particularly at higher temperatures across all moisture levels (J. Kaur et al., 2023).

The darkening effect observed in germinated chickpeas and all associated products may be attributed to the enzymatic breakdown of proteins, sugars, and polyphenols during sprouting, with outcomes influenced by germination conditions (Mencin et al., 2023; Navarro et al., 2024; Olaerts et al., 2018). Although phytase may release bound phenolics and potentially alter MRPs, phytate content remained unchanged between untreated and germinated flours in our study. However, total polyphenols increased by more than 40%, indicating that enzymatic changes during germination may still influence product colour outcomes.

Table 5.4. Colour measurements for pre-treated chickpea products

	L*	%D	a*	%D	b*	%D
Bread						
Control	79.39 (0.88)[a]	-12.6	3.97 (0.30)	849.2	17.31 (0.35)[a]	68.4
Untreated	76.38 (0.44)[b]	-15.2	5.12 (0.20)	514.9	18.57 (0.14)[ab]	20.1
Germinated	74.65 (1.56)[c]	-17.1	5.33 (0.43)	1255.0	18.16 (0.41)[ab]	33.8
Roasted	78.16 (0.26)[ad]	-12.8	4.35 (0.10)	603.2	19.69 (0.68)[b]	25.5
Micronized	77.52 (0.15)[bd]	-13.2	4.44 (0.00)	575.7	18.81 (0.15)[ab]	22.5
Pasta						
Control	88.07 (0.49)[a]	3.9	0.94 (0.16)[a]	-59.6	22.11 (0.07)[a]	-32.7
Untreated	80.54 (0.06)[b]	-7.3	5.40 (0.00)[b]	112.3	25.82 (0.54)[b]	-4.7
Germinated	80.56 (0.07)[b]	-7.2	4.79 (0.13)[b]	277.4	24.78 (0.30)[c]	3.9
Roasted	84.84 (0.16)[c]	-1.0	2.51 (0.02)[c]	19.9	25.21 (0.07)[bc]	-10.1
Micronized	83.71 (0.55)[d]	-0.6	2.63 (0.16)[d]	18.1	24.63 (0.11)[c]	-13.7
Extrudate						
Control	83.43 N/A	2.3	2.91 N/A	-60.4	29.48 N/A	-24.6
Untreated	83.20 (1.44)[a]	-4.5	2.26 (0.51)[b]	-24.3	26.58 (1.11)[b]	3.8
Germinated	78.31 (1.35)[c]	-10.0	4.37 (0.54)[a]	143.7	28.98 (0.35)[a]	32.3
Roasted	80.94 (0.01)[bc]	-5.8	3.67 (0.22)[ab]	50.2	30.73 (0.19)[a]	17.8
Micronized	79.09 (0.14)[bc]	-5.8	4.25 (0.05)[a]	55.5	28.90 (0.31)[a]	5.2

%D, Difference from raw ingredient or mixture; L*, darkness (0) to brightness (+100); a*, greenness (−60) to redness (+60); b*, blueness (−60) to yellowness (+60); Significant differences in means ($P < 0.05$) are indicated by different letters within each column for a given product; Standard deviation in brackets

5.4.6: *Protein content, amino acid composition and scores*

The protein content of chickpea flours across pre-treatments ranged from 19.00% to 20.69%, with the highest observed in germinated chickpeas, followed by micronized chickpeas, and similar protein levels in roasted and untreated samples (Table 5.12). The AAS ranged from 0.93 to 0.99, limited by tryptophan, with germinated chickpeas exhibiting the lowest values (Table 5.13). The other three treatments showed higher AAS values, which are consistent with untreated chickpeas reported in our previous work (Guldiken et al., 2022) and by other authors (Bekele et al., 2021). The lower AAS of germinated chickpeas is likely attributable to the increase in protein content, which dilutes the relative ratio of limiting amino acids to total protein. This increase in protein concentration has been previously observed in chickpea sprouting (Guardado-Félix et al., 2020), which may be attributed to carbohydrate consumption during germination. Previous research has demonstrated that roasting chickpeas can significantly reduce lysine and tryptophan content while increasing the levels of other amino acids relative to untreated samples (Xu et al., 2016), an effect likely associated with the much higher roasting temperatures (400°C) employed.

Each bread product contained significantly more protein content than the control bread, ranging from 16.53% to 18.69% (Table 5.5). Relative to their raw flours, the chickpea pre-treatments increased in protein content by 13.3 to 16.0%, whereas the control bread increased by 2.1%. Lysine remained the limiting amino acid across all bread products, ranging from 24.28 to 32.78 mg lysine/g protein, corresponding to AAS values of 0.42 to 0.57 (Table 5.3). The content of lysine decreased across all chickpea breads relative to their raw flour mixture by 8.6% to 28.2%, whereas the control observed a marked increase of 7.9%. Each chickpea bread exhibited a significantly greater lysine content, as reflected by higher AAS values, compared to the control

bread. No significant differences in protein content or AAS were observed among the chickpea pre-treatment breads. Incorporating whole or germinated chickpeas into bread at 15% has been shown to significantly improve both total protein and lysine content compared to a refined hard wheat control (Guardado-Félix et al., 2020). Higher inclusion levels of 20% and 40% have also reliably resulted in significantly greater total protein contents, supporting their use as a form of wheat bread fortification (Utrilla-Coello et al., 2007). Notably, chickpea pre-treatments yielded total protein levels comparable to those of breads fortified with 10–15% salmon fish powder and exceeded their lysine amino acid scores across all inclusion rates (Desai et al., 2018).

Pasta products exhibited a narrow range of protein contents, from 19.53% to 21.22%, and were similarly limited in lysine content, with AAS ranging from 0.36 to 0.73. The protein content across all pastas increased from 13.2% to 16.4%, whereas the lysine content decreased by 6.2% to 16.1% relative to their raw flour mixture. Incorporation of chickpea pre-treatments significantly increased the lysine content, nearly doubling the AAS compared to the control. However, neither total protein nor lysine content significantly differed among the chickpea-fortified pasta products, except for the untreated chickpea pasta, which did not differ in protein content relative to the control pasta. The inclusion of as little as 10% chickpea flour has been shown to significantly increase the protein content relative to semolina pasta (Schettino et al., 2019; Wood, 2009). Furthermore, fortification with up to 30% desi chickpea resulted in lysine contents comparable to those observed in the present study (Bayomy & Alamri, 2022; Wood, 2009). Notably, this inclusion rate is lower than that used here, as Kabuli chickpeas typically contain less total protein than Desi varieties (Wood et al., 2011).

The protein content in the extrudates ranged from 7.88% to 16.16%, with pre-treated chickpea extrudates exhibiting an increase in protein content of 5.3% to 15.9% relative to their

raw flour, while the control increased by 21.2%. The control extrudate contained less than half the protein content of the chickpea extrudates. Although slight decreases in protein content were observed among the pre-treatments relative to the untreated chickpea, these differences were not significant. Higher AAS were achieved in the chickpea extrudates, with the limiting amino acid shifting from 0.23 for lysine in the corn control to tryptophan—0.83 to 0.86—in the chickpea products. The markedly low lysine AAS in the corn extrudate—nearly half the value reported for extruded whole maize (Wang et al., 2020)—may reflect the specific extrusion parameters used in this study, particularly since the raw maize flours had similar protein and lysine levels. Relative to their raw flour mixtures, tryptophan content decreased by 8.1 to 12.5% in the chickpea extrudates, whereas the control decreased in lysine by 47.8%. Wang et al. (2020) also evaluated extruded whole Kabuli chickpeas and reported similar AAS values (0.84–0.87), although valine was identified as the limiting amino acid. Other studies have demonstrated that lower-moisture extrusion of Kabuli chickpeas can yield even higher AAS values (up to 0.97), which are influenced by varietal and growing conditions (Nosworthy et al., 2020). Several studies have demonstrated that fortifying corn extrudates with chickpea improves both protein and amino acid composition (Milán-Carrillo et al., 2007; Serrano-Sandoval et al., 2022; Shah et al., 2022). In addition to improved nutrient profiles—including fiber, iron, zinc, and vitamins A and C—these formulations also exhibited changes in amino acid composition. Notably, Shah et al. (2022) reported significant reductions in cysteine across all extrudates compared to their raw blends, while tryptophan decreased the least. Although protein quality indices were not calculated, these findings underscore the importance of indispensable amino acids relative to human requirements. Despite being less affected by processing, tryptophan remained the most limiting amino acid in the chickpea–corn extrudates examined in this study.

Table 5.5. Protein (N x 6.25) and amino acid composition (mg AA/g protein) of pre-treated chickpea products

	Protein	%D	Thr	%D	Val	%D	M+C	%D	Ile	%D	Leu	%D	P+T	%D	His	%D	Lys	%D	Trp	%D
Bread																				
Ctrl	16.53(0.22)[b]	2.1	26.27(0.07)	12.4	38.37(0.28)	5.6	32.56(0.80)	4.6	35.18(0.47)	5.9	66.00(0.01)	3.9	75.74(0.17)	0.02	22.60(2.62)	-0.6	**24.28(0.89)**	7.9	10.28(0.17)	-3.7
Untr	18.63(0.18)[a]	16.0	24.39(0.19)	-30.5	37.45(0.47)	-17.3	32.73(0.10)	0.6	35.13(0.71)	-21.0	66.07(0.35)	-9.8	63.10(0.50)	-40.7	24.32(0.44)	-8.2	**31.26(0.42)**	-24.8	10.86(0.15)	-2.2
Grm	18.69(0.00)[a]	13.3	24.56(0.21)	-24.8	38.06(0.42)	-13.1	32.77(0.34)	2.1	35.01(0.48)	-17.1	65.97(1.30)	-7.1	63.31(1.85)	-37.7	23.30(0.88)	-10.9	**30.78(0.40)**	-22.8	10.60(0.16)	-1.1
Rst	18.44(0.09)[a]	13.9	24.43(0.65)	-42.0	37.79(0.68)	-26.6	33.56(1.21)	3.7	35.66(0.12)	-29.2	66.13(0.85)	-16.2	63.07(0.85)	-55.2	24.70(0.91)	-8.4	**32.78(0.64)**	-28.3	10.86(0.13)	-0.4
Mcr	18.56(0.18)[a]	13.4	24.74(0.22)	-26.5	37.94(0.58)	-13.4	33.11(0.02)	6.8	35.03(0.42)	-16.9	66.45(0.90)	-6.5	62.99(2.26)	-37.7	23.70(0.66)	-7.2	**32.31(0.16)**	-18.6	10.59(0.07)	-1.6
Pasta																				
Ctrl	19.53(0.40)[b]	14.9	23.90(3.24)	-3.2	39.16(5.09)	-3.2	34.71(0.38)	-0.6	35.05(4.55)	-3.6	69.33(8.89)	-3.1	71.38(7.77)	-5.1	21.56(3.23)	-18.7	**21.00(2.60)**	-6.2	9.10(0.13)	-1.9
Untr	20.66(0.04)[ab]	15.2	29.21(0.15)	-5.6	41.27(0.08)	-4.9	30.20(0.38)	-10.0	38.81(0.04)	-4.0	71.62(0.19)	-5.5	77.87(0.53)	-6.2	25.48(0.70)	1.8	**40.46(0.24)**	-15.2	9.67(0.36)	-9.7
Grm	21.16(0.04)[a]	13.6	29.25(0.10)	-5.3	41.67(0.01)	-3.9	30.23(0.33)	-3.2	39.47(0.28)	-3.2	72.64(0.15)	-2.3	78.71(0.25)	-4.3	24.73(0.38)	0.2	**41.24(0.36)**	-16.1	9.49(0.51)	-9.1
Rst	21.03(0.04)[a]	16.4	28.97(0.31)	-6.7	41.24(0.06)	-4.6	28.70(2.28)	-10.3	38.48(0.32)	-5.8	71.45(0.29)	-4.5	76.48(0.36)	-6.6	24.33(1.03)	-3.6	**42.42(0.06)**	-10.2	9.75(0.46)	-5.5
Mcr	21.22(0.40)[a]	13.2	30.46(0.18)	-0.4	41.99(0.25)	-1.8	30.45(0.57)	-0.1	39.58(0.03)	-1.2	70.80(0.20)	-4.5	77.41(0.05)	-4.1	24.61(0.05)	-0.3	**40.70(0.13)**	-14.1	10.02(0.08)	-1.3
Extrudate																				
Ctrl	7.88N/A	21.2	27.54N/A	-19.7	40.09N/A	-12.3	35.65N/A	-23.6	30.41N/A	-18.1	124.78N/A	-11.2	74.54N/A	-21.5	33.18N/A	77.0	**13.48**N/A	-47.8	5.82N/A	19.2
Untr	16.16(1.11)	15.9	33.46(0.67)	-24.0	42.93(1.05)	-17.9	27.27(2.31)	-14.7	40.03(0.77)	-21.0	82.51(1.75)	-11.5	81.53(0.26)	-33.6	29.01(1.11)	0.5	55.64(0.76)	-11.8	**9.27(0.78)**	-11.3
Grm	15.69(0.00)	9.6	33.52(0.15)	-35.0	42.77(0.18)	-27.8	25.97(1.13)	-9.1	40.30(0.40)	-31.3	83.58(0.18)	-16.6	80.23(1.14)	-46.8	26.40(1.44)	-0.2	52.83(2.71)	-22.6	**9.47(0.14)**	-12.0
Rst	15.22(0.57)	7.3	34.22(1.58)	-17.5	43.68(1.89)	-12.9	28.01(0.31)	-4.1	40.89(1.34)	-18.1	85.08(3.39)	-5.9	81.94(1.83)	-27.2	28.84(1.77)	2.1	56.19(2.21)	-11.1	**9.34(0.02)**	-8.1
Mcr	15.53(0.04)	5.3	33.36(0.70)	-18.9	42.35(0.80)	-16.4	23.85(0.41)	-18.6	39.88(0.39)	-19.3	82.56(1.37)	-9.9	79.63(0.05)	-27.9	25.44(1.89)	-5.3	51.50(1.33)	-22.3	**9.12(0.27)**	-12.5
Reference Pattern (FAO/WHO 1991)																				
			34		35		25		28		66		63		19		58		11	

Ctrl, control; Untr, untreated; Rst, roasted; Mcr, micronized; %D, Difference from raw ingredient or mixture; Thr, Threonine; Val, Valine; M+C, Methionine & Cystine; Ile, Isoleucine; Leu, Leucine; P+T, Phenylalanine & Tyrosine; His, Histidine; Lys, Lysine; Trp, Tryptophan; Limiting amino indicated in bold; Significant differences in mean protein content ($P < 0.05$) are indicated by different letters within each column for a given product; Standard deviation in brackets

5.4.7: *In vitro protein digestibility*

True protein digestibility was evaluated using two *in vitro* methods: the pH drop method (pIVPD) and gastro-intestinal digestion (gIVPD). Across all chickpea-derived products and their respective controls, pIVPD values ranged from 90.93 to 93.70%, while gIVPD values ranged from 81.05 to 87.58% (Table 5.6). Casein, used as an inter-day reference protein (Table 5.13), yielded protein digestibility values of 98.10% (pIVPD) and 90.34% (gIVPD). Although casein generally achieves 100% true protein digestibility *in vivo*, the lower *in vitro* value observed with gIVPD reflects a potential method-specific constraint. To highlight protein-specific differences between the two methods, additional reference proteins were evaluated. Soy protein isolate achieved 95.33% digestibility by pIVPD, but only 79.50% digestibility with gIVPD. Gelatin achieved 94.31% and 92.60%, respectively, with a notably higher protein digestibility than casein by gIVPD. Protein digestibility values were not truncated to 100%, both to allow transparent comparisons across products and treatments, and to reflect the divergence observed among highly digestible proteins.

Among bread products, higher protein digestibility ($P < 0.05$) was observed in the control compared to the untreated and germinated chickpea breads, as measured by pIVPD, with no such differences detected using gIVPD. When comparing finished breads to their raw flour mixtures, both *in vitro* methods generally indicated reduced protein digestibility. Values measured by pIVPD declined between 0.4 and 1.7%, and gIVPD values ranged from 0.7 to 2.9%. Roasted chickpea bread under pIVPD was the exception, exhibiting a small relative increase of 0.6%. Fortified breads containing 15% whole chickpea flour have previously demonstrated protein digestibility (as assessed by pIVPD) comparable to wheat bread (Guardado-Félix et al., 2020). In contrast, breads with 15% inclusion of whole germinated chickpeas exhibited a significant

decrease in protein digestibility. In gluten-free bread formulations composed solely of raw, germinated, toasted, or cooked chickpeas, only cooking resulted in a significant increase in protein digestibility compared to the other pre-treatments (Ouazib et al., 2016). Additional work on gluten-free breads formulated from rice flour with 25% chickpea inclusion—using raw, roasted, or dehulled chickpeas—showed that roasted chickpeas produced higher protein digestibility than rice flour alone (Kahraman et al., 2022). Independent assessments of dry- and wet-roasted chickpeas have also demonstrated improvements in IVPD (Xu et al., 2016). Taken together with our findings, these results suggest that water tempering (via cooking or roasting), when combined with thermal treatment, is necessary to enhance protein digestibility in chickpea-based breads. Although statistically significant, improvements in protein digestibility are generally modest—typically around a 2% absolute increase. Fermentation may also contribute to increased protein digestibility, though its effects appear relatively consistent across different pre-treatments. In peas fermented with *L. plantarum*, for example, protein digestibility increased over time despite concurrent rises in phenolics and tannins. These improvements were attributed to reductions in chymotrypsin and trypsin inhibitors (Çabuk et al., 2018)—which may particularly influence pIVPD in raw pulses (Ene-Obong, 1995)—but are likely less impactful when used alongside, or following, thermal processing. Similarly, black chickpeas subjected to spontaneous or *L. plantarum* fermentation exhibited improved protein digestibility following *in vitro* gastric–intestinal digestion, which coincided with decreases in raffinose, condensed tannins, TIA, saponins, and free phenolics (De Pasquale et al., 2021). Whether these fermentation-driven improvements carry over to end products like bread remains uncertain. In Ouazib et al. (2016), breads made from raw chickpea flour and fermented with baker's yeast showed protein digestibility comparable to raw chickpeas, suggesting limited benefit from

fermentation alone. In our study, all breads exhibited small decreases in protein digestibility despite substantial reductions in ANFs. Notably, colorimetric indicators of MRPs were significantly associated with protein digestibility; pIVPD was negatively correlated with a* and positively correlated with L*, while gIVPD showed a positive correlation with b* (Table 5.7). While few studies have examined the impact of chickpea fortification in wheat breads on protein digestibility, it is also important to consider that differences among wheat cultivars may contribute to variability in IVPD observed in bread products (Lavoignat et al., 2022). Moreover, the inclusion of wheat or wheat gluten itself imposes an upper limit on protein digestibility, as a portion of gluten proteins will remain resistant to enzymatic breakdown, limiting the ability to maximize protein digestibility (Guillin et al., 2020; Laleg et al., 2019).

Pasta samples also revealed differences depending on the *in vitro* method. Both the control and roasted chickpea pasta exhibited higher ($P < 0.05$) protein digestibility by pIVPD compared to pastas prepared from untreated and germinated chickpeas. In contrast, gIVPD identified roasted chickpea pasta as greater ($P < 0.05$) than the control. Relative to their raw flour mixtures, gIVPD consistently decreased across treatments (0.4 to 3.1%), whereas pIVPD mainly increased (0.1 to 4.6%), except for micronized chickpea pasta, which showed a slight reduction (0.7%). Reports assessing true protein digestibility of finished pasta products using *in vivo* methods are rare, although semolina pasta has been evaluated at 84.51 ± 3.88% (Torres et al., 2006)—similar to our reports for protein digestibility as assessed by gIVPD, but less than pIVPD. In the same study, fortification with 10% fermented pigeon pea significantly increased protein digestibility to 89.62 ± 0.69%, indicating that pulse fortification, combined with pre-treatment through fermentation can enhance IVPD in pasta. This is further supported by findings from a separate *in vivo* study, in which semolina pasta fortified with 35% faba bean and 6%

gluten powder achieved a true protein digestibility of 98.3%, highlighting the potential of pulse flours to improve the nutritional quality of pasta products substantially (Laleg et al., 2019). The level of inclusion, though, may also play a critical role. El-Sohaimy et al. (2020) reported an incremental increase in protein digestibility with increasing levels of chickpea flour inclusion at 2.5%, 5%, and 7.5%, using a modified pIVPD approach. At a 10% inclusion rate, a sharp decrease in protein digestibility was observed, resulting in pasta that was less digestible than the semolina control. The authors attributed this reduction to potential increases in covalent protein crosslinking, driven by the higher protein content at elevated levels of chickpea flour. Additional insights were gained from pastas made with chickpea protein isolates, which demonstrated similar protein digestibility regardless of the inclusion rate. Notably, ANFs were not evaluated in the study, yet the use of protein isolates may contribute to ANF content and influence protein digestibility within a pasta matrix (Manzanilla-Valdez et al., 2024). In contrast, semolina pasta fortified with a greater inclusion level—15% black chickpea—and assessed using an *in vitro* gastro-intestinal model, showed no reduction in protein digestibility relative to a semolina control (De Pasquale et al., 2021). The inclusion of an equal amount of fermented black chickpea flour was also observed to increase protein digestibility further.

In extruded products, no differences in protein digestibility were identified between treatments using pIVPD. The gIVPD method, however, detected a higher value ($P < 0.05$) in roasted chickpea extrudates relative to germinated chickpeas. All extruded formulations demonstrated increased protein digestibility compared to their raw mixtures, whether measured by pIVPD or gIVPD, apart from micronized chickpea evaluated with pIVPD, which experienced a minor decline (0.1%). Improvements in protein digestibility among chickpea pre-treatments ranged from 1.9 to 7.3% for pIVPD and from 1.6 to 5.9% for gIVPD—in addition to increases in

the control extrudates of 6.4% and 5.9%, respectively. The pIVPD methods have previously been employed and compared to whole extruded chickpeas (composite samples) using *in vivo* true protein digestibility assessment, reporting values of 87.1 ± 0.09% *in vitro* and 86.6 ± 1.0% *in vivo* (Nosworthy et al., 2020). These values are somewhat lower than those observed in our study, which may reflect differences in the regression equations used to calculate pIVPD. Germinated and dehulled chickpea blends, incorporated at 10%, 20%, and 30% inclusion levels, all demonstrated significantly greater protein digestibility compared to a corn control (Hegazy et al., 2017). These increases were consistent with improved protein digestibility relative to raw flours, when assessed by pIVPD. Although the study employed a method adjacent to an *in vitro* gastro-intestinal digest, the highest inclusion level yielded the greatest protein digestibility. Still, it not significantly different from the lower inclusion levels. A linear increase in protein digestibility—assessed by pIVPD—with increasing chickpea inclusion was observed in maize extrudates containing 10%, 20%, 30%, and 40% germinated Kabuli chickpea flour (Serrano-Sandoval et al., 2022). Protein digestibility was lowest in maize grits, and values at 20–40% inclusion were not significantly different from those observed in 100% chickpea extrudates. Although dehulling was not indicated in this study or used in the study assessed *in vivo*, baseline improvements in IVPD are likely influenced by the inclusion of a dehulling step. Dehulling alone has been shown to significantly improve protein digestibility relative to both whole and germinated whole chickpeas (Ghavidel & Prakash, 2007). Our previous work, however, found no additional effect of pre-treatments applied prior to roller milling—specifically scouring and moisture conditioning—on pIVPD in Kabuli chickpeas (Franczyk et al., 2025), suggesting that once dehulled, further refinements to the process may not enhance protein digestibility. Chickpea inclusion up to 80% may represent an optimal threshold for protein digestibility and quality,

based on findings from extruded blends of nixtamalized maize flour and chickpea flour formulated for infant nutrition (Milán-Carrillo et al., 2007). In that study, the combined extrudate exhibited greater protein digestibility than either product alone, suggesting that this ratio may maximize nutritional benefit. The inclusion of alkaline treatment associated with nixtamalization, however, may introduce lysinoalanine, which decreases both protein digestibility and amino acid availability (Gilani et al., 2012). Extrusion parameters—particularly in the lower moisture range of 14–20%—should also be emphasized as potential sources of variability in chickpea-based products. Differences in moisture content have been associated with absolute differences in protein digestibility of up to 10%, as observed in chickpea–barley and sorghum–chickpea extrudates (Guldiken et al., 2020; J. Kaur et al., 2023).

The impact of ANFs, MRPs and protein secondary structure offers some explanation through correlation (Table 5.7) on the outcome of protein digestibility across all products. Forgoing the impacts of colour, both methods harmonized on polyphenols, with a negative association observed in both pIVPD ($P < 0.05$) and gIVPD ($P < 0.01$) assessments. Strangely, TIA did not affect pIVPD—where the influence of TIA may be expected—and gIVPD observed a positive association ($P < 0.5$). The content of phytate from products was also positively associated with gIVPD ($P < 0.5$). A previous association with pIVPD and phytate in Kabuli chickpeas, including raw and processed samples (with moisture and thermal treatments), revealed a significantly negative correlation (Xu et al., 2016). Importantly, although products, such as extrusion, elicit decreases in phytate (Abd El-Hady & Habiba, 2003), across all products, an important consideration may be the form of phytate reduced (e.g. from Inositol-6 phosphate to Inositol-5 phosphate), as total reduction in phytate without contrasting form (Nikmaram et al., 2017), may not fully elucidate changes in protein digestibility, as IP6 will more strongly reduce

protein digestibility than IP5 (Knuckles et al., 1989). This may further be observed in studies where increases in phytate or total phenolic content did not always infer improvements in protein digestibility, following the inclusion of cookies formulated with apple, lemon, wheat or wheat bran fibre, i.e. phytate content may decrease, but protein digestibility also decreased in wheat, but from wheat bran, phytic acid increased and total phenolics increased and protein digestibility decreased (Bilgiçli et al., 2007). Additional fibre from different sources, such as apple, lemon, wheat and wheat bran, is a more reliable way of reducing protein digestibility than changes in phytate or polyphenolic compounds across the fibre types, which is an important consideration for the food matrix, where our scope focused on chickpeas. Protein secondary structure elements identified no associations in pIVPD, whereas previous research had identified this relationship using a modified pIVPD method, with changes in pH after 20 minutes relative to 10 (Carbonaro et al., 2012). Additionally, this discrepancy may be attributed to differences in the selection of proteins evaluated and their treatments. Specific structures, demonstrated negative associations with gIVPD ($P < 0.0001$), including intermolecular β sheets (1620–1630 cm-1), side chains and protein aggregates (1610-1615 and 1690-1695 cm-1), whereas positive associations in gIVPD ($P < 0.0001$) with β sheets (1630-1638 cm-1) —where a negative association may be expected— and random coil (1640-1648 cm-1) are also observed.

Table 5.6. Protein digestibility, amino acid scores and protein quality of pre-treated chickpea products

	pIVPD	%D	gIVPD	%D	AAS	pIVPDCAAS	%D	gIVPDCAAS	%D
Bread									
Control	92.79 (1.22)[b]	-1.7	81.20 (0.99)	-2.6	0.42 (0.02)[b]	38.86 (1.94)[c]	6.1	34.00 (1.67)[c]	5.1
Untreated	91.11 (0.00)[a]	-0.7	82.02 (2.16)	-1.7	0.54 (0.01)[a]	49.11 (0.66)[ab]	-25.3	44.21 (0.57)[ab]	-26.1
Germinated	90.93 (0.25)[a]	-0.6	81.85 (0.01)	-1.5	0.53 (0.01)[a]	48.26 (0.76)[b]	-23.4	43.44 (0.56)[b]	-23.9
Roasted	92.12 (0.58)[ab]	0.6	83.36 (1.04)	-0.7	0.57 (0.01)[a]	52.06 (1.34)[a]	-27.8	47.11 (1.51)[a]	-28.7
Micronized	91.72 (0.36)[ab]	-0.4	81.73 (1.33)	-2.9	0.56 (0.00)[a]	51.09 (0.46)[ab]	-19.0	45.53 (0.52)[ab]	-21.0
Pasta									
Control	93.70 (0.00)[b]	0.1	81.05 (0.28)[b]	-3.1	0.36 (0.04)[b]	33.92 (4.20)[b]	-6.1	29.34 (3.53)[b]	-9.1
Untreated	91.97 (0.15)[a]	4.6	82.96 (0.83)[ab]	-0.7	0.70 (0.00)[a]	64.16 (0.28)[a]	-11.3	57.87 (0.92)[a]	-15.8
Germinated	91.08 (0.11)[a]	3.4	82.96 (0.69)[ab]	-0.4	0.71 (0.01)[a]	64.77 (0.64)[a]	-16.7	58.99 (0.03)[a]	-16.5
Roasted	92.40 (0.18)[ab]	0.7	84.35 (0.11)[a]	-0.4	0.73 (0.00)[a]	67.58 (0.23)[a]	-5.8	61.69 (0.18)[a]	-10.6
Micronized	92.07 (0.29)[a]	-0.7	83.33 (0.42)[ab]	-1.0	0.70 (0.00)[a]	64.61 (0.01)[a]	-14.7	58.48 (0.11)[a]	-15.0
Extrudate									
Control	91.21 N/A	6.4	86.07 N/A	5.9	0.23 (0.00)	21.20 N/A	-44.3	20.01 N/A	-44.5
Untreated	92.33 (0.07)	6.8	85.82 (0.07)[ab]	2.2	0.84 (0.07)	77.85 (6.64)	-5.2	72.35 (6.05)	-9.3
Germinated	92.00 (0.18)	7.3	84.86 (0.15)[a]	1.6	0.86 (0.01)	79.19 (0.98)	-10.3	73.03 (0.92)	-10.6
Roasted	91.92 (0.36)	1.9	87.58 (1.12)[b]	5.3	0.85 (0.00)	78.02 (0.51)	-1.5	74.34 (1.14)	-3.3
Micronized	92.02 (0.07)	-0.1	86.35 (1.20)[ab]	4.8	0.83 (0.02)	76.29 (2.20)	-12.6	71.57 (1.13)	-8.3

pIVPD, pH drop *in vitro* protein digestibility; gIVPD, gastro-intestinal digestion *in vitro* protein digestibility; AAS, Amino acid score; pIVPDCAAS, pH drop *in vitro* protein digestibility corrected amino acid score; gIVPDCAAS, gastro-intestinal digest *in vitro* protein digestibility corrected amino acid score; %D, Difference from raw ingredient or mixture; Significant differences in means ($P < 0.05$) are indicated by different letters within each column for a given product; Standard deviation in brackets

Table 5.7. Correlations across all pre-treated chickpea products for IVPD and IVPDCAAS with intrinsic and extrinsic factors

	pIVPD		gIVPD		pVPDCAAS		gIVPDCAAS	
TIA	-		0.45	*	0.83	****	0.82	****
Polyphenols	-0.49	**	-0.40	*	-		-	
Phytate	-		0.67	****	0.86	****	0.87	****
SC+AGG1	-		-0.72	****	-0.75	****	-0.77	****
I-β sheet	-		-0.68	****	-0.55	**	-0.58	***
β-sheet	-		0.72	****	0.68	****	0.71	****
RC	-		0.43	*	-		-	
α-Helices	-		-		-		-	
β-T1	-		-		-		-	
β-T2	-		-		-		-	
AP-β sheet	-		-		-		-	
SC+AGG2	-		-0.66	****	-0.65	****	-0.67	****
L*	0.66	****	-		-		-	
a*	-0.69	****	-		-		-	
b*	-		0.83	****	0.64	****	0.67	****

pIVPD, pH drop *in vitro* protein digestibility; gIVPD, gastro-intestinal digestion *in vitro* protein digestibility; pIVPDCAAS, pH drop *in vitro* protein digestibility corrected amino acid score; gIVPDCAAS, gastro-intestinal digest *in vitro* protein digestibility corrected amino acid score; TIA, Trypsin inhibitor activity; SC+AGG, Sidechain aggregates; I-β sheet, Intermolecular beta sheet; RC, Random coil; β-T, Beta turn; AP-β, Anti-parallel beta sheet; L*, darkness/brightness; a*, greenness/redness; b*, blueness/yellowness; P values (* = 0.05; ** = 0.01, *** = 0.001, **** = 0.0001)

5.4.8: *In vitro protein digestibility corrected amino acid score & protein content claims*

Protein quality of bread products, as evaluated by pIVPDCAAS, ranged from 38.86 to 52.06, while gIVPDCAAS had a lower range of 34.00 to 47.11 (Table 5.6). In both assays, bread made from roasted chickpeas had significantly higher ($P < 0.05$) protein quality than bread made from germinated chickpeas. All chickpea pre-treatments produced significantly higher ($P < 0.05$) values than the wheat control. Compared to their raw flour mixtures, pIVPDCAAS values for chickpea breads declined by 19.0 to 27.8%, while the wheat control increased by 6.1%. Similarly, gIVPDCAAS values decreased by 21.0 to 28.7% for chickpea breads, with a 5.1% increase observed in the control. Breads prepared with 15% whole chickpea flour demonstrated lower protein quality when germinated chickpea flour was used; however, both germinated and non-germinated chickpea breads achieved higher IVPDCAAS values than refined hard wheat bread (Guardado-Félix et al., 2020). Interestingly, breads fortified with salmon powder—despite including only 5–15%—showed lower pIVPDCAAS, suggesting that pulse fortification may be equally or more effective than animal protein fortification in improving protein quality (Desai et al., 2018). The control bread in our study had a pIVPDCAAS of 39%, lower than the 46% reported by Guardado-Félix et al. (2020), however, both were much higher than the 15% observed by Desai et al. (2018)—likely due to differences in the amino acid scoring reference pattern, wheat cultivar and/or breadmaking methods. Notably, fermentation with *L. plantarum* and pea protein concentrate over 0–11 hours reduced indispensable amino acid content and pIVPDCAAS (Çabuk et al., 2018), suggesting that similar microbial activity from baker's yeast could also lower amino acid availability.

For pasta, pIVPDCAAS values ranged from 33.92 to 67.58, with gIVPDCAAS values also from a lower range of 29.34 to 61.69. All chickpea pre-treatment pastas resulted in significantly greater ($P < 0.05$) protein quality in both assays compared to the semolina control. No significant differences were observed among the chickpea pre-treatments, although the roasted pre-treatment yielded the highest protein quality value. Protein quality declined relative to the raw flour mixtures for all pasta products, ranging from 5.8 to 16.7% for pIVPDCAAS and 9.1 to 16.5% for gIVPDCAAS. Although PDCAAS was not used, semolina pasta fortified with 35% faba bean and 6% gluten achieved a protein efficiency ratio equivalent to that of casein (Laleg et al., 2019). Similarly, fortification with 10% fermented pigeon pea improved protein quality relative to unfortified semolina pasta, though it did not match the quality of casein (Torres et al., 2006).

In extrudates, pIVPDCAAS values ranged from 21.20 to 79.19, while gIVPDCAAS maintained a lower range among the products, from 20.01 to 74.34. Chickpea pre-treatments produced protein quality values more than three times greater than those of the corn control extrudate; however, statistical comparison by ANOVA was not possible. Among chickpea treatments, germinated chickpea extrudates had the highest values in both assays, though the differences were not statistically significant. Compared to their raw flour mixtures, chickpea extrudates had a relative decline in pIVPDCAAS of 1.5 to 12.6% and in gIVPDCAAS of 3.3 to 10.6%. In contrast, the corn control extrudate observed a large relative reduction of 44.3% and 44.5%, respectively, due to the decrease in the limiting amino acid, lysine. Whole extruded chickpeas have demonstrated high protein quality, with pIVPDCAAS values reaching 84.3%, closely aligning with *in vivo* PDCAAS results of 83.8% (Nosworthy et al., 2020). In contrast, corn-based extrudates exhibited lower pIVPDCAAS values (Wang et al., 2020). Adjustments to

extrusion parameters, such as increased shear or temperature, may lead to lower pIVPDCAAS in Kabuli chickpeas, with reported values around 67–71%.

Interestingly, among the ANFs, only TIA and phytate showed associations that carried over from gIVPD to both measures of protein quality (Table 5.7), resulting in significantly higher correlations. In contrast, the negative associations of polyphenols with protein digestibility did not translate to their corresponding measures of protein quality. A similar pattern was observed for most protein secondary structures previously associated with gIVPD, except for the random coil, and with a weakening association observed for intermolecular β -sheets. Regarding colour parameters, neither L* nor a* were associated with pIVPDCAAS or gIVPDCAAS, while b* remained associated with both measures of protein quality but with reduced strength relative to gIVPD.

The highest potential claim (Table 5.8) for any of the evaluated products is "a good source of protein." Both measures of IVPDCAAS provided similar opportunities for establishing content claims across products. Due to the more conservative TPD values estimated from the gIVPD method, each product's claim is scored lower—whether using the Canadian calculation for protein rating (PR) or the percent daily value (%DV) system in the U.S. All chickpea pastas qualify for a claim in both Canada and the U.S., while only chickpea breads qualify in Canada. Chickpea extrudates do not meet the criteria for a claim in either jurisdiction. Although chickpea extrudates had the highest IVPDCAAS and a high protein content, they are constrained by lower serving size standards—Schedule K in Canada and the reference amount customarily consumed (RACC) in the U.S.—compared to the larger reference amounts assigned to bread and pasta. The ready-to-eat designation also affects how moisture content is considered. Bread is evaluated as-is, pasta is evaluated in its dry form (or also hydrated in the US), and extrudates, such as

cereal puffs or extruded snacks, are also measured as-is (i.e., already with low moisture). These moisture-based differences further influence claim eligibility. While IVPD methods are not currently recognized for substantiating official protein content claims in either jurisdiction, selecting an appropriate method can support early-stage assessment of a product's potential prior to *in vivo* validation.

Table 5.8. Estimated protein content claims in Canada and the US for pre-treated chickpea products

	Protein	pIVPDCAAS	gIVPDCAAS	Canada			US					
				Schedule K (g)	pPR	gPR	RACC (g)	CR Protein	pIVPDCAAS CR	gIVPDCAAS CR	p%DV	g%DV
Bread												
Ctrl	0.123	0.389	0.340	150	7.1	6.2	50	6.13	2.38	2.08	4.8	4.2
Untr	0.137	0.491	0.442	150	**10.1**	**9.1**	50	6.85	3.36	3.03	6.7	6.1
Grm	0.132	0.483	0.434	150	**9.6**	**8.6**	50	6.61	3.19	2.87	6.4	5.7
Rst	0.131	0.521	0.471	150	**10.3**	**9.3**	50	6.57	3.42	3.09	6.8	6.2
Mcr	0.128	0.511	0.455	150	**9.8**	**8.7**	50	6.40	3.27	2.91	6.5	5.8
Pasta												
Ctrl	0.195	0.339	0.293	85	5.6	4.9	55	10.74	3.64	3.15	7.3	6.3
Untr	0.207	0.642	0.579	85	**11.3**	**10.2**	55	11.36	7.29	6.57	**14.6**	**13.1**
Grm	0.210	0.648	0.590	85	**11.6**	**10.5**	55	11.57	7.49	6.82	**15.0**	**13.6**
Rst	0.212	0.676	0.617	85	**12.2**	**11.1**	55	11.64	7.86	7.18	**15.7**	**14.4**
Mcr	0.212	0.646	0.585	85	**11.7**	**10.5**	55	11.67	7.54	6.82	**15.1**	**13.6**
Extrudate												
Ctrl	0.079	0.212	0.200	14	0.2	0.2	30	2.36	0.50	0.47	1.0	0.9
Untr	0.162	0.778	0.724	14	1.8	1.6	30	4.85	3.77	3.51	7.5	7.0
Grm	0.152	0.792	0.730	14	1.7	1.6	30	4.57	3.62	3.33	7.2	6.7
Rst	0.157	0.780	0.743	14	1.7	1.6	30	4.71	3.67	3.50	7.3	7.0
Mcr	0.155	0.763	0.716	14	1.7	1.6	30	4.66	3.55	3.33	7.1	6.7

Ctrl, control; Untr, untreated; Rst, roasted; Mcr, micronized; pIVPDCAAS, pH drop *in vitro* protein digestibility corrected amino acid score; gIVPDCAAS, gastro-intestinal digest *in vitro* protein digestibility corrected amino acid score; IVPDCAAS values / 100; RACC, Recommended amount customarily consumed; PR, Protein rating, ≥ 8 good source of protein, ≥ 16 excellent source of protein; pPR, pH drop calculated protein rating; gPR, gastro-intestinal calculated protein rating; %DV, Daily value, ≥ 10% good source of protein, ≥ 20% excellent source of protein; p%DV, pH drop calculated daily value; g%DV, gastro-intestinal calculated daily value; Protein content (N x 6.25) % / 100, bread products modified for ready-to-eat starting moisture; CR, protein corrected RACC; Bolded PR or %DV identify protein content claim meets threshold

5.5: CONCLUSION

This study demonstrates that incorporating chickpea in place of cereal flours significantly improved the protein content and amino acid score of bread, pasta, and extrudates, an outcome congruent with the nutritional profile of pulse flours. Among pre-treatments, roasting yielded the most consistent improvements in protein quality across all products, followed by micronization. Germination was generally less effective due to increased polyphenol content and variable impacts on ANFs. The in vitro methods also highlighted several key correlates of protein digestibility, where both measures of digestibility showed negative associations with polyphenols, while specific protein secondary structure features—intermolecular β-sheets, β-sheets, random coil, side chains, and aggregates—were correlated only with the gastro-intestinal digest. Digestion method-dependent relationships were also observed with colour metrics, phytate, and trypsin inhibitor activity. While amino acid score contributed substantially to protein quality outcomes, the use of pIVPD generally resulted in greater values for potential content claims, suggesting that the gIVPD method may offer a more conservative assessment. These method-dependent patterns underscore that the choice of *in vitro* assay influences which determinants of digestibility appear most important, reinforcing the need to consider assay-specific strengths and limitations in protein quality evaluation, such as physiological relevance, duration, cost, and practical scalability, while maintaining that no single assay fully captures the complexity of digestion across food matrices. The correlations identified here provide a useful foundation for refining *in vitro* models and for developing multivariate approaches that better capture aspects of protein digestion typically resolved through *in vivo* assessment.

Importantly, product matrix, serving size standards, and regulatory criteria shaped the extent to which improvements in protein quality translated into claim eligibility. Extrudates

achieved the highest protein quality scores but were limited by low reference serving sizes, precluding a content claim. Bread products exceeded protein quality thresholds for a content claim under Canadian regulations, but not under U.S. regulations, where serving size standards were more favorable. Conversely, pasta products consistently met thresholds for a "good source of protein" claim in both jurisdictions. Taken together, these findings reinforce the value of evaluating final food products, not just raw flours, for protein quality. Insights into the impact of intrinsic and extrinsic factors enhance our understanding of how formulation and processing influence protein digestibility, quality, and regulatory outcomes. More broadly, this study underscores how formulation decisions can help meet regulatory thresholds for protein content claims in both Canada and the United States—an important step toward supporting national dietary goals aimed at increasing plant-based protein consumption.

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5.7: AUTHORSHIP

A.F. Conceived and designed the study, carried out the research for each component, analyzed the data and wrote and reviewed each draft of the manuscript. N.B. and J.C. assisted all collections of *in vitro* protein digestibility data and amino acid hydrolysis, which was analyzed by J.N. Anti-nutritional factors were also assisted, collected, and calculated with the aid of J.C. Sample preparation, including pre-treatments and milling was completed by L.B. M.N. was the nominated principal investigator and J.H., E.S. and J.P. were co-investigators involved in conceiving and designing the study, reviewing data analysis, and approving the final draft of the manuscript. With the loss of J.H., at the time of the manuscript preparation, R.A. also assisted with reviewing data analysis and approving the final draft of the manuscript.

5.8: CONFLICT OF INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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5.10: SUPPLEMENTARY DATA

Table 5.9. Straight grade flour yield (total product basis %) for pre-treated chickpea flours

	SG (%)	Shorts (%)	Hulls (%)
Untreated	83.9	11.3	4.9
Germinated	90.9	3.2	6.0
Roasted	83.5	3.0	13.4
Micronized	79.0	15.5	5.5

SG, Straight grade; Milling in singlet

Table 5.10. Dry matter (%) of raw flour mixtures, products, and bread after freeze drying

	Raw Flour	Product	Freeze Dry
Bread			
Control	94.67 (0.50)	96.41 (0.61)	71.46 N/A
Untreated	91.73 (0.35)	97.19 (0.54)	71.42 N/A
Germinated	91.51 (0.21)	96.68 (0.08)	69.28 (1.13)
Roasted	91.03 (0.45)	97.00 (0.07)	68.13 (0.18)
Micronized	91.90 (0.12)	96.37 (0.63)	66.48 (1.87)
Pasta			
Control	87.36 (0.24)	97.28 (0.23)	
Untreated	89.65 (0.01)	97.25 (0.12)	
Germinated	90.04 (0.30)	97.46 (0.44)	
Roasted	91.07 (0.67)	97.81 (0.08)	
Micronized	91.60 (0.03)	97.68 (0.03)	
Extrudate			
Control	91.35 (0.36)	93.39 (0.07)	
Untreated	91.77 (0.21)	94.61 (0.14)	
Germinated	92.06 (0.27)	94.64 (0.08)	
Roasted	91.41 (0.37)	94.15 (0.03)	
Micronized	93.71 (0.28)	94.97 (0.21)	

Mean values with standard deviation in brackets; Standard deviation of raw flours from replication, products and freeze dry from duplicates and replication

Table 5.11. Dry matter (%), colour, trypsin inhibitor activity (TUI/g sample), polyphenols (GAE/g sample) and phytate (phytate/g sample) of 100% straight grade pre-treated chickpeas and soy protein isolate

	DM	**L***	**a***	**b***	**TIA**	**Polyphenols**	**Phytate**
Untreated	91.67 (0.14)	88.22	2.22	24.24	13569.95 (447.63)	1.13 (0.01)	11.58 (0.21)
Germinated	92.18 (0.14)	88.43	0.61	19.67	11812.94 (446.99)	1.60 (0.02)	11.80 (0.09)
Roasted	92.62 (0.05)	86.71	1.71	25.37	2229.38 (131.65)	0.89 (0.00)	10.57 (0.40)
Micronized	93.21 (0.03)	84.56	1.83	26.52	2138.29 (144.54)	1.04 (0.05)	9.28 (0.23)
Soy Isolate	93.76 (0.02)	86.63	0.47	15.84	3826.02 (174.54)	2.30 (0.08)	25.61 (1.27)

DM, Dry matter; TIA, Trypsin inhibitor activity; L*, darkness (0) to brightness (+100); a*, greenness (−60) to redness (+60); b*, blueness (−60) to yellowness (+60); Standard deviation from replication in brackets

Table 5.12. Protein (N x 6.25) and amino acid composition (mg AA/g protein) of 100% straight grade pre-treated chickpeas, soy protein isolate, gelatin and casein

	Protein	**Thr**	**Val**	**M+C**	**Ile**	**Leu**	**P+T**	**His**	**Lys**	**Trp**
Untreated	19.00	34.94	45.35	28.50	43.50	75.06	80.09	27.90	72.27	**10.93**
Germinated	20.69	34.52	45.18	29.23	43.25	74.15	75.22	28.52	70.73	**10.27**
Roasted	19.00	34.77	45.33	28.46	43.48	75.59	78.66	28.21	71.62	**10.73**
Micronized	19.69	35.52	45.95	29.63	43.88	76.04	78.10	32.28	73.05	**10.84**
Soy Isolate	100.00	30.63	41.28	21.09	39.83	67.51	67.32	20.07	56.19	11.88
Gelatin	86.25	19.89	26.70	14.10	15.30	34.67	23.96	4.98	40.81	**0.04**
Casein	85.06	47.32	69.97	31.95	58.88	104.62	101.74	29.80	89.25	12.03
Reference Pattern (FAO/WHO 1991)										
		34	**35**	**25**	**28**	**66**	**63**	**19**	**58**	**11**

Thr, Threonine; Val, Valine; M+C, Methionine & Cystine; Ile, Isoleucine; Leu, Leucine; P+T, Phenylalanine & Tyrosine; His, Histidine; Lys, Lysine; Trp, Tryptophan; Limiting amino indicated in bold

Table 5.13. *In vitro* protein digestibility, quality and amino acid score of 100% straight grade pre-treated chickpeas, soy protein isolate, gelatin and casein

	pIVPD	**gIVPD**	**AAS**	**pVPDCAAS**	**gIVPDCAAS**
Untreated	85.92 (0.27)	86.48 (0.83)	0.993	85.33	85.89
Germinated	85.51 (0.39)	83.52 (0.30)	0.934	79.84	77.99
Roasted	90.59 (0.64)	86.85 (0.30)	0.975	88.35	84.70
Micronized	92.38 (0.49)	86.32 (1.71)	0.986	91.05	85.08
Soy Isolate	95.33 (0.54)	79.50 (0.15)	1.080	103.00	85.89
Gelatin	94.31 (0.80)	92.60 (0.42)	0.003	0.30	0.30
Casein	98.10 (0.67)	90.34 (0.99)	1.094	107.30	98.81

pIVPD, pH drop *in vitro* protein digestibility; gIVPD, gastro-intestinal digestion *in vitro* protein digestibility; AAS, Amino acid score; pIVPDCAAS, pH drop *in vitro* protein digestibility corrected amino acid score; gIVPDCAAS, gastro-intestinal digest *in vitro* protein digestibility corrected amino acid score; Standard deviation from replication in brackets

CHAPTER 6.

6.1: CONCLUSION

This thesis addressed the evaluation of protein digestibility and quality in pulse-based ingredients and products, with a focus on the development and application of *in vitro* methods to supplement or potentially replace *in vivo* assays. Across three research studies, this work highlighted the complexity of factors influencing protein quality in pulses, particularly green lentils, yellow peas, navy beans and Kabuli chickpeas.

The first two studies (Chapters 3 and 4) demonstrated that pre-milling treatments such as moisture conditioning and mechanical scouring, while effective for modifying by-product yields and flour compositions, exerted limited effects on *in vitro* protein digestibility, with an effect observed only in lentils among the four pulse types evaluated. Improvements in protein quality, when present, were primarily attributable to changes in protein content and amino acid composition, both of which influenced the AAS, rather than protein digestibility itself. Across pulse types, minor ingredient-level modifications offer limited practical relevance for improving protein quality. Pulse selection, though, remains important, as certain pulse types—such as lentils—show specific potential for targeted improvements. An important caveat is that while efforts to enhance dehulling through pre-milling treatments did not yield protein digestibility improvements in navy bean, chickpea, or pea, dehulling itself—relative to whole pulses with intact hulls—remains effective at improving digestibility, as supported by the broader literature, despite whole samples not being directly assessed in these studies. Additionally, the use of two distinct regression equations for calculating IVPD highlights the need to consider their origin and intended application. Although only a few treatment differences were unique to one regression, true protein digestibility estimates are better suited for calculating IVPDCAAS. Accordingly, the

regression that most closely approximates PDCAAS would be preferred when validating an *in vitro* method intended to replace *in vivo* assays for protein content claim substantiation.

The third study (Chapter 5) shifted the analytical focus toward finished products, revealing that thermal pre-treatment of whole seeds and product formulation significantly influenced protein quality outcomes. Importantly, intrinsic and extrinsic factors exhibited method-dependent relationships with protein digestibility, but their impact diminished when evaluated in the context of regulatory protein content claims, which are strongly shaped by serving size and compositional factors like total protein and amino acid content.

Collectively, the findings suggest that while processing can induce measurable changes in protein digestibility and quality at the ingredient level, it is the formulation and composition of final products that exert the most substantial influence on consumer-relevant protein quality metrics. This work emphasizes the necessity of evaluating protein quality within the context of actual consumption forms, rather than solely at the raw ingredient stage. The methodological work presented here supports the broader application of standardized *in vitro* methods as practical, reproducible, and scalable tools for assessing protein quality, particularly for plant-based proteins where *in vivo* methods may be impractical or ethically constrained.

Moving forward, the adoption of *in vitro* methods within regulatory frameworks in Canada and the US could help bridge the gap between product development and substantiation of protein content claims, ultimately facilitating the promotion of plant-based proteins as viable, high-quality protein sources within national dietary guidelines. The findings from these studies contribute to this broader effort by supporting the applicability of *in vitro* methods for evaluating protein quality in pulse-based ingredients and products.

6.2: MAJOR CONTRIBUTIONS

The following contributions collectively advance the understanding of protein quality assessment in pulse-based ingredients and products. Through a combination of ingredient-level and product-level analyses, these works address key gaps in existing literature, including the limited focus on milling practices, the predominance of ingredient-based evaluations, and the isolated study of intrinsic or extrinsic factors. Together, these contributions support the broader adoption of *in vitro* methods for regulatory and product development applications in plant-based protein foods.

- Utility of *in vitro* protein digestibility methods for pulses: Demonstrated that static *in vitro* digestion methods provide consistent, low-variance measurements of protein digestibility in pulse flours and products, thereby contributing to the continued development and refinement of these methods as potential alternatives to *in vivo* bioassays.
- Novel investigation of milling practices and protein quality: Addressed a critical gap in current literature by evaluating the effects of milling practices—including pre-milling treatments—on the protein quality of pulse flours. Previous research has largely focused on functional properties (e.g., particle size, hydration, pasting) and milling efficiency, with comparatively little attention to how milling influences protein composition, amino acid profiles, and protein quality outcomes relevant to regulatory assessment and product development.
- Comparative evaluation of pre-milling treatments: Clarified the limited but specific role of moisture conditioning and mechanical scouring in modifying protein quality outcomes in roller-milled pulse flours, emphasizing that pre-treatment impacts are more

compositional than protein digestibility-driven. While dehulling itself remains beneficial for improving protein digestibility relative to whole seeds, efforts to enhance dehulling through pre-milling treatments demonstrated limited effectiveness in yellow pea, navy bean, and Kabuli chickpea with meaningful improvements observed primarily in green lentils.

- Identification of amino acid content as a key driver of protein quality: Demonstrated that variation in protein content and amino acid composition influences protein quality more strongly than changes in digestibility. This becomes especially evident in finished products, where formulation often reduces total protein relative to the raw materials, amplifying the impact of compositional changes on protein quality outcomes..
- Assessment of intrinsic and extrinsic factors in finished products: Provided a rare, holistic evaluation of how ANFs (e.g., phytate, trypsin inhibitors, polyphenols), protein secondary structures, and Maillard reaction products interact with processing and product formulation to affect protein digestibility and quality in chickpea-fortified foods. This comprehensive approach is distinct in that both intrinsic and extrinsic factors were assessed concurrently in finished products, rather than ingredients alone or in isolated pre-treatment studies—reflecting a more realistic and applied evaluation of protein quality outcomes.
- Methodological insights on *in vitro* protein digestibility assessment: Demonstrated that both the choice of regression equation and the type of *in vitro* digestibility method can influence treatment differences observed in identical samples. Regressions derived from true protein digestibility values are preferable for estimating PDCAAS, as alternative regressions may undervalue protein quality. Although method selection affected IVPD

outcomes, both digestion models were generally sufficient for determining content claim eligibility, however, products just above a regulatory threshold offer more flexibility for formulation. These findings reinforce the need for continued methodological standardization and validation when applying *in vitro* approaches to protein quality evaluation within regulatory and product development contexts.

- Bridging raw flour and product-level protein quality evaluation: Demonstrated that differences observed in raw flours are often reduced or no longer evident in finished products, underscoring the importance of assessing protein quality at the product level for accurate estimation.
- Framework for future regulatory and industry applications: Positioned *in vitro* models as a practical, cost-effective, and easily scalable method that minimizes the need for live-animal assays for supporting protein content claims under Canadian and US regulatory frameworks.

6.3: Prospects

This body of work advances the understanding of protein quality evaluation in pulse-based ingredients and products, while identifying key methodological and practical considerations for future research and industry application. Together, these findings support the broader adoption and refinement of *in vitro* methods for regulatory, nutritional, and product development purposes, contributing to the positioning of plant-based proteins as viable, high-quality protein sources.

- Emphasize the need for more product-level evaluations: Much of the existing research focuses on raw ingredients, simple cooking methods, or processing steps designed for

studying mechanistic change or animal feed applications, rather than methods intended for human consumption. Future works should expand to include food matrices.

- Acknowledge the limitations of protein quality assessment in guiding product development: While pre-treatments and processing strategies can modify protein quality outcomes, assessing protein quality alone may provide a superficial understanding of product value. The findings from this work suggest that product development decisions based solely on protein quality improvements may overlook other critical factors, including consumer preferences and sensory quality. Future studies should integrate protein quality evaluation with sensory, functional, and consumer acceptance assessments to more holistically evaluate the practical benefits of processing interventions such as pre-roasting or moisture conditioning.
- Reassess the necessity of increasingly sophisticated digestibility assays: The development of dynamic systems (e.g., TIM-1) and standardized static digestion models (e.g., INFOGEST) has largely been driven by the goal of more closely simulating human digestion. Their sophistication requires additional processing steps, specialized equipment, and increased time and cost. While these systems have demonstrated good associations with TPD and even standardized ileal or amino acid digestibility, their direct applicability to regulatory protein quality evaluation remains unclear. Standardized animal-based assays (rat, pig, human) evaluate protein quality under controlled, low-protein and low-fat conditions, directly influencing digestive physiology and outcomes. In contrast, INFOGEST and TIM-1 protocols simulate typical meal digestion—characterized by moderate protein and fat intakes and consequently elevated digestive inputs—which may not reflect the controlled physiological conditions underpinning

protein quality determinations such as PDCAAS or DIAAS. Bailey et al. (2023) demonstrated that dynamic digestion using TIM-1 offered no clear advantage over static methods in predicting TPD in raw and thermally treated potatoes. Similarly, comparisons between the pH drop method and INFOGEST (Bui, 2025) showed equally strong associations with TPD outcomes. These findings suggest that for protein quality evaluation aimed at regulatory protein content claims, simpler static methods may remain both appropriate and sufficient. Nonetheless, more sophisticated models retain value, especially in the context of future DIAAS-based frameworks, where amino acid-specific digestibility and ileal-level assessments will necessitate more robust digestion and analytical procedures. In the interim, simpler static assays remain practical for high-throughput screening, guiding when more complex methods like INFOGEST or TIM-1 should be employed.

- Consider the role of amino acid composition and infant-specific evaluation in method development: A recent consensus report (2025) on *Protein Quality and Growth Monitoring Studies: Quality Factor Requirements for Infant Formula* published in the National Academies identified key limitations of IVPD methods when applied to infant formulas, primarily due to their lack of infant-specific physiological relevance. Notably, the report positions amino acid composition itself as a potential interim replacement for the Protein Efficiency Ratio, suggesting that amino acid composition alone may serve as a sufficient baseline indicator of protein quality. This perspective highlights two important directions for future work: (1) the need to develop IVPD methods specifically aligned with infant physiology, and (2) the question of whether amino acid composition

alone can adequately communicate protein quality, at least in certain regulatory or nutritional contexts.

- Re-evaluate protein quality classification using *in vitro* TPD-based DIAAS estimation and updated reference amino acid patterns: Protein quality assessment of the studied flours and products could be advanced by estimating DIAAS using *in vitro*-derived TPD as a provisional substitute for ileal amino acid digestibility, consistent with FAO recommendations where direct ileal values are unavailable. This approach offers a practical means to estimate protein quality until more precise data can be generated. Consideration should also be given to how these products score against updated reference amino acid patterns. Newer patterns, such as those in the 2013 FAO report, generally feature lower amino acid requirement values (e.g., lysine decreasing from 58 mg/g protein in 1991 to 48 mg/g in 2013), potentially improving DIAAS outcomes for plant-based proteins. Research in lentils (Sá et al., 2023) similarly demonstrated that updated patterns can elevate plant proteins into higher quality categories, highlighting the value of applying contemporary standards. Future work could evaluate how the products investigated here rank using both historical and modern scoring patterns to better position them as quality protein sources in current nutrition frameworks.
- Consider whether additional chemical or structural parameters could better explain outcomes: Factors discussed in the literature review—but not explored experimentally in these studies—may offer further explanatory power for understanding protein quality outcomes. Future analyses could benefit from evaluating:
    - Changes to sulfur-containing amino acids and their availability.
    - Protein solubility as an indicator of structural changes.

    - Direct measurement of reactive (available) lysine.
    - Specific quantification of tannins.
    - Characterization of phytate forms (IP1 to IP6).
    - Analysis of saponin content.

- Advance and refine standardized *in vitro* methods for protein quality evaluation: The recent adoption of the pH drop (Ka 1b-2024) and pH stat (Ka 1a-2024) methods as standardized assays by the American Oil Chemists' Society—a process in which the author contributed directly (outside the scope of this thesis)—marks an important step toward harmonizing *in vitro* protein digestibility assessment. While this thesis did not contribute directly to the method standardization, the findings provide complementary insights that support method refinement. Specifically, these works highlight the need for assay-specific regression development, ideally supported by expanded *in vivo* to *in vitro* comparisons. Future work should also explore whether separate regressions are warranted based on protein origin (animal vs. plant), processing (e.g., germination, roasting, protein concentrates), or untreated ingredients. In addition, the method itself requires critical evaluation to determine whether procedural modifications could improve its relevance and performance. Areas for potential refinement include enzyme concentrations, sourcing (e.g., non-animal enzymes), salt composition (including calcium additions to enhance trypsin activity), and digestion duration, as some studies suggest extended digestion times (up to 20 minutes) may improve accuracy. Work is also needed to determine the relative

utility of the pH drop versus pH stat methods, including whether one approach offers practical or analytical advantages over the other for specific applications. Together, these considerations could inform further standardization and optimization of *in vitro* protein digestibility methods for diverse protein sources and applications.

An additional avenue to enhance protein digestibility estimation could involve integrating supplementary chemical measures via multiple regression analysis. However, given the objective of maintaining simplicity and broad applicability of the pH drop and pH stat methods across laboratories, introducing an additional analytical step would only be warranted if the measure is easily performed and standardized. Based on regression analyses presented in this thesis, colorimetric parameters or phytate content may represent practical candidates, as both can be measured with relatively straightforward, cost-effective methods. Alternatively, techniques such as FTIR may offer stronger predictive capacity due to their ability to capture broader compositional and structural information. Yet, the higher instrumentation costs and the need for specialized expertise in spectral interpretation may limit the practicality of FTIR integration for routine application. Future research should evaluate whether the predictive gains from such supplementary measures justify their inclusion, particularly in contexts where rapid, accessible assessment methods are prioritized.

CHAPTER 7. APPENDIX

7.1: Phytate analysis (detailed)

Prepared from: “Sensitive Method for the Rapid Determination of Phytate in Cereals and Cereal Products” Wolfgang Haug and Hans-Joachim Lantzsch

Written September 9, 2022

Determine content of phytic acid salt (Sigma: 68388) used for stock via direct measure

- Description from Sigma: “≥90% phosphorus (P) basis (dry basis)” Assumed P:Phytate is 1:1; thus can use certificate of analysis from Sigma to assume content of phytic acid. Currently (Feb 23, 2022) this is 93%.

Sodium Phytate Stock Solution & Reference Standard

1. Stock: Weigh 150mg phytic acid sodium salt hydrate (Sigma: P8810) and bring to volume in 50mL volumetric using reverse osmosis water at room temperature
2. Reference Standard: target between 300-30mcg/mL (93% phosphorus)
   a. 25mL volumetric diluted stock (with reverse osmosis water)
      i. 2.7mL of phytate stock (1) and 5mL 1M HCl; final [HCl] is 0.2M
   b. Prepare serial dilution of diluted stock in 2mL microcentrifuge tube

	1	2	3	4	5	6	7	8	9	10
HCl [0.2M]; mL	0.00	0.05	0.10	0.15	0.20	0.25	0.30	0.35	0.40	0.45
Solution 2. a; mL	0.50	0.45	0.40	0.35	0.30	0.25	0.20	0.15	0.10	0.05
[phytate; mcg/mL] 93% phosphorus	300	270	240	210	180	150	120	90	60	30

Ferric Stock Solution

1. Dissolve 0.1g ammonium iron (III) sulphate (Sigma: F3629) in 50mL 2M HCl
2. Bring to final volume of 500mL with MQ water (0.415mM solution)

Bipyridine Solution

1. Dissolve 5g 2,2′-Bipyridyl (Sigma: D216305) with 5mL Thioglycolic acid (Sigma: T3758)
2. Bring to final volume of 500mL with MQ water

Stock solutions good for several months

Procedure

1. Weigh 25-750mg (see notes) of sample into a 12mL hydrolysis tube
2. Extract phytate from sample with 5.0mL 0.2M HCl rotating at 40 RPM for 3 hours at room temperature
    a. Prepare diluted reference standard prior to extraction finishing to save time, see "Sodium Phytate Stock Solution & Reference Standard" above
3. Vortex sample for 6 seconds at 3000 RPM then allow sample to rest on a test tube rack for 10 minutes
    a. Turn on heat block to 112°C at this time
4. Pipette 0.5mL of sample from the interface (i.e. separation of clear/opaque and turbid layer of sample) to a 2mL microcentrifuge tube
    a. For thick samples, cut a pipette tip. Determine appropriate volume via scale
5. Add 1mL of ferric stock solution to the sample and reference standard tubes and vortex for 6 seconds at 3000 RPM
6. Heat all tubes on a heat block set to 112°C (100°C inside the tube; check) for 30 minutes and leave a microcentrifuge rack(s) in the freezer for this duration
7. Carefully transfer tubes using forceps to a cooled rack, leaving 1 space open between samples and place back in the freezer for 5 minutes
    a. Avoid rupturing the tubes by lightly grabbing below the lid and lifting directly up on the sample; do not pinch
    b. Wear safety goggles
8. Continue to cool samples by transferring to a fridge or ice bath for 5 minutes
9. Transfer tubes to a new rack at room temperature and wait an additional 10 minutes to allow samples to come to room temperature
10. Centrifuge for 30 minutes at 3000g at room temperature
11. Prepare spectrophotometer for absorbance at 519nm and blank against 0.5mL of 0.2M HCl and 0.75mL bipyridine solution
    a. 1.25mL total 0.2HCl prepares a similar blank
12. Transfer 0.5mL of the supernatant (sample extract or reference standard) to a micro cuvette (Fisher: 14-955-127; 1.5mL, 340-750nm) and add 0.75mL of bipyridine solution; this begins the reaction
13. Measure absorbance at 519nm after 30-60 seconds

7.2: Gastro-intestinal digest (detailed)

Updated A.J. Franczyk 2018 (MSc. Thesis); Written August 3, 2022

Modified from Boisen & Fenandez 1995 and Boisen 2007

- Weigh out 150mg (+/- 5) of protein for the test sample in a 50mL flask with screw cap
- Add 2.5cm magnetic stirring rod to flask
- Alternatively: Seal with cap and leave in freezer at -20°C for next day digestion

Phase 1 – Gastric Digestion

1. Warm up water bath to 39°C and a single hot plate to 50°C
2. Add 18.75mL of 0.1M pH 6.0 potassium phosphate buffer to the sample flask
3. Mix at 130 RPM on the multi-position stir plate (to hydrate sample and reduce clumping)
4. Add 7.5mL of 0.2M HCl and place samples in water bath to bring temperature to 39°C
5. On a hot plate at 50°C, balance pH to 2.0 (+/- 0.05) using 1M HCl
    a. Record volume of solution added to balance
    b. Actively stirring may improve accuracy, as temperature of solution mixes more readily, impacting pH
    c. Temperature of hot plate =/= temperature of digestion, should maintain 39°C
6. Add 375µL of 0.5% chloramphenicol solution to each digestion flask
7. Add 750µL of pepsin solution containing 5mg pepsin
8. Seal flasks and add to water bath to shake for 6 hours at 75 RPM and 39°C

Phase 2 – Intestinal Digestion

1. Stop water bath and leave samples inside at 39°C
2. Add 7.5mL of 0.2M pH 6.8 potassium phosphate buffer and 3.75mL of 0.6M NaOH to the sample flask
3. While stirring at 130 RPM and 50°C, balance pH to 7.0 (+/- 0.05) using 1M NaOH
    a. Record volume of solution added to balance
4. Add 750µL of pancreatin solution containing 50mg pancreatin
5. Seal flasks and add to water bath to shake for 18 hours at 75 RPM and 39°C

Phase 3 (next day) – Sample Preparation

1. Prior to removal of samples, load 2mL amber centrifuge tubes with 200uL of 60% TCA solution
2. While actively stirring at 130 RPM remove 1.4mL digestate from the flask and transfer to the centrifuge tube (final [TCA]: 7.5%)
3. When all sample aliquots have been transferred to centrifuge tubes, vortex each for 6 seconds at 3000RPM
4. Leave samples in refrigerator (4°C) for 1 hour
5. Centrifuge the sample at 17000g for 15 minutes
6. Transfer ~1mL of the supernatant to a labelled 2mL cryogenic vial and store at -20°C for hydrolysis

Enzyme Solutions

- Prepared fresh
- Pepsin (5mg/mL or 3200-4500 units/mL):
    - 0.75mL transfer to each flask
    - 1mg = 3200-4500 units/mg protein
    - <u>85mg in 12.75mL 0.1M HCl; mixed at 300RPM and 35°C</u>
        - Enough for 14 samples, with some extra
    - P6887 - Pepsin from porcine gastric mucosa lyophilized powder, 3,200-4,500 units/mg protein
    - Note: Require ~3200 units to *maximize* digest of 250mg casein (Gauthier 1986). Max TCA-Soluble N demonstrated with 100mg casein and ~3200 units.
- Pancreatin (66.7mg/mL):
    - 0.75mL transfer to each flask
    - <u>850mg in 12.75mL water; mixed at 300-700RPM and 35°C</u>
    - P7545 - Pancreatin from porcine pancreas 8 × USP specifications

Digest Stock Solutions (15 Flasks/10 Experiments)

- Potassium phosphate monobasic 1M (500mL clear bottle)
    - Stock solution stored at room temperature
    - 51g of potassium phosphate monobasic in 324mL MQ water
- Potassium phosphate dibasic 1M (250mL clear bottle)
    - Stock solution stored at room temperature
    - 27g of potassium phosphate dibasic in 128mL MQ water
- Potassium phosphate buffer 0.1M pH 6.0 (1000mL clear bottle)
    - Stock solution stored at room temperature
    - 13.2mL of potassium phosphate dibasic 1M mixed with 86.8mL of potassium phosphate monobasic 1M and 900mL MQ water
- Potassium phosphate buffer 0.2M pH 6.8 (1000mL clear bottle)
    - Stock solution stored at room temperature
    - 99.4mL of potassium phosphate dibasic 1M mixed with 100.6mL of potassium phosphate monobasic 1M and 800mL MQ water
- Chloramphenicol 0.5% (100mL amber bottle)
    - Stock solution stored in refrigerator @ 2-8°C
    - 500mg chloramphenicol solid in 100mL ethanol
- Trichloroacetic acid (TCA) 60% (100mL amber bottle)
    - Stock solution stored in refrigerator @ 2-8°C
    - 60g TCA mixed with 40mL of MQ water at room temperature
- 0.1M HCl (250mL clear bottle)
    - Stock solution stored at room temperature
    - 4mL 6M HCl added to 236 of MQ water
- 0.2M HCl (1000mL clear bottle)
    - Stock solution stored at room temperature
    - 29mL 6M HCl added to 841 of MQ water
- 0.6M NaOH (500mL clear bottle)
    - Stock solution stored at room temperature
    - 12g of NaOH solid brought to 500mL in water

Digestion Notes

- Amount of sample protein to weigh = (150 / %protein in test sample) x 100
- Maximum number of flasks / clips for digestion is 15
  - 3 samples in triplicate, 2 enzyme blanks, 1 water only blank
  - Water blanks are run similar to the test sample, where 150µL of MQ water replaces the test protein
  - Water blanks should match for total volume, minus balance requirements
  - No addition of enzyme is made to the water only blank
- The same single pH probe is used to pH balance all samples in both digestive phases
- The pH probe calibrated once a day
  - Ensure pH calibration solution and probe storage solution is changed each week
  - The mV reading from calibration is recorded and monitored digest to digest for potential variability
- Samples should be warmed prior to pH balance to account for temperature influence on pH
  - With samples in triplicate, only one of the triplicates needs to be measured to balance, to which the other two can have the same amount of volume needed to balance added
- During phase 1, the total volume is too low for the pH probe to measure accurately, so stirring is momentarily stopped and the probe is set inside the flask, during phase 2, however, the probe may be maintained in the solution while stirring
- During phase 3, only 15 samples are collected, thus a 16th sample or an adjustment needs to be made to maintain proper balance in the centrifuge (24 spots total)
- Sample buildup on sides of flask may occur for some samples. Swirling the sample, or 'rinsing' with potassium phosphate buffer during phase 2, or the digest solution in phase 3 is not unusual

Changes from Original Method

- All volumes are reduced by 25%
  - Enzyme concentrations maintained
- Boisen 2007 updated pH used in phase 2 from 6.8 to 7.0
- TCA replaces sulfosalicylic acid
- Centrifugation replaces paper filtration
- OPA + regular amino acid hydrolysis replaces Kjeldahl nitrogen (alpha amino nitrogen) determination
  - No phenol in regular amino acid hydrolysis
  - Not brought to volume

Regular Amino Acid Hydrolysis (modified AOAC method)

Phase 1

1. Begin warming oven to 110°C and thaw test samples for approximately 30 minutes at room temperature
2. Prepare hydrolysis tubes with 4-5 wraps of Teflon tape over the glass threads
3. Vortex thawed sample at 3000RPM for 6 second and transfer 200µL to a hydrolysis tube
4. Add two drops of octanol (from a 200 µL pipette)
5. Add 3.8mL of 6.3N HCl
6. Add 10 seconds of nitrogen gas into the tube and cap; seal the tube immediately with the cap afterwards
7. In a heat resistant polypropylene rack, add copper jacket over test sample tubes
8. Place samples in the oven with a metal sheet overtop, for 24 hours

Phase 2 (next day)

1. Remove from oven and remove copper jackets
2. Allow sample tubes to cool for 10 minutes
3. When cool, remove Teflon tape; while maintaining order of caps to sample tube
4. Add 4.0mL of 25% NaOH to each sample tube
5. Pour out contents of hydrolysis tube to a 50mL beaker with magnetic stir bar
6. Add 2mL of MQ water to the sample tube, seal tightly and shake vigorously or vortex
7. Transfer these contents to the same 50mL beaker and repeat 2 more times for a total of 3 rinses
8. Stir sample briefly at 300 RPM
9. Remove approximately 1.5-2mL of solution using a 5mL syringe from the beaker
10. Apply a 0.22µm filter and transfer approximately 1-1.5mL of the syringe contents to a centrifuge tube
11. Samples are to be measured via OPA analysis prior to being stored at -20°C (may not maintain nitrogen content through storage)

OPA Analysis (Church et al. 1983)

OPA Reagent Preparation

1. Add 25mL of 0.1M sodium tetraborate, 2.5mL of 20% SDS and 21.4mL (or bring to volume in volumetric flask) of MQ water to a 150mL amber flask and stir (2.5cm stirring rod; 150-200 RPM) at room temperature
2. Add 100µL of β-mercaptoethanol under a fume hood and allow solution to stir again at room temperature with non-translucent stopper
3. Add 1mL of OPA solution containing 40mg phthaldialdehyde in methanol to flask with stopper
    a. See 'Analysis solutions'
4. Allow at least 1 hour for final solution to stir at room temperature prior to use

L-Leucine Standard Curve Solutions

1. Transfer 1mL of 10mM stock leucine solution to 9mL of 0.1M sodium tetraborate buffer to produce 1mM solution
2. Label 9 centrifuge tubes 10 to 2
3. From 10-2, pipette increasing volumes of 1mM L-Leucine solution being at 100µL and ending with 900µL (increments of 100µL)
4. From 10-2, pipette increasing volumes of MQ water beginning at 900µL and ending with 100µL (increments of 100µL), with the total volume of each test tube brought to 1mL

Analysis Solutions

- Leucine 10mM
    - Refrigerated stock solution stored in amber bottle @ 2-8°C
    - 131.17mg target
    - Brought to volume with 100mL 0.01N HCl
- Sodium Tetraborate 0.1M
    - Stock solution stored at room temperature
    - 76.27g sodium tetraborate decahydrate
    - Brought to volume; 2L of MQ water
    - 300RPM at 50°C until solution is clear
- Sodium dodecyl sulfate (SDS) 20%
    - Stock solution stored at room temperature
    - 10g SDS in 40mL MQ water
    - 300 RPM at 30-50°C until bubbles are reduced and solution is clear

- Phthaldialdehyde (OPA)
    - 1mL transfer
    - Made fresh in brown plastic vial
    - 60mg phthaldialdehyde in 1.50mL methanol for 50mL final solution
    - 300 RPM for 30 minutes

Notes

- Preparing the OPA solution (i.e. phthaldialdehyde and methanol) may take different times to dissolve based on crystal size
- Note changes in amount of OPA based on number of samples to be analyzed
    - Be sure to have extra to makeup for errors; if preparing a 100mL solution weight 100mg of phthaldialdehyde in 2.5mL of methanol
- During OPA spec analysis, the timer begins at the time when mixing has finished
- OPA pipette tips should be changed for each sample with mixing
- SDS is very soapy and can get sucked into the pipette or produce a lot of bubbles. Care must be taken to pipette mix effectively, by having the pipette tip at least mid-way into the cuvette. Discharge within the solution and remove pipette tip to reduce bubbles and damage to the pipette.
- Each sample reading is approximately 5-8 seconds
- The OPA solution may be temperature sensitive; placing the solution and samples to be read in the spectrophotometer room for 30-60 minutes prior to reading may eliminate this potential source of error

Sample Calculation (e.g. Casein)

Protein target (mg) =	150	
CP (%) =	84.88	N × 6.25
Actual sample weight (mg) =	177.16	
Max protein digested (mg) =	150.37	Actual sample weight x (%CP/100)
Max nitrogen digested =	24.06	Max protein digested / 6.25
Nitrogen content (μmol N) =	1718.55	Max nitrogen digested / 14 (MW of nitrogen) x 1000
Gastric HCl balance (mL) =	0.150	Adjustment from experimental run
Intestinal NaOH balance (mL) =	0.450	Adjustment from experimental run
Final digest volume (mL) =	39.975	Total from method + above
Digest concentration (μmol N / mL) =	42.99	Nitrogen content / final digest volume
[1]Dilution 1 (D1; μmol N / mL) =	37.62	(Digest concentration x 1.4) / 1.6
[2]Dilution 2 (D2; μmol N / mL) =	1.881	(D1 × 0.2) / 4
[3]Dilution 3 (D3; μmol N / mL) =	0.537	D2 / (14/4)
[4]Dilution 4 (D4; μmol N / mL) =	0.107	D3 / 5
Spec Measurement 1 & 2 average =	0.504	Measured
Regression x (Abs340) =	0.504	Spec average
Regression m =	5.1216	Given/measured; leucine regression
Regression b =	-0.00776	Given/measured; leucine regression
Casein, y [μmol N] =	0.097	x + b / m
*Enzyme Blank, y [μmol N] =	0.0168	x + b / m
Initial digest nitrogen (μmol N) =	0.107	D4
Final digest nitrogen (μmol N) =	0.0776	y value from regression – y value from enzyme blank
Initial *in vitro* Digestibility =	72.5	(Final digest nitrogen / initial digest) x 100
Final *in vitro* Digestibility =	89.02	37.58+0.7095(Digest); 2018 Thesis Regression

[1]D1 = 200uL TCA in 1.4mL aliquot of final digest volume / 1.6 mL (final volume); 2mL centrifuge tube

[2]D2 = 200uL of D1 (1/5 of 1mL) in 4 mL (final volume; 3.8mL 6N HCl, hydrolysis step)

[3]D3 = 4 mL (above) in addition to 10mL (4mL 25% NaOH; 3x 2mL MQ water; end of hydrolysis)

[4]D4 = 200uL D3 (1/5 of 1mL) added to cuvette (spectrophotometric analysis)

*Enzyme blank follows same regression equation from leucine run on same day as sample run & casein