

ABSTRACT

BALFANY, CONNOR MITCHELL DODDS. Advancing the Utilization of Leaf Proteins: Optimization of Extraction Protocols and Comparative Functional Characterization (Under the direction of Dr. Slavko Komarnytsky).

The world's most abundant source of protein is found in green leaves. This is a powerful fact, especially in our modern food ecosystem; As the global population continues to increase, the pressure to secure sustainable and nutritious proteins intensifies, driving research and innovation in alternative protein sources. Consumers are increasingly using their purchasing power to support sustainable and cruelty-free practices, and proteins derived from green leaves align perfectly with this shift towards increasingly mindful dietary choices and are widespread enough to be an asset in sustainable protein sourcing. The inherent abundance of leafy material containing extractable proteins combined with the increasing global demand for novel protein sources and advancements in extraction and processing technologies has positioned leaf proteins as a promising consideration for sustainable food systems.

While the global demand for food is constantly evolving, the concept of leaf protein extraction is not a new one, with the first recorded recovery of leaf protein dating back to 1773. However, its widespread use has not been adopted due to challenges in extraction efficiency, cost-effectiveness, and consistency in protein quality deemed desirable by the market. This thesis aims to address these concerns by outlining innovative methods to improve extraction efficiency, reduce costs, and ensure consistent protein quality from leaf sources. By developing scalable extraction techniques and exploring their relationship to functional characteristics, this research seeks to unlock the potential of leaf proteins as a viable and sustainable alternative to conventional protein sources.

In the first chapter, a comprehensive overview of the current state of research on leaf proteins is presented, emphasizing leaf proteins comparative nutritional value with other protein standards and their potential applications in the food industry. This chapter first highlights conventional proteins available on the market, then explores the history of various types of leaf species that have been studied as input material and the methods in which the proteins can be extracted; A display of the diversity of both biomass sourcing and processing methods is ascertained when utilizing leaf proteins for commercial food sources. Through this exploration, a baseline understanding is created that serves as a starting point to further the development of leaf protein extraction and utilization, thereby setting the stage for the experimental investigations presented in subsequent chapters.

The second chapter takes discussed extraction parameters of the first, combines them with cited techno-economic feasibility, and continues to optimize a cost-effective baseline leaf protein extraction from *Atriplex hortensis*. Outlining the systematic approach taken to optimize various extraction parameters, factors such as pH levels, temperature, and additive type are adjusted in relation to one-another to maximize the yield and purity of recovered leaf protein. Alkaline pH pretreatment of leafy tissue was observed to have a positive effect on protein extraction, and when used in tandem with a preliminary heating step, there was a synergistic effect on the amount of soluble protein recovered when compared to single or non-pretreatment extractions. The isoelectric point of the recovered soluble proteins was found to exhibit a tight tolerance of 4.1- 4.4 pH, which was further influenced by the acidifying agent used.

The third chapter evaluates the functional properties of extracted leaf proteins, including solubility, emulsification, and foaming, as well as looking at the preservation of nutritional

attributes such as each extracted proteins antioxidant potential, and quantifying the pigments contained within. The study found that leaf proteins exhibit competitive functional properties compared to conventional proteins, but also exhibited a very wide range of functionality dependent on extraction conditions. This range of functional attributes allows for the consideration of tailoring the leaf protein extraction process for specific downstream product applications. Additionally, when compared to conventional protein sources, leaf proteins exhibited very high radical scavenging ability, indicating they may be of interest to products leveraging nutritional claims, or functioning as an antioxidant preservative in food matrices. The pigmentation of the leaf-based samples was found to be greatly influenced by extraction type, exhibiting hyper concentration in some proteins while exhibiting the ability for di-pigmentation in others.

The fourth chapter concludes this thesis with a summary of the findings and postulates future directions that will likely play a vital role in leaf proteins commercial success.

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Advancing the Utilization of Leaf Proteins: Optimization of Extraction Protocols and
Comparative Functional Characterization for Food Industry Applications

by
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DEDICATION

I dedicate this work to those who have encouraged me, no matter what I pursued; who answered my questions, no matter what I asked; and who stood by me, no matter the challenges I faced. Thank you for making this program as enlightening on the importance of supporting others as it was on aspects of leaf protein.

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To my advisor Slavko, your trust in me was something that turned an educational program into a life changing journey. You defined what it meant to be a professional researcher all those years ago, and since then I have drawn inspiration from your seamless blending of scientific curiosity and real-world application. The autonomy you gave me to make my own blend was given in parallel with lessons that extend far beyond the confines of any lab, and will be carried with me long after the ink of these pages fade.

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CHAPTER 1: A Review of the Current Status and Nutritional Value of Green Leaf Protein

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Abstract

Green leaf biomass is one of the largest underutilized sources of nutrients worldwide. Whether it is purposely cultivated (forage crops, duckweed) or upcycled as a waste stream from the mass-produced agricultural crops (discarded leaves, offcuts, tops, peels, or pulp), the green biomass can be established as a viable alternative source of plant proteins in food and feed processing formulations. Rubisco is a major component of all green leaves comprising up to 50% of soluble leaf protein and offers many advantageous functional features in terms of essential amino acid profile, reduced allergenicity, enhanced gelation, foaming, emulsification, and textural properties. Nutrient profiles of green leaf biomass differ considerably from those of plant seeds in protein quality, vitamin and mineral concentration, and omega 6/3 fatty acid profiles. Emerging technological improvements in processing fractions, protein quality, and organoleptic profiles will enhance the nutritional quality green leaf proteins, as well as address scaling and sustainability challenges associated with the growing global demand for high quality nutrition.

1. Introduction

Globalization is intertwined with socioeconomic growth, and the expanding populations in both emerging and advanced markets demand more protein that is affordable, sustainable, and nutritious [1]. Health attributes, with a particular focus on increased consumption of plant-based proteins, are rapidly becoming major product traits sought by the consumers [2]. To stay ahead in the food value chain, the industry movement toward increased protein production is gradually matched with consumer demands. Among the newly advancing protein technologies, cultivated meats (animal cell culture), fermentation protein (bacterial, fungal, or algae biomass), precision fermentation (recombinant protein expression), novel animal sources (i.e. insect protein), and

alternative plant proteins have recently reached either commercial or late research stages [3]. The global alternative protein market for animal feed, whether in the form of novel feed ingredients (i.e., oilcake proteins) or novel forage crops that bring additional agronomic or ecological value, is also expected to have a positive impact in all areas of modern food production. The conventional protein markets could be seriously disrupted by these technologies, especially as production prices drop. The stakes are high as global protein ingredient market was capitalized at \$38B in 2019 and projected to grow 9% per year in the next 10 years [4]. Ongoing research therefore aims to identify “new” and reasonably priced sources of plant protein, including agricultural production waste streams in the form of fruit pomaces, distiller’s grains, as well as discarded seeds, offcuts, peels, pulp, and green leaf biomass. Extracted plant proteins can be utilized in the form of flours (20%+), concentrates (70%+), isolates (90%+), or hydrolysates of various degree and may contain other plant-derived confounders such as fibers, starches, and bioactive phytochemicals that hold the potential to modify energy density and organoleptic properties of the final product. When produced at competitive prices, these proteins hold high potential to offset the market share from conventional animal and plant proteins (meat, dairy, eggs, and whole legumes). The raising popularity of vegan, vegetarian, and flexitarian diets provides additional support to this trend, and creates a unique opportunity to develop and manufacture a wide range of novel protein products. Alternative plant protein ingredients may also provide additional functional features such as decreased allergenicity (when compared to dairy, eggs, and soy-based products), or additional clean label opportunities to achieve structure, stability, emulsification, and flavor enhancement goals. Equivalency or superiority of novel alternative proteins or protein feed ingredients must be extensively demonstrated before they are expected to gain market success. This includes development of efficient protein extraction and manufacturing strategies that ensure competitive

high yields and enhanced preservation of functionality traits. Recent technological advances begin to position plant proteins as viable alternatives to support stabilization (milk casein), viscosity (whey proteins), foaming networks (egg white proteins), and gelation (muscle myofibrillar, sarcoplasmic, and stromal proteins) goals critical to many food processing and manufacturing industries. The texture and flavor challenges must be overcome or utilized to their advantage in novel ingredients with enhanced health promoting or sensory profiles. This review looks into a number of underutilized plant protein sources, describes possible extraction strategies to enhance their yields and preserve functionality, and discusses factors that influence acceptance and demand for novel food protein ingredients with a particular focus on green leaf biomass.

2. Conventional Protein Sources and Their Alternatives

The recommended dietary allowance (RDA) of protein for a healthy adult is in the range of 0.8-1.6 g protein/kg body weight per day depending on the level of physical activity, and rates in excess of 2 g/kg/day are not generally recommended [5]. Often overlooked is the fact that most of this protein comes from plants globally (57%), with meat (18%), dairy (10%), fish (6%), and other animal products trailing behind [6]. This ratio is skewed in favor of animal protein (55-60%) in Oceania, Europe, and Americas [7]. Animal proteins score higher on protein content, digestibility, net protein utilization, biological value, and protein digestibility-corrected amino acid score that accounts for human amino acids requirements (PDCAAS), and digestible indispensable amino acid score that incorporates information on digestible amino acids and their ileal digestibility (DIAAS) (**Table 1**). This is explained in part by increased incidence of hydrophobic β -sheet protein structures, polysaccharide fibers, and antinutritive (or rather antidigestive) factors in plant tissues [8]. Additional treatments during the processing (soaking for phytic acid, heating for

protease inhibitors and cyanogens, etc.) or manufacturing of protein concentrates and isolates can improve their digestibility closer to that of the animal products [9]. Accurate flavor profiling of plant protein products during the manufacturing process may also allow for complete removal of the undesirable off-flavors in the future [10].

With consumer demand unlikely to fall, animal meat alternatives with improved undeniable environmental and animal welfare impacts will be essential. Several emerging technologies are positioned to become mainstream when price parity and nutritional parity are reached. While the former is projected to become possible within a few years, the latter is lagging behind due to ultra-processed nature of these products, and will require an unconventional paradigm shift away from mimicking animal products (meat analogues) to creating its own category that can address the nutritional challenges of the current agricultural production systems [3]. The emerging animal-based and plant-based industries are also projected to generate a new series of waste streams that need to be recognized and utilized.

Table 1. Protein content, digestibility, and essential amino acid profiles of major conventional and alternative protein sources.

Protein type	Protein quality						Essential amino acid composition (% protein)														
	100 g	%Dig	%Biolog	%Net	%	%	Arg	Cys	His	Ile	Leu	Lys	Met	Phe	Tre	Trp	Tyr	Val	Met	Phe	
	fresh	estibil	ical	utilizati	PDC	DI	*	*	**	**	**	**	**	**	**	**	*	**	+	+	
	weight	ity	value	on	AAS	AAS														Cys	Tyr
<i>Animal sources</i>																					
Beef	22.7	92%	80%	73%	92%	112%	6.3	1.4	2.7	4.9	8.5	8.3	2.4	3.8	4.0	1.3	3.0	5.3	3.8	6.8	
Pork	16.9	98%	-	-	70%	-	6.6	1.3	2.8	4.8	7.0	7.2	2.4	4.1	5.0	1.3	2.8	4.2	3.7	7.0	
Chicken	20.8	95%	79%	80%	91%	108%	7.0	1.2	4.7	3.8	12.2	5.6	6.8	4.7	3.3	7.4	3.8	5.5	8.1	8.7	
Fish ^a	17.8	94%	67%	64%	106%	100%	4.5	2.0	2.3	3.1	4.7	6.2	3.6	5.5	3.4	0.9	5.7	3.8	5.6	11.2	
Eggs ^a	12.6	98%	100%	94%	100%	113%	5.4	2.2	2.3	5.7	8.5	6.9	3.4	5.8	4.6	1.2	3.9	6.4	5.6	9.7	
Milk ^a	3.3	96%	91%	82%	100%	114%	3.33	0.9	3.6	4.0	8.8	7.7	2.9	4.6	4.8	1.4	5.0	4.7	3.7	9.6	
Whey PI	0.9	100%	104%	92%	100%	125%	1.8	2.1	1.3	5.6	10.3	9.7	1.7	2.6	7.9	1.9	2.7	5.9	3.8	5.3	

Table 1. (continued)

<i>Pulse (legume) seeds</i>																				
Soybean ^a	13.0	97%	73%	66%	100%	100%	6.2	2.1	3.0	5.3	7.1	6.1	2.7	3.9	3.7	7.6	4.1	5.2	4.8	8.0
Soy flour ^a	37.8	80%	-	-	93%	105%	7.6	1.5	2.6	4.9	7.8	6.4	1.4	5.2	3.6	1.4	3.8	4.7	2.9	9.0
Soy PI ^a	88.3	98%	74%	61%	100%	98%	7.6	1.2	2.6	4.8	7.7	6.0	1.3	5.2	3.6	1.3	3.7	4.7	2.5	8.9
Pea, yellow	22.3	87%	64%	56%	78%	65%	8.4	1.4	2.5	4.2	7.1	7.2	1.0	4.7	3.8	0.9	3.1	4.8	2.4	7.8
Pea PI	-	99%	65%	-	89%	-	7.4	0.7	2.0	3.8	7.2	5.8	0.7	4.6	3.0	0.7	3.2	4.0	1.4	7.8
Bean, kidney	22.5	64%	-	-	68%	59%	5.7	0.9	2.7	4.5	7.6	5.5	1.2	5.1	3.4	7.5	4.2	5.0	2.1	9.3
Chickpea	20.5	89%	68%	58%	74%	-	14.1	0.6	4.5	5.1	8.8	10.5	1.4	5.1	3.6	1.0	3.6	5.0	2.0	8.7
Fava bean	26.1	95%	-	-	69%	-	9.0	1.2	2.6	4.1	7.1	6.3	0.8	4.0	3.5	0.8	2.7	4.6	2.0	6.7
Lupin	36.2	76%	83%	-	81%	-	11.0	1.5	2.7	4.2	6.9	4.7	0.7	4.0	3.4	0.8	3.6	3.9	2.2	7.6
<i>Cereal seeds</i>																				
Wheat, grain ^a	9.6	86%	80%	-	42%	54%	2.4	0.7	1.4	3.0	5.0	1.1	0.7	3.7	1.8	0.3	2.4	2.3	1.4	6.1
Barley, grain	12.5	99%	81%	-	61%	51%	6.0	1.5	2.2	3.6	4.6	0.8	0.7	3.6	1.9	0.7	1.6	3.5	2.2	5.2
Oats, grain	13.5	90%	-	-	-	77%	9.7	1.7	3.6	4.4	9.1	3.7	1.7	5.5	4.3	3.6	2.6	6.0	5.4	8.1
Rice, white	6.8	92%	-	-	63%	64%	5.9	0.2	1.6	2.3	5.7	4.7	0.3	3.7	2.3	1.0	2.6	2.7	0.5	6.3
Rice, brown	7.5	79%	-	-	-	-	7.6	1.2	2.6	4.2	8.3	3.8	2.2	5.1	3.7	1.3	3.8	5.8	3.4	8.9
Corn, grain	9.4	-	-	-	60%	48%	1.7	0.3	1.1	1.7	8.8	1.0	1.1	3.4	1.8	0.6	2.7	2.1	1.4	6.1
Corn, distillers	27.1	-	-	-	-	-	3.4	2.0	2.4	3.5	12.0	2.6	1.9	4.6	3.2	0.5	4.1	4.4	3.9	8.7
Sorghum, grain	10.6	-	-	-	20%	29%	4.1	1.7	1.9	3.1	13.0	2.0	1.2	5.0	3.2	1.8	4.0	4.3	2.9	9.0
<i>Oilseed cakes or meals</i>																				
Canola	39.0	-	-	-	-	-	5.9	2.5	2.6	4.0	6.8	5.6	2.0	3.9	4.2	1.2	2.9	4.9	4.5	6.8
Rapeseed	38.3	-	-	-	92%	70%	6.1	2.3	2.6	4.0	6.7	5.5	2.1	3.9	4.4	1.3	3.1	5.1	4.4	7.0
Sunflower	37.7	-	-	-	99%	97%	8.5	1.7	2.5	4.1	6.2	3.5	2.3	4.4	3.6	1.2	2.4	4.9	4	6.8
Hemp	33.4	87%	-	-	48%	-	12.4	1.8	3.0	3.9	6.9	3.9	2.4	4.7	3.8	1.1	3.2	5.1	4.2	7.9
Flax	34.2	-	-	-	-	-	2.81	2.0	2.7	3.7	5.8	3.6	1.0	5.2	3.7	0.5	2.4	4.7	3	7.6
Cotton	45.0	85%	-	-	-	-	11.1	1.6	2.9	3.2	5.9	4.2	1.4	5.1	3.3	1.1	2.9	4.2	3	8.0
<i>Green leaf or forage crops</i>																				
Alfalfa	5.2	76%	-	-	57%	72%	4.4	1.8	3.0	4.8	6.9	4.8	1.9	3.9	1.7	1.3	3.5	4.1	3.7	7.2
Spinach	2.9	74%	-	-	51%	-	5.6	1.3	3.0	3.7	7.0	5.5	1.2	4.5	4.0	1.6	6.1	5.0	2.4	10.5
Sugar beet	2.2	72%	-	-	-	-	5.3	0.3	1.7	6.2	6.8	5.8	1.5	6.0	4.2	0.7	3.3	5.6	1.8	9.3
Cabbage	1.0	82%	-	-	57%	-	4.0	2.4	1.4	3.1	4.1	2.1	4.2	2.8	3.4	1.0	2.1	4.6	6.6	4.9
Lettuce	1.1	91%	77%	-	19%	-	6.3	1.3	1.9	4.5	5.9	6.4	1.5	5.3	4.7	6.1	1.9	6.9	2.8	7.3
Sweet potato	2.5	73%	-	92%	70%	-	6.0	3.8	1.4	3.7	8.6	3.6	1.1	7.0	5.0	0.9	4.1	5.7	4.9	11.1
Cassava	1.8	68%	57%	40%	-	-	5.9	3.6	2.2	5.2	10.5	6.2	1.0	5.7	5.1	1.0	3.3	5.3	4.6	9.0

Table 1. (continued)

Duckweed	3.0	65%	-	-	45%	75%	6.6	1.2	1.6	3.6	6.6	4.7	1.4	4.4	3.5	1.4	2.8	4.5	2.6	7.2
Grass, orch.	4.0	69%	-	-	-	-	0.9	0.2	0.3	0.8	1.4	0.8	0.3	0.9	0.7	0.2	0.5	1.0	0.3	1.5
<i>Others (nuts, tubers, etc.)</i>																				
Almonds ^a	21.2	-	-	-	23%	-	9.3	0.2	2.1	2.7	5.8	2.4	0.4	4.5	1.9	0.9	1.2	3.2	0.7	5.4
Peanuts ^a	25.8	95%	54%	47%	52%	43%	10.6	1.1	2.2	2.9	6.0	3.4	1.0	4.7	0.1	0.8	3.4	3.6	2.1	8.1
Potato	2.1	82%	-	-	82%	-	3.3	0.3	1.4	3.1	6.7	4.8	1.3	4.2	4.1	0.1	3.8	3.7	1.6	8
Brewer's yeast	48.6	-	-	-	-	-	4.4	0.9	2.0	4.6	6.2	6.3	1.5	3.6	4.4	1.1	2.7	4.9	2.4	6.3
Mushroom, butt.	3.1	-	-	-	-	-	4.1	0.1	1.6	4.6	7.9	8.1	1.4	4.7	5.6	0.1	2.9	5.9	1.4	7.6

PI, protein isolate (typically less allergenic and digested more slowly); (a) Major 8 food allergens as identified by the FDA; (*) Conditionally indispensable amino acids: tyrosine, cysteine, and arginine; (**) Indispensable amino acids: valine, tryptophan, threonine, phenylalanine, methionine, lysine, leucine, isoleucine, and histidine; (-) Data not available.

2.1. Cultivated protein

The category of cultivated proteins encompasses predominantly cultivated or cultured meats, however algal and insect protein cultivation can be loosely associated with this group as well (all these cultivation techniques require oxygenation). These proteins are produced with the major goal of achieving parity with expensive animal products like whole or minced meats, and with most efforts focused in the development of acceptable ingredients that mimic meat texture and flavor profiles. Achieving parity with eggs and dairy is not expected short-term, as these products cost less and the key proteins responsible for their functionality can be successfully produced using various fermentation technologies as discussed below.

2.1.1. Cellular agriculture of animal protein

Cellular *in vitro* agriculture of animal muscle and fat tissues have been envisioned as early as 1930s and reduced to a routine practice in 2013. Significant investments from both industry and

academic stakeholders led to rapid technological advances that improved product quality and accelerated the time to parity from \$1.2M/lb in 2013 to \$50/lb in 2021, although costs, cell sourcing and culture medium composition, scaleup (bioreactor capacity), and biological limitations of the animal cell culture systems remain a challenge [11]. The consumer appeal and private interest in the technology is substantial, as the consideration for improved animal welfare [12] and environmental impact [13] continue to drive modern purchasing decisions in food products [14]. Policy surrounding cellular agriculture is also in its infancy, with regulations and legislation focusing more on labeling, branding, and food safety restrictions as consumer perception changes under the different names and marketing strategies [15]. Nutritional parity of cultured meats is difficult to achieve since conventional meats are enriched with often highly bioavailable minerals (iron, zinc, and selenium) and vitamins (A, B9, B12, D, and E) [16].

2.1.2. Algal bioreactors

Algae is a diverse polyphyletic group ranging from the largest single cell organism *Caulerpa taxifolia* (M.Vahl) C. Agardh to the largest cloned seagrass *Posidonia australis* Hook. F, however algal protein manufacturing currently focuses on unicellular microalga. Rapid doubling times as low as 1.5 h for chlorella and the naturally high protein content ranging from 40-60% are the key metrics in creating sustainable and highly efficient algal protein production systems [17]. Algal bioreactors show one of the lowest environmental footprints with an average of 2.5 m² land area use per kg protein produced, which compares favorably not only with animal production systems (42-258 m²), but also grains and beans (22-46 m²) [18]. Algal protein is a complete protein with adequate amounts of every amino acid, however aspartic and glutamic acids can constitute 22-44% of amino acids in some brown seaweed species, which could lead to systemic acidosis if consumed in high enough quantities [19], and it's lower digestibility is critically dependent on the

rigid properties of the microalgal cell wall [20]. Alginates in brown seaweed and carrageenan in red seaweed further decrease efficiency of protein extraction [21]. Algal protein has a stronger flavor profile and is associated with higher energy costs due to rigidity of algal cell walls [22]. The combined technological challenges are therefore the major reasons why most of the algal biomass production is currently geared towards animal feeds [23].

2.1.3. Insect protein farms

Insect protein has served and continues to serve a large role in many nonwestern diets, and rejection of insect-based foods is largely a learned behavior [24]. Insect protein production industry has a unique opportunity to circumvent this pushback by producing the majority of insect protein for animal feed, while developing advanced technological solutions to use insect in human food production [25]. Depending on the production species, both high value food sources and biowaste or agricultural waste streams can be used for rearing [26]. Insect protein is complete and ranges 35-60% dry weight or 10-25% fresh weight [27], however research into its production, processing, and application to food matrices to achieve parity with other alternative proteins is critically lacking. Protein production by insect cells and their potential application in large-scale manufacturing faces the same challenges and bottlenecks that apply to animal cells even though insect cell lines with high protein yield and greater passaging stability are readily available [28].

2.2. Fermentation protein (traditional, biomass, and precision)

Microbial fermentation cultures can produce protein products in the absence of oxygen. Traditionally used in preservation of animal (cheese, yoghurt, kefir) and plant (tempeh, tofu, sauerkraut, wine) foods [29], fermentation technologies can be extended to target total protein biomass (marmite, mycoprotein) [30] or a particular protein based on the synthetic DNA

expression in the host microbial cells [31]. Fermentation also has the capacity to improve incomplete protein profiles of fermentation substrates and achieve high 40-75% dry weight protein yields [32]. Recently many industry players focused their efforts on advancing fermentation technologies to produce bulk protein (Quorn Foods), or functional proteins such as casein (Perfect Day), leghemoglobin (Impossible Foods), egg white (Every Food), or collagen (Geltor). Strain screening, early-stage bioprocess development, growth optimization, and substrate selection are critical for achieving commercial grade biomass yield and productivity [33]. Fermentation is one of the most interesting alternative protein markets within the agricultural food systems to watch, and the one with higher chances to achieve price parity with conventional meats in the short term.

3. Plant-based alternatives and undervaluation of green leaf biomass

Vegetal sources of protein dominate the global protein supply and account for as much as 60% protein provided [6]. However, plant proteins may not contain all the essential amino acids in the required proportions (**Table 1**). The bulk of this protein comes from consumption of plant seeds as a part of the omnivore human diet. Seed storage proteins naturally accumulate in the cotyledons and embryos of dicots (i.e., pulses) or the endosperm of monocots (i.e., cereals). These can be mechanically fractionated and therefore enriched for downstream protein processing using milling, air classification, and steeping [34]. In this group, soy, pea, chickpea, and bean proteins are the most widely used. Additionally, oilseeds provide protein-enriched press cakes (meals) following the initial dehulling and extraction of vegetable oils [35]. These waste products can be upscaled to produce bulk protein as shown for rapeseed, sunflower, and hemp crops, among others (**Figure 1**). Green biomass or green leaf protein remains a largely unexplored option in this landscape. Traditionally, these materials are used in the direct foraging by ruminants or as a part of the animal feeds. Novel forage crops with improved protein and essential amino acid profiles

suited for marginal or environmentally challenged soils, as well as novel byproduct fractions produced from the green leaf biomass with improved digestibility or nutritional quality profiles represent an untapped opportunity to provide additional sustainable sources of plant proteins for human and animal diets.

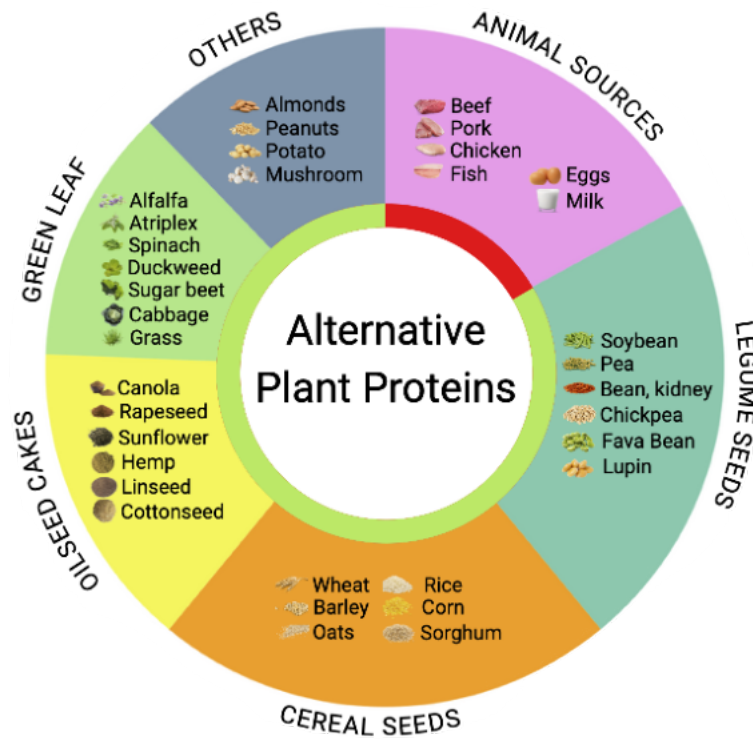


Figure 1. Major groups of alternative plant-based proteins (green) contrasted with the traditional proteins from animal sources (red).

3.1. Green leaf protein, brief history and past uses

Anthropoids include at least some plant foliage in the diet by balancing intake of scarce, higher quality (low fiber, higher carbohydrate) fruits with abundant, lower quality (high fiber, higher protein) leaves, and this process seems to be reinforced by an active selection for soluble protein [36]. Historical consumption of plant leaves also explains the evolutionary diversity of the

human TAS2R bitter taste receptors [37], as well as emergence of the umami TAS1R1/TAS1R3 heterodimer receptor highly and specifically sensitive to L-glutamate as an indicator of proteinogenic leaf substrates [38]. Green leaves are nutrient rich foods that have been an important part of the traditional human diet, and their potential contribution to protein intake is often overlooked. First isolation of green leaf proteins was achieved by Rouelle in 1773 in the form of “*matiere glutineuse ou vegeto-animale*” ‘with a burned feathers smell’ (proteins have not been discovered or named until 1838). The method used involved many of the modern concepts of protein isolation and included pulping of the leaves, straining the juice, heating to obtain a green coagulum, and decolorizing it with alcohol. A similarity to coagulation of egg whites and blood was proposed in 1792 by Beddoes, with possible application to human nutrition, but supposedly ridiculed. Alkali extraction of dried and ground leaves was pioneered by Winterstein in 1901 and Osborne in 1920, while the optimized leaf protein extraction technology using heat precipitation was disclosed by Ereky in 1927 [39]. The early commercial application of green leaf protein was achieved in the form of the PRO-XAN protein-xanthophyll concentrate from alfalfa *Medicago sativa* L., developed as poultry feed, with the green fiber fraction used for ruminant feed [40]. The protein concentrate from the mixed grasses and berseem clover *Trifolium alexandrinum* L. was fed to 100 children for 8 months and shown to be comparable to milk protein supplementation [41]. These studies have indicated proteins from green biomass as a viable alternative to soy and other seed proteins for food and feed applications (**Figure 1**).

3.2. Composition of green leaf proteins

The large-scale screening effort to evaluate the green leaf protein content of 500 plants species harvested prior to flowering was undertaken by the USDA Tropical Agriculture Research Station in Puerto Rico in the 1970s. This study reported the green leaf dry matter in the range of

10.2-34.9% and the crude protein content of 10.8-35.7% dry weight [42]. When crudely processed, green leaves produce 50% leaf liquid (95% moisture content), 45% fiber (55% moisture), and 5% leaf protein concentrate (55% moisture) [43]. As such, the amount of protein in green leaves varies between 1.2-8.2% fresh weight depending on the species and cultivation settings, and in many plants, compares favorably to that of milk (3.5%) even when extracted at 50% efficiency (**Table 1**). Depending on the plant species, the green leaf protein can be further fractionated (*see section 3.3*). While plants are very diverse, they all share a common set of proteins in the photosynthetic tissues that enables capture of sunlight energy and its use in carbohydrate synthesis [44]. Because of this, 75-80% of C3 plants total nitrogen is found in the green chloroplasts, with the CO₂-fixing protein Rubisco accounting for 10-30% of total leaf nitrogen, and up to 50% of soluble leaf protein [45]. Rubisco is a complex but very conservative protein consisting of 8 small and 8 large subunits (12.5 and 55 kDa, respectively) that, in a model spinach *Spinacia oleracea* L. plant, is characterized by the isoelectric point of pH 6.0-6.1 and the denaturation temperature of ~65C [46]. Rubisco has advantageous functional features in terms of enhanced gelation, foaming, emulsification, and textural properties [47]. During the leaf protein fractionation, Rubisco together with the other soluble proteins form a more desirable beige fraction of the leaf protein (white protein) [48].

The bulk of the remaining proteins are hydrophobic in neutral solutions, and include cell wall proteins, cell membrane proteins, leaf storage proteins, and lectins. Many of these proteins have an increased capacity to bind polyphenols and fibrous polysaccharides due to their structural integration into cell walls and membranes, associate with thylakoid membranes of plant chloroplasts, and precipitate together with chloroplast fragments at a lower coagulation temperature of ~55C, thus forming a less desirable green fraction of leaf protein (green protein)

[49]. Additionally, selected plant species produce a minor fraction of tan protein at a higher coagulation temperature of ~82C, or show amorphous coagulation [42].

3.3. Extraction and concentration

Crude isolation of green leaf proteins is achieved by pulping the leaves, pressing the pulp to obtain green leaf juice, then quickly bringing the green liquid to the boiling point to coagulate leaf proteins and to partially pasteurize the final product. When starting with 2 kg of fresh green leaves, the process produces ~100 g of filtered green curd that contains ~50 g of crude protein and generates 1 kg of wet fiber and 1 L of liquid as byproducts [43]. The green leaf protein concentrate (LPC) can be used in animal feed as a part of the biomass utilization biorefinery setup [50], however the lack of many organoleptic and functional properties prevents its wider use in food product manufacturing.

In contrast with pulse, cereal, or oilseed protein extraction technologies, mechanical separation and concentration steps of dry materials to achieve a protein-rich fraction are not applicable to the green plant biomass. For this reason, in depth studies into further fractionation and isolation of green leaf proteins focused on a small number of green crops such as alfalfa (lucerne), sweet potato *Ipomoea batatas* (L.) Lam. [51], or sugar beets *Beta vulgaris* L. [52] and narrowed down the juicing technology to the use of twin-screw extrusion as one of the most effective strategies [53]. Several downstream technological solutions exist to increase yield and functionality of leaf proteins albeit none of them can address both issues simultaneously (Supplementary Table 1).

3.3.1. Thermal-assisted extraction

Heat and pressure could be applied to the plant biomass to increase green juice recovery from spinach, however it was associated with partial denaturation of plant proteins and

functionality loss [54]. This application, however, could be used for a partial *in tissue* precipitation of the green protein to assist with fractionation of the white protein.

3.3.2. Alkaline extraction

Alkali extraction solvents are used to increase recovery of proteins from plant biomass of many established agricultural crops, including soybeans *Glycine max* (L.) Merr. [55], peas *Pisum sativum* L. [56], and barley *Hordeum vulgare* L. [57], among many others. High amounts of basic OH⁻ ions partially degrade β -1-4 glycosidic linkages in cellulose and saponify lipids within the cell membrane, thus aiding in cellular disruption and increasing protein recovery [58]. Basic pH also disrupts disulphide bonds and increases protein solubility. This is achieved by adding sodium hydroxide, potassium hydroxide, calcium hydroxide or ammonia, although the latter requires an additional stripping column and therefore is more expensive [59]. Alkalization at lower temperatures decreases solubility, but improves protein structure and functionality [60]. Dry and processed leaf tissue can be also extracted with alkali treatments, yet at lower efficiency [61]. However, basic extractions may lower the overall quality and applicability of the isolated protein by decreasing its lysine and cysteine content [58].

3.3.3. Enzyme-assisted extraction

External enzymes can be used to aid in degradation of cell wall components (carbohydrases and cellulases that target hemicellulose, cellulose, or pectin) and enhanced release of plant proteins, alone or in combination with other mechanical pre-treatments [62]. Although added in relatively small amounts (0.2-5%), they change the economics of the protein extraction process due to large volumes of treated biomass [58] and additional control of pH ranges [63]. These may also include proteases to enhance fractionation (hydrolysis) of high molecular

weight proteins, increase solubility, and alter the final functionality of the resulting protein hydrolysates [64]. Protease mixtures may also aid in a partial breakdown of native protein complexes with carbohydrates, phytates, and chlorophylls [65], and may be more important in this process than carbohydrases [66]. The drawback of uncontrolled proteolytic hydrolysis is generation of low molecular weight peptides and newly exposed hydrophobic amino acid clusters that result in increased bitterness and limited protein applicability.

3.3.4. Ultrasound-assisted extraction (sonication)

Liquified green biomass can be also subjected to ultrasonic treatment to aid in cellular disruption and protein release [67]. This treatment is often combined with alkaline and enzymatic-assisted extraction to allow for more extensive penetration and increased surface area, as well as reduction undesired enzymatic activity due to direct inactivation of enzymes [68]. Technical challenges related to uneven distribution of energy and high costs of operation currently limit the commercial application of this approach, although recent studies with cauliflower byproducts [69] and blanched alfalfa [70] warrant further investigation on the subject.

3.3.5. Pulse electric field-assisted extraction (PEF, electroporation)

Permeability of plant biomass could be increased by rapid and repeated exposure to electrical pulses that cause partial disruption of cell walls and pore formation in cell membranes [71]. Similarly to ultrasound, PEF treatment can aid in inactivation of enzymes [72] and increase protein yields as shown for alfalfa [73], albeit at a lower efficiency.

3.3.6. Heat precipitation

Green leaf protein must be concentrated before it can be successfully used in food manufacturing, and thermally induced precipitation continues to be a cost-effective method in

modern day recovery systems by using heat exchangers or steam injectors. Due to inherent differences in denaturation temperatures, green proteins predominantly associated with membranes and chloroplasts can be separated from white (beige) soluble proteins in a two-step heat treatment at ~55C and ~65C, although exact temperatures must be established anew for each green biomass and manufacturing process [42] (**Figure 2**). Heat-coagulated protein, however, comes at a relatively high energy consumption and lower water solubility that hampers its application to food systems [74].

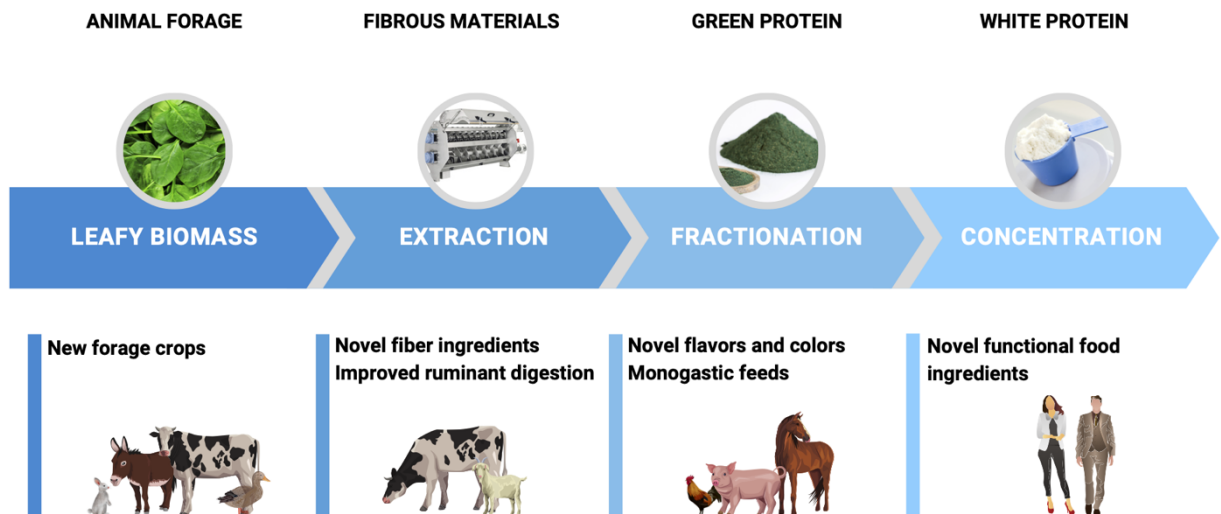


Figure 2. Schematic processing of green leaf biomass into various fractions with different application to animal forage, ruminant animal feeds, monogastric animal feeds, and human food systems.

3.3.7. Acid precipitation (isoelectric)

Alternatively, isoelectric precipitation by adjusting plant juice within the pH 3.0-5.0 range to precipitate proteins with hydrochloric acid [75], anaerobic fermentation including lacto-

fermentation [76], or as a part of the cellulosic ethanol production process [61] may result in higher quality protein ingredients, especially when the pelleted proteins are neutralized before use [77]. This process takes advantage of the proteins aggregating at their isoelectric points. This is equally applicable to seeds as shown for peas [56], and both high moisture (i.e., spinach) [78] and low moisture (i.e., cassava) [79] green leaves. Moreover, this process can be combined with prior thermal precipitation of green protein (wet fractionation) to obtain a more functional white protein enriched with the globulin fraction [80]. Variations of this process allowed for the initial attempts at commercial scalability of green leaf proteins with sugar beet tops (Cosun Beet, Netherlands), cabbage trimmings (Naylor Farms, UK), and duckweed (Plantible, USA).

3.3.8. Ultrafiltration

Ultrafiltration or diafiltration allow for more gentle recovery of the target proteins with enhanced functionality, and for enrichment of both globulin and albumin fractions of plant proteins [81]. This step can be also applied to proteins that were salted out from solution without heating or pH change [82]. This allows for higher solubility, emulsification, and foaming capacity of the resulting ingredients, yet comes with higher costs due to membrane manufacturing and fouling [83].

3.4. Scaleup and technological concerns

The economics of extracting green leaf biomass for protein production was reviewed several times in the 1980s, 2010s, and as recent as 2021 [42,59,84,85]. As many green leaf protein extraction techniques were validated only at the lab scale, they lack the cost analysis required to implement them commercially. Wet fractionation (alkaline extraction followed by isoelectric precipitation) was estimated to recover 85% of proteins with a purity of 52% and to cost €0.102

per kg of green protein in 2014 [86]. The economic feasibility of green protein or combined (green and white) protein was based on the breakeven price of €2 per kg of bulk ingredient in 2021 when accounted for agricultural production and fertilization [84]. More expensive fractions may be justified based on the functionality profile and market application, as seen in whey protein and its application to sport nutrition [83].

The agricultural aspect of green biomass protein production is often overlooked and will likely require a vertical integration of a single agricultural entity that ensures crop selection, coordination of harvesting and production, processing of green biomass adjacent to crop land to minimize the transport and storage, and integration of livestock to capture fibrous materials and green protein (**Figure 2**). This may require development of novel agricultural food production systems based on partially controlled environment agriculture [3]. Duckweed, due to its unique nature of cultivation, may offer some logistical advantages to this approach [87]. Target crops production should also be optimized for leaf rather than stem, and young rather than old leaf production, to limit processing of more fibrous plant tissues. Focus on utilization of agricultural byproducts of existing crops available year-round (fruit pomaces, distiller's grains, discarded seeds, offcuts, peels, pulp, or tops) may offer additional advantages. For processes that include spent green biomass such as tea leaves, a reversed biorefinery approach starting with ethanol-assisted extraction of phytochemicals, followed by mild (55C, pH 9-11) and severe (95C, pH 13) protein extraction, and the ultimate harsh hydrolysis to release monosaccharides (100C, 0.5M alkali) may be considered [88].

4. Leaf protein quality and nutritional outcomes

Nutritional and functional profiles of green leaf proteins vary depending on the source of the protein (**Table 1**), the degree of purification, and the extraction strategy used. Likewise, the

different extraction steps may be used to intentionally modify the physicochemical and nutritional properties of these alternative proteins. Additional processing steps that may influence the protein profile include the use of organic solvents (ethanol, acetone, 1-butanol) to remove chlorophyll and phytochemicals [89], flocculants that may remain in the final product [90], spray drying that may concentrate minerals and salts in the protein powder [91], and the duration of treatment that may increase endogenous proteolysis and oxidation [92]. This intrinsic complexity of green leaf protein ingredients does not allow for a straightforward comparison of their functional and nutritional values among different agricultural crops and their byproducts. The following sections will therefore briefly summarize the key factors critical for evaluation of protein ingredients as it applies to green leaf proteins, while the comprehensive summaries can be found in other recent reviews [9,93].

4.1. Functionality of protein ingredients

Structural properties of the food matrix define its palatability, texture, digestibility, bioavailability of nutrients, and shelf life of the final product [94]. Protein ingredients are inherent food structures that have capacity to form gels that entrap liquid, stabilize emulsions and enhance encapsulation of oils, increase stability of foams, and modulate texture (hardness, mouthfeel) [95]. These properties are largely defined by the amino acid composition, the presence of intermolecular bonds, and the structural confirmation of the protein [96,97].

4.1.1. Solubility

The solubility of a protein is the most important determinant in its application to food systems, as it directly impacts protein-protein interactions and modulates many functional outcomes discussed in this section. The overall charge of the protein defines its solubility in

aqueous solutions of various pH and ionic strength. The solubility of green leaf proteins is at minimum in the pH range close to the isoelectric point of Rubisco (pH of 3.5-5.0), slightly increases at pH 2, and reaches maximum solubility at pH 9-11 [52]. The majority of luminal and cytosolic leaf proteins are highly soluble, while the proteins associated with thylakoid and plasma membranes show decreased solubility [87]. This can be partially improved by a successful pH-shift approach that solubilizes proteins at alkaline pH 11-12 with chemical additives such as NaOH and NH₄OH before neutralizing the protein solution at pH 7 with HCl [98]. Protein solubility can be further improved with high pressure homogenization [99], glycation-induced structural modification [100], ultrasonic treatment [101], or enzymatic hydrolysis [102].

4.1.2. Gelation

The partial unfolding of protein structures to allow for formation of intermolecular polymer bonds results in establishment of elastic protein aggregate networks (gels) that capture liquids, dissolved food ingredients, and flavors. This is achieved by heating the soluble protein to a dissociation temperature of 70-90C and cold-setting [103]. Green leaf proteins form strong gels at relatively low concentrations (2-10%) that perform better than whey protein, soy protein, or egg white protein as shown for sugar beets [52], duckweed [87], or spinach [46]. Gelation can be partially improved by the high temperature, short time extrusion of the protein ingredients [104] or the enzymatic treatment with transglutaminase [105].

4.1.3. Foaming and stability

Foaming of the protein depends on its ability to position at the air-solution interface and to maintain this configuration. This is achieved by interactions of polar and hydrophobic amino acid regions with the respective media, and also defines the water and oil absorption capacity of the

protein, respectively. Green leaf proteins form better foams at lower pH 2-3 [106] than neutral pH 7 [52]. Foaming and absorption capacities of protein ingredients may be improved by homogenization [107] and acetylation [108], while hydrolyzation is generally detrimental [109].

4.1.4. Emulsifying properties

A good balance of polar and hydrophobic regions is also required for the protein to interact well with both water and oil to form emulsions. This can be achieved by partial denaturation of the proteins to expose the hydrophobic regions, however the degree of denaturation that produces smaller and more desirable micelle size is specific to the target protein ingredient and difficult to control [110]. While certain plant proteins are good emulsifiers as shown for soy [111] and potato [112], the emulsifying properties of green leaf proteins are not well studied. Recent studies indicated that Rubisco protein showed average emulsification properties [52], which seem to improve with a more alkaline pH in case of alfalfa [113]. A partial denaturation increases emulsification when caused by high pressure homogenization [99], glycation [100], and a combination of pH shifting and ultrasonication [101].

4.2. Nutritional aspects of green leaf protein

Nutrient profiles of green leaf biomass (plant leaves) differ from those of plant seeds in protein quality, vitamin and mineral concentration, and omega 6/3 fatty acid profiles [114]. In contrast to cereal grain proteins deficient in lysine and/or tryptophan, or legume seed proteins deficient in methionine and/or cysteine, green leaf proteins nearly match the FAO standard of a complete protein (**Table 1**). Green leaf concentrates (green crude preparation with approx. 50% protein content) are a good source of vitamins such as β -carotene (provitamin A), B6, B12, E, and K, as well as several micronutrients including iron, calcium, and magnesium [43] that are regarded

as common mineral inadequacies in modern diets [115]. In contrast to edible seeds enriched with omega 6 fatty acids, green leaves accumulate more omega 3 fatty acids including α -linolenic acid, a major precursor to the EPA and DHA metabolites [116]. Nutritional value is therefore clearly on the side of increasing the proportion of green leaf biomass-derived food ingredients in human diet, yet some challenges remain as briefly described below.

4.2.1. Amino acid composition

When considering alternative proteins in human diets and animal feeds, aspects such as protein content, amino acid profile, digestibility, essential amino acid (EAA) deficiencies, anti-nutritional factors and palatability must be addressed. Imbalances in essential amino acid (EAA) profiles are common for many plant proteins. They are defined by the indispensable amino acid content of a protein (mg/g) versus a theoretical reference (complete) protein, and the lowest ratio delimits the most limiting amino acid [117]. Legumes are often lacking in methionine, while grains are generally poor in lysine. Green leaf proteins satisfy the FAO standard of a complete protein similar to animal foods (**Table 1**). Its completeness is mostly defined by high Rubisco and other chloroplast protein content which is highly conserved at both gene and protein level [118]. When fractionated, Rubisco is expected to end up in the white fraction of green leaf proteins [42].

4.2.2. Digestibility and antinutritional components

To further standardize the nutrient analysis of the protein, it is corrected for the fecal true digestibility (PDCAAS) or ileal digestibility (DIAAS). While most of the animal protein have PDCAAS at or very near 1.0, plant protein scores are usually lower due to amounts of one or more indispensable amino acids, and the presence of antidigestive (antinutritional) factors. In general, the digestibility of green leaves improves with processing, and is highest in the white fraction

[119], while higher temperatures used during the concentration, isolation, or drying steps decrease leaf protein digestibility [120].

4.2.3. Applications to feed and foraging systems

Current conversion rates of plant to animal protein in the conventional agricultural food systems were estimated at only 3-13% [125]. Therefore, reallocating the agricultural land used for beef feed to production of alternative feeds may be environmentally sound and economically feasible. Additionally, focusing on green leaf crops that can be produced in the extreme or marginal ecological niches not utilized by current agricultural practices due to temperature, precipitation, or salinity, may allow for generation of additional revenue streams. Together, these factors hold the potential to disrupt the current economic cycle for using plant seeds as international commercial commodities, feeding seed protein to livestock, and using excess seed oils in fast-food industry. Marginal, dry, or high salinity pastures are limited in many nutrients including vitamin E, resulting in nutritional myopathy and less hydrated carcasses. These can be partially compensated for with introduction of alternative forages such as saltbush (*Atriplex cinerea* Poir.) [126] or orache (*Atriplex hortensis* L.) [127] as unexplored sources of green leaf protein. During the first step of green leaf protein isolation (**Figure 2**), near half of the protein with a comparable amino acid profile is captured in the insoluble green fibrous material (pulp) suitable for high quality ruminant feed production [128]. The use of novel forage crops and their green leaf proteins in animal feed can help to improve the nutritional value of the feed, enhance animal performance and reduce the reliance on traditional protein sources, especially on marginal soils and in areas affected by salinity.

4.2.4. Agrochemicals and reuse of treated wastewater

Current agricultural practices include widespread use of agrochemicals, pesticides, fertilizers, and treated wastewater that resulted in global exposure of agricultural crops and natural ecosystems to many synthetic chemicals and nanomaterials [129]. Some of these chemicals may impact human health, physiology, reproduction, and development through a variety of neuroendocrine effects [130]. Green leaf biomass, whether produced in conventional, greenhouse, or vertical farming settings, remains at risk of a direct exposure and transfer of these chemicals when integrated into the food manufacturing processes. Precise management of environmental and health risks, sustainable and safe use of agrochemicals, internationally-adopted maximum residue limits, and integration of alternative agricultural management strategies will ensure that consumers can be confident that their food meets the agreed standards for safety and quality.

5. Conclusions

Green leaves are also a diverse source of secondary metabolites and other antinutritional (yet possibly health-promoting) [3] factors that may decrease protein digestibility, biological value, and net utilization by various mechanisms such as cross-linking and reduced solubility. These mechanisms include direct oxidation and formation of quinone complexes and dark melanin pigments mediated by the chloroplast-derived polyphenol oxidase, peroxidases, or laccase enzymes [121]. The resulting protein-phenol complexes showed a marked decrease in free amino groups, increased protein derivatization that reached maximum at pH 10, and changes in protein digestibility that inhibited pepsin and favored trypsin digestion [122]. This can be partially prevented with addition of higher amounts of sulfites [123] or several alternative treatments [72]. Among the other secondary plant metabolites of concern, phytates generally do not accumulate in green leaves, however tannins, saponins, lectins, chlorophyllides, protease inhibitors, oxalates, and phytoestrogens may decrease the quality of leaf protein concentrates and need to be addressed in

a species-specific approach. While these purported antinutrients may decrease protein digestibility and absorption of certain minerals, recent findings in their application to other human health outcomes and metabolism by human microbiome may warrant a reevaluation of current guidelines on their safety and use in foods [124].

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CHAPTER 2. Baseline optimization of leaf protein extraction from a resource-tolerant garden orache crop (*Atriplex hortensis* L.)

Abstract

The rising global demand for protein, driven by increased purchasing power and consumer preference for protein-fortified foods, has heightened the need for alternative sustainable protein sources. Garden orache or mountain spinach (*Atriplex hortensis* L. cv. Golden), a drought-tolerant leafy green crop with a rich history of human use, offers a promising solution due to its ability to thrive in poor soil conditions and marginal environments. This study focused on refining methods to maximize the quantity and quality of *Atriplex* leaf protein using scalable production techniques. The most effective approach combined alkaline pretreatment with mild heating and isoelectric coagulation, yielding the highest protein recovery. Findings also revealed that *Atriplex* leaf proteins exhibited isoelectric points between pH 3.0 and 5.5, with hydrochloric acid being the most effective for maximizing protein recovery in this range. These results highlighted the potential of *Atriplex* as a sustainable, resource-tolerant crop for protein production, offering a viable alternative to traditional animal-based proteins and contributing to global food security in the face of climate change.

1. Introduction

As the global population is projected to reach over 8 billion by 2050, the demand for dietary protein will continue to increase [1]. Current food sources, particularly animal-based proteins, are not sustainable enough to meet the growing needs without significant societal and environmental impacts [2]. This is particularly true for less privileged populations with limited access to nutritious and affordable commodities, with over 122 million people entering hunger since 2019. This alarming increase has pushed the total number of people experiencing hunger to 735 million, or

10% of the global population [3]. Protein has always been identified as the key macronutrient most closely linked to malnutrition, as it is essential for maximizing growth, development, and health [4].

Adding to this urgency are shifting socio-economic trends that have accelerated the global demand for protein. Rising purchasing power worldwide is directly correlated with increased protein consumption [1]. Additionally, food products are increasingly marketed with additional protein fortification to appeal to consumers [5]. However, consumer appeal is not driven solely by protein content; ethical considerations regarding sourcing also play a significant role [6]. As the climate continues to change, environmentally harmful practices have come under scrutiny, and animal-based protein sources have been identified as a significant contributor to environmental degradation. As much as 18% of anthropogenic global greenhouse gas emissions have been attributed to livestock. When considering that global pastures are twice the size of cropland, with one-third used for animal feed [7], the cumulative impact of greenhouse gas emissions from animal-based proteins raises this figure to 32% [8]. All these reasons make it apparent that increasing global food security is closely linked to sustainable (and likely plant) protein production practices.

Green leaf biomass has been heralded for a long time as the most abundant source of protein in the world [9]. This is largely due to the high stromal content of ribulose-1,5-bisphosphate carboxylase-oxygenase (Rubisco), an enzyme that plays a critical role in photosynthesis. Because all photosynthetic biomass contains Rubisco, and given the sheer volume of greenery on Earth, the global dry weight of Rubisco has been estimated to exceed 30 billion tonnes [10]. Green leaf biomass also offers a wealth of opportunities as a bio-renewable resource, making it a critical

component in sustainable agriculture, energy production, and environmental management. It is a natural, continuous, and readily available material that presents an attractive option for renewable resource development [11]. Furthermore, the global availability of green leaf biomass makes it a resource that can be locally sourced, reducing transportation costs and promoting regional economic development [12].

The extraction of leaf protein has been a topic of interest since the early 1900s, with the first patent for making food from green leaves appearing in 1927 [13]. However, it wasn't until the 1940s and 50s that industrial manufacturers began exploring the commercial potential of scalable leaf protein extraction [14]. Notably, the first commercial use of green leaf protein was seen in the development of PRO-XAN, a protein-xanthophyll concentrate derived from alfalfa (*Medicago sativa* L.) for poultry feed [15]. In addition, a protein concentrate made from a blend of mixed grasses and berseem clover (*Trifolium alexandrinum* L.) was given to 100 children over an 8-month period, demonstrating nutritional value comparable to milk protein supplementation [16]. While production demonstrated technical feasibility, leaf protein was subsequently deemed less profitable and appealing to consumers compared to traditional seed crops [17]. Consequently, green leaf protein fell by the wayside, and aside from a few niche applications, technological limitations and cost inefficiencies hindered its commercial viability.

In the last decade, with modern technological advances that enhance the quantity and quality of leaf protein products, and the growing need for sustainable protein sources, research into leaf protein extraction is experiencing a resurgence [18]. Contemporary efforts to re-commercialize leaf protein extraction can be seen in the emerging large-scale facilities utilizing cabbage (Naylor Farms, UK), sugar beets (Cosun Beet Company, Netherlands), alfalfa (Leaft Foods, New Zealand), and duckweed (Plantible Foods, USA). The need for new crops cultivated

for green leaf protein production therefore becomes increasingly evident. Traditional plant protein sources such as soy, corn, and wheat dominate the current market but often lack a complete amino acid profile and carry allergenic risks, thus limiting their use in diverse diets. Developing new crops for green leaf protein production can provide a more balanced protein source with a complete amino acid profile similar to animal proteins, especially in regions where protein deficiency is prevalent and agricultural lands are limited or marginalized [18].

Orache, mountain spinach, or saltbush (*Atriplex hortensis* L.; лобода, лобідка, лутига in Ukrainian) is a green leaf crop with an extensive history of prior human use. It includes several varieties distinguished by their leaf color that were widely cultivated in Europe and Asia since roman and medieval times [19]. Apart from direct use of orache green leaves in salads, soups, and stews similar to spinach, *Atriplex* was also a popular famine food and a traditional remedy [20]. The plant can be cultivated both as garden greens or forage due to its ability to thrive in challenging environments with poor soil quality, higher salinity, or limited water resources. This trait contributes to its classification as a resource-tolerant species, offering a sustainable option for food production in marginal, drought-prone, and coastal areas.

Atriplex hortensis accumulates a high level of raw protein (16 to 25.2 g/100 g) in the leafy tissue [21]. This yield is achieved at the age of 14 weeks, when the plant produces up to 14 tonnes/ha dry matter and 1,200 kg/ha total protein [22]. Approximately 60% of the plant proteins can be extracted into the plant juice, from which about 30% can be obtained as a white protein fraction [23]. Enhancing this extraction process could significantly improve the recovery rate of high-quality protein, making the orache crop a more viable and sustainable source of green leaf protein. In this study, we applied an integrated approach combining alkaline pretreatment, mild

heating, and isoelectric coagulation, tailored to maximize protein yield and preserve key functional properties of *Atriplex* leaf proteins.

2. Materials and Methods

2.1. Plant materials and growing conditions

Six cultivars of orache (*Atriplex hortensis*) distinguished by their leaf color — Aurora (mixed colors), Green Velvet (dark green), Golden (pale green), Magenta Magic (purple), Ruby Gold (dark red), and Triple Purple (dark purple) — were procured from Wild Garden Seeds (Philomath, OR, USA). Seeds were germinated at the NC State University Piedmont Research Station (Salisbury, NC, USA) and transplanted into the white plastic-covered, irrigated beds that had been supplemented with standard fertilizer in early July. Cultivars were evaluated based on their propagation rates and vegetation stand height over 10 weeks of cultivation, and the variety with best agricultural characteristics (Golden) was harvested in mid-September for further analysis.

2.2. Plant samples

Leaves were stripped from the stalks manually and kept under 3 different storage conditions. Fresh leaf samples were vacuum sealed in the airtight plastic bags and stored in the 4 °C refrigerator. Frozen leaf samples were vacuum sealed in the airtight plastic bags, flash-frozen in liquid nitrogen, and stored in the -20 °C freezer for 48 h before testing. Flash-frozen leaves were also freeze dried in a Labconco Freezone 2.5 Benchtop freeze dryer (Kansas City, MO, USA) for 56 h before pulverization to fine powder using a Cuisinart SG-10 Spice and Nut Grinder (Stamford, CT, USA). The freeze-dried powders were then transferred to an airtight container that was kept at -20 °C until further processing.

2.3. Crude protein determination

Leaf moisture content was determined in fresh leaves kept at 4 °C using an Ohaus MB-23 moisture analyzer (Parsippany, NJ, USA). Uniform 20 mg leaf sections were heated to 160 °C and the moisture content was recorded automatically when the analyzer detected the weight loss has ended. Next, 300 mg dry leaf samples were loaded in triplicate in an Elementar Rapid N Exceed nitrogen analyzer (Langenselbold, Germany) and the nitrogen content in samples was calculated based on the Dumas combustion method. The equipment was calibrated with an aspartic acid standard, and an N-prot conversion factor of 6.25 was used to obtain the protein content of the samples as described previously for *Atriplex lampa* [24].

2.4. Atriplex leaf protein concentrate by wet fractionation

The recovery process of heat coagulation (separation of insoluble leaf proteins), isoelectric precipitation, and density separation (soluble leaf proteins) proves to be a cost effective and scalable way to recover green leaf proteins [25]. In this study, we applied the small-scale protein isolation protocol that used 20 mg dry leaf tissue (an equivalent of 100 mg fresh leaf weight) homogenized in 500 µl alkaline reagent as described below. The samples were further solubilized in a Branson CPX5800H ultrasonic bath (Brookfield, CT, USA) set at 24 °C at 40 khz frequency for 30 min. Heat coagulation of the membrane-bound leaf proteins was achieved in a hot water bath set to 95 °C for 15 min. Coagulated proteins were removed by centrifugation at 15,000 g for 10 min, resuspended in 500 µl of 0.1 N sodium hydroxide (green protein), and the insoluble precipitate was removed by centrifugation. The supernatant proteins were precipitated with 10% final concentration of trichloroacetic acid at 4 °C for 1 h, centrifuged, and the resulting pellets were resuspended in 500 µl distilled water (white protein).

2.5. Quantification of soluble proteins

Bradford protein assay was performed with 5 μ l of protein samples, 5 μ l of distilled water, and 300 μ l of the Pierce Bradford Plus Protein Assay reagent (Thermo Fisher Scientific, Pittsburg, PA) in a 96-well microplate gently shaken for 1 min and incubated for additional 10 min at room temperature, and protein concentrations were determined spectrophotometrically at 595 nm against the appropriate blank using a BioTek Synergy H1 spectrophotometer (Winooski, VT, USA). Serial dilution of Pierce Bovine Serum Albumin was used to generate a standard calibration curve.

2.6. Optimization of protein solubilization in alkaline reagent

Small scale protein isolation protocol was performed using 500 μ l of 0.025 (control) or 0.025, 0.05, 0.1, 0.20, 0.25, and 0.30 N sodium hydroxide and potassium hydroxide as specified in each experiment. All other extraction parameters remained unchanged.

2.7. Optimization of heat-assisted extraction

Small scale protein isolation protocol was performed using 500 μ l of 0.05 (control) or 0.1, 0.25, 0.5, 0.75, 1.0, and 2.0 N sodium hydroxide as specified in each experiment. At the heat coagulation step, samples were kept in the 95 °C water bath for 0 (control) or 2, 10, 15, 30, 60, 120, or 180 min as specified in each experiment. All other extraction parameters remained unchanged.

2.8. Optimization of alkali reagent and heat-assisted extraction

Small scale protein isolation protocol was performed using 500 μ l of sodium hydroxide, potassium hydroxide, and calcium hydroxide reagent using a narrower dose range of 0.025 (control) or 0.05, 0.1, 0.15, 0.2, 0.25, 0.3 and 0.35 N concentrations as determined in a previous optimization step (section 2.6). The heat coagulation step of green protein was performed in the

95 °C water bath for 15 min as determined in a previous optimization step (section 2.7). All other extraction parameters remained unchanged.

2.9. Optimization of isoelectric precipitation of white protein fraction

Small scale protein isolation protocol was performed with 0.35 N sodium hydroxide for solubilization followed with 95 °C water bath incubation for 15 min for heat coagulation of the green protein (as determined in a previous optimization steps 2.6-2.8). Isoelectric precipitation of the white protein was evaluated using a pH range of 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, and 6.0 adjusted with 1 N hydrochloric acid. All other extraction parameters remained unchanged.

2.10. Optimization of the acid reagent

Small scale protein isolation protocol was performed with 0.35 N sodium hydroxide for solubilization followed with 95 °C water bath incubation for 15 min for heat coagulation of the green protein (as determined in a previous optimization steps 2.6-2.8). Isoelectric precipitation of the white protein was evaluated at a pH of XXX (as determined in a previous optimization steps 2.9) using hydrochloric, acetic, or citric acid. All other extraction parameters remained unchanged.

2.11. Statistical analysis

Data was analyzed by one-way ANOVA followed by Dunnett's multiple-range tests using Prism 8.0 (GraphPad Software, San Diego, CA). All data was presented as means \pm SEM. Significant differences were accepted when the p-value was <0.05 .

3. Results and Discussion

While plants are diverse, they share a major common set of proteins in their photosynthetic tissues, which capture sunlight energy and synthesize carbohydrates. These proteins can be divided

in two functional groups based on their solubility and spatial distribution. The larger group (50-60%) consists of hydrophobic and less soluble proteins in neutral solutions that directly interact with chloroplast and cell membranes, as well as have the capacity to bind fibrous polysaccharides, pigments, and other plant secondary metabolites [26]. This fraction is particularly enriched with structural proteins that include photosystem I and II, ATP synthases, chlorophyll A/B binding proteins, and ribosomal proteins. Due to their spatial distribution within cell membranes and polysaccharide networks, these proteins precipitate together with chloroplast fragments at a lower coagulation temperature of about 55 °C (a range of 40-60 °C), and are therefore designated as insoluble or green proteins [27].

The remaining stromal and cytoplasmic proteins form a more soluble fraction with a higher coagulation temperature of about 65 °C (a range of 60-80 °C) [28]. This fraction is designated as soluble beige (white proteins) dominated by Rubisco that accounts for up to 50% of soluble leaf protein [29]. Rubisco has advantageous functional features in terms of enhanced gelation, foaming, emulsification, and textural properties [30]. When green leaf juice is non-selectively boiled at temperatures that exceed 90 °C, 1 kg of leaves produces about 50 g of green curd (25 g of crude protein of low functionality), 500 g of wet fiber, and 450 g of brown liquid as byproducts [31]. Thus, efficient, and cost-effective wet fractionation of green and white proteins enhances the usability and value of leaf-derived proteins by separating them into distinct fractions with specific functional properties, thereby improving their application in food, feed, and industrial products.

3.1. Compositional parameters

Among six *Atriplex* cultivars used in this study, Golden variety showed better propagation rates and vegetation stand height after 10 weeks of cultivation (Figure 1) and was therefore selected

for the in-depth study. A chromosome-scale genome assembly for this cultivar has been also reported recently [32].

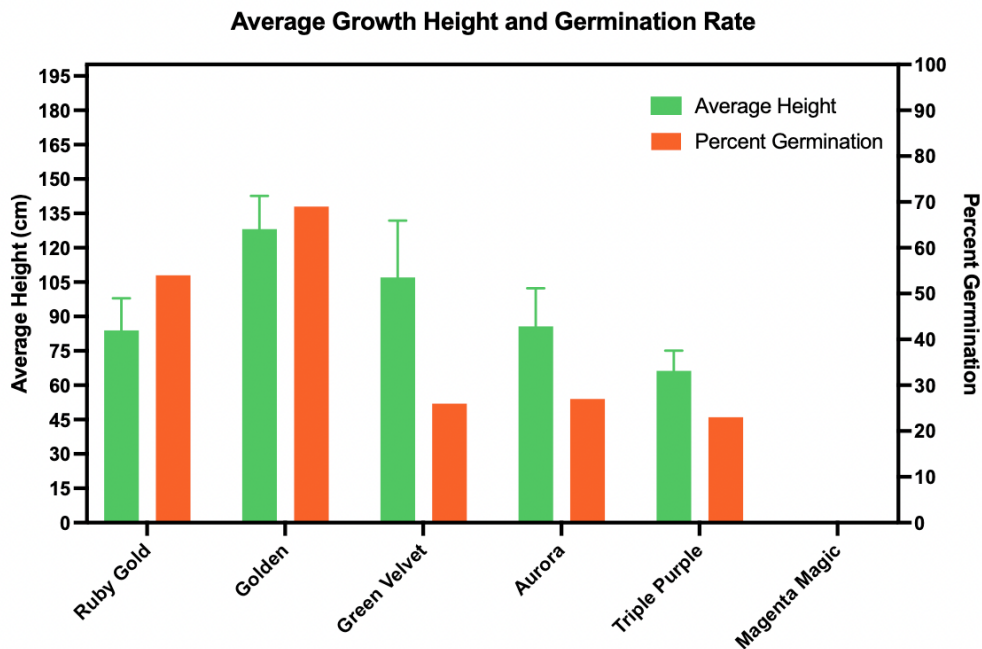


Figure 1. Average plant height and germination rate of six cultivars of *Atriplex hortensis*, Ruby Gold, Golden, Green Velvet, Aurora, Triple Purple, and MM.

This *Atriplex* variety had a dry matter content of 22.8 ± 3.3 %. The protein content of dry leaves was about 21.5 ± 1.4 % (or 4.9 ± 0.3 % on fresh weight basis). This compares similarly with other green leaf crops evaluated for leaf protein production, including sugar beet leaves (18%) and spinach leaves (28%) on dry weight basis [33].

3.2. Soluble protein extraction

The optimal extraction methodology of soluble proteins from leafy biomass is highly influenced by the manipulation of pH and temperature. Alkali extraction solvents are commonly

used to enhance the recovery of proteins from plant biomass across various established agricultural crops, including soybeans [34], peas [35], and barley [36], among many others. The process involves the use of high amounts of basic hydroxide ions, which play a crucial role in breaking down the cell structure to release proteins more efficiently. These ions partially degrade the glycosidic linkages in polysaccharides, saponify lipids within the cell membrane, and consequently aid in cellular disruption, thereby increasing protein recovery.

Sodium hydroxide was selected as the alkaline reagent for the initial extraction process due to its cost-effectiveness and potential for commercial application [37]. Although other studies have explored the use of ammonia for food-grade protein production, achieving profitability at a pilot or commercial scale with ammonia requires a stripping column to remove and recover the ammonia [38]. This adds significantly to the initial setup cost for both pilot and commercial operations. If the alkaline solution is strong enough, it can also hydrolyze the protein structure into smaller, more soluble amino acid fragments [39]. Figure 2 shows the effect of sodium hydroxide applied in the dose range of 0 (distilled water) or 0.05, 0.1, 0.25, 0.5, 0.75, 1.0, and 2.0 N during the first step of the small-scale protein isolation protocol from *Atriplex* leaves.

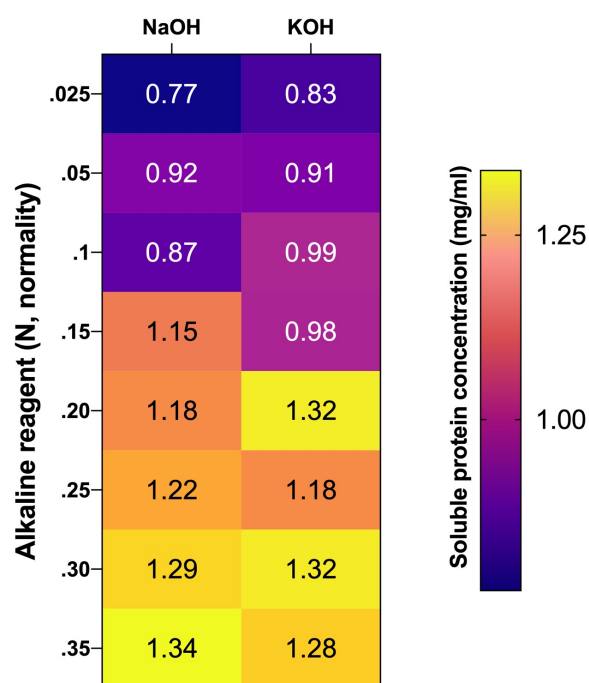


Figure 2. Extraction performance of sodium hydroxide (NaOH) and potassium hydroxide (KOH) over the dose range of 0.025 - 0.3 N in the first step of the small-scale protein isolation protocol from Atriplex leaves.

Starting at 0.15N sodium hydroxide, the soluble protein extraction is enhanced 2-fold. Higher normality of potassium hydroxide was required to achieve similar results, and both alkali agents showed highest soluble protein recovery from Atriplex leaves at the upper concentrations used (0.3-0.35 N), thus suggesting that higher alkali concentrations may further increase recovery of leaf proteins (Figure 2).

Based on this data, sodium hydroxide was chosen for optimization of the next extraction step that included additional application of heat during the initial leaf protein solubilization. The use of heat pretreatment has been explored in various applications of protein recovery because it

can significantly enhance the extraction process. Heat pretreatment helps precipitate and remove a substantial portion of insoluble plant-cell proteins, secondary metabolites, and disrupts cell structure. This process improves the efficiency of subsequent purification steps and enhances the quality of the purified soluble protein [40].

When optimizing for soluble protein recovery, the interplay between pH and temperature was explored to develop an efficient and cost-effective method for isolating leaf protein. Using freeze-dried *Atriplex* leaves, assays determined that brief exposure to elevated temperatures (10 min at 95 °C) combined with moderate alkalinity (0.75N sodium hydroxide) enhanced the recovery rate of the soluble protein fraction to 72.6%, when compared to a control sample with 0.05N sodium hydroxide and no heat treatment. At each normality, there was a specific heat treatment that maximized soluble protein recovery. At lower normality, longer exposure to heat resulted in the greatest soluble protein recovery. This U-shaped response indicated two areas of higher soluble protein recovery in the ranges of 0.25 N sodium hydroxide solubilization at 95 °C for 30 min (peak 1), and 0.75 N sodium hydroxide solubilization at 95 °C for 10 min (peak 2). Curiously, there was a third area of enhanced protein solubilization observed at 0.25 N sodium hydroxide solubilization and no heat treatment (Figure 3), and the significance of this finding remains elusive.

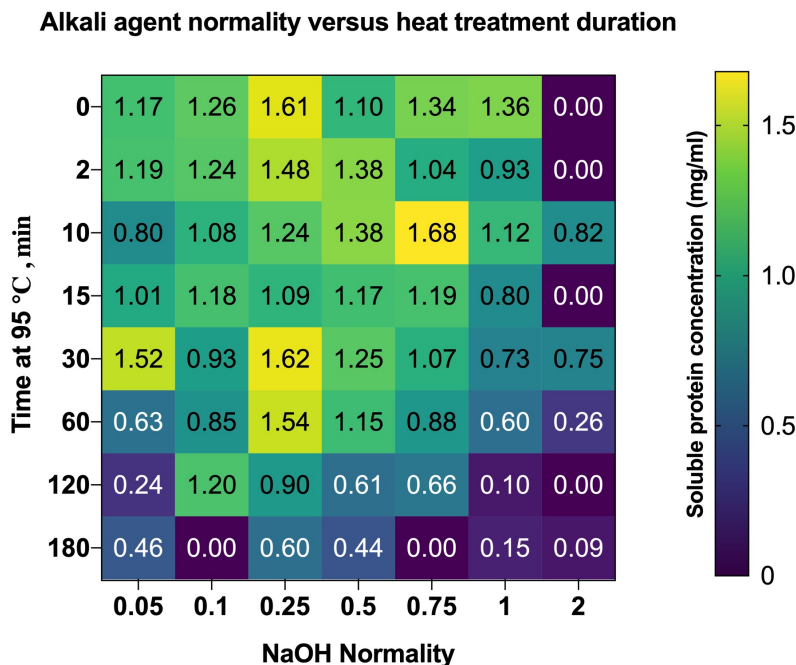


Figure 3. Extraction performance of sodium hydroxide (NaOH) over the dose range of 0.05 - 2 N when combined with the 95 °C heat treatment for 0-180 min in the first step of the small-scale protein isolation protocol from Atriplex leaves.

Heat can be effectively applied during the extraction process to enhance the recovery of soluble leaf proteins. By applying heat to the input biomass, the cell structure is disrupted, which releases compartmentalized proteins and makes them more accessible for subsequent purification and extraction. This disruption facilitates a higher yield of soluble proteins [41]. However, excessive heat and alkaline treatment can negatively impact protein recovery. High temperatures can alter protein structures by breaking molecular bonds, exposing hydrophobic interior regions, which can lead to aggregation and loss of solubility [42], as evident at higher normality (1-2N) of sodium hydroxide used in this study (Figure 3). The combination of low normality and short heat treatment was further evaluated both for sodium hydroxide and potassium hydroxide (Figure 4).

Alkali agent normality at a set heat treatment duration

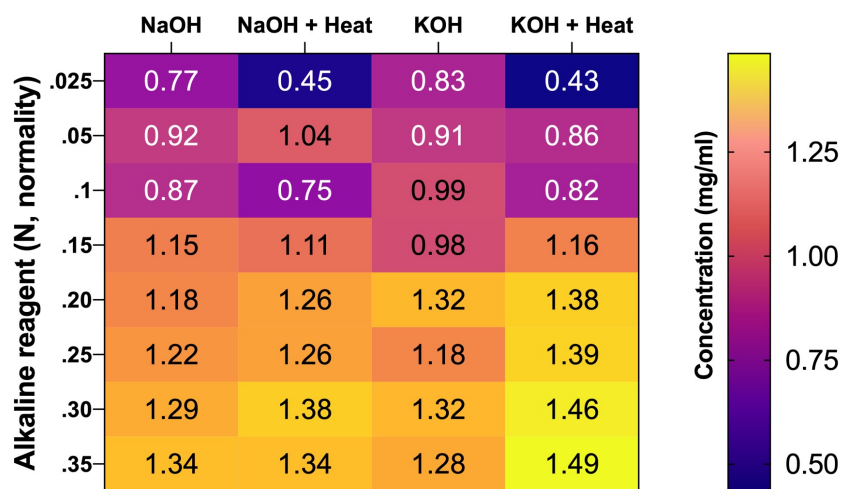


Figure 4. Extraction performance of sodium hydroxide (NaOH) and potassium hydroxide (KOH) over the dose range of 0.025 - 0.3 N when combined with heat treatment (95 °C for 15 min) in the first step of the small-scale protein isolation protocol from Atriplex leaves.

When optimizing a bi-phasic protein extraction protocol, it's important to consider whether specific temperatures or pH levels are required for sterilization or functional modifications. Once these requirements are established, the pH or temperature can be adjusted to maximize the recovery of soluble proteins. In this study, a threshold likely occurred beyond 30 min of heat treatment, where a significant decrease in soluble protein recovery is observed for every normality treatment except for 0.25N. This observation could indicate that at this point, the soluble proteins were likely denatured and removed during centrifugation along with other cellular debris, which significantly reduced downstream protein recovery.

The area of 0.25N sodium hydroxide application, however, remained effective at recovering similar amounts of soluble Atriplex leaf proteins whether combined with heat treatment or without it, and this phenomenon was once again observed not only for sodium hydroxide, but also for potassium hydroxide. This observation supports the presence of the third area of enhanced protein solubilization observed when using 0.25 N alkali agent in the earlier experiments (Figure 3) and warrants further investigation into the mechanism of these effects, as well as their application to the large-scale protein isolation from Atriplex leaves. It is possible that heat application might have led to excessive protein denaturation due to the lack of ionic stabilization, causing the soluble proteins to aggregate and become insoluble at low alkali normality when combined with heat treatment, as observed previously [43].

3.3. Soluble protein precipitation

Protein solubility is also significantly affected by the presence of bases or acids during the extraction steps. Proteins are generally less soluble at their isoelectric point (IEP), where the protein's surface charge is neutralized by ions, and this characteristic is often used for selective and abundant precipitation of the target proteins from the extracted plant liquids. This neutralization reduces electrostatic repulsion between proteins, leading to aggregation and precipitation, and the further the pH of protein solution deviates from this point, the more soluble the protein tends to become [44]. Most leaf proteins, including Rubisco, have acidic isoelectric points. For example, in spinach Rubisco is characterized by the isoelectric point of pH 6.0-6.1 and the denaturation temperature of about 65 °C [45].

Because Rubisco makes up a majority portion of the soluble fraction of leaf protein content, the expected IEP of solubilized Atriplex extractions was expected to mimic that of Rubisco. However, the IEP of Rubisco can be influenced by secondary metabolites present in the leaf tissue

[18] that act as natural buffers [46], and therefore varies over a significant range of pH 4.4 and 6.0 [47]. Figure 5 shows the recovery of Atriplex soluble leaf protein from the supernatant after pH adjustment with hydrochloric acid.

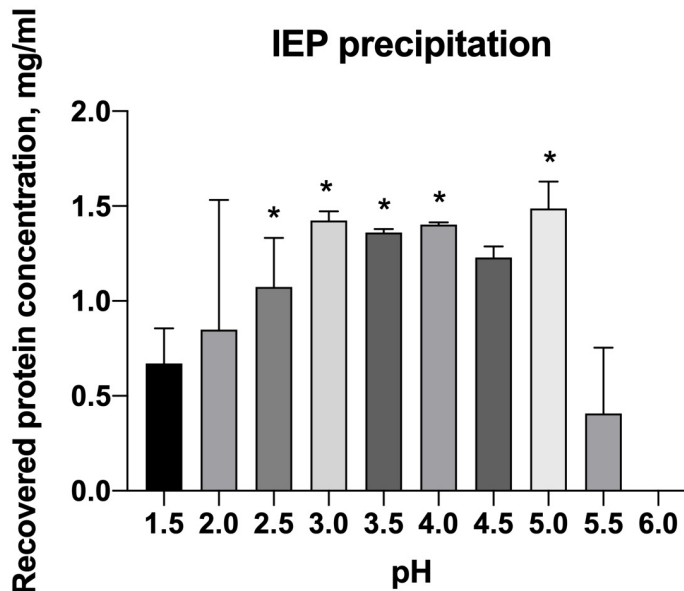


Figure 5. IEP precipitation of Atriplex leaf protein over the pH range of 1.5-6.0. Data were analyzed using one-way ANOVA followed by Dunnett's multiple comparisons, * $p < 0.05$ versus pH 6.0 control.

At a low pH of 1.5-2.5, protein recovery was decreased, suggesting that extreme acidity below the average Atriplex leaf protein IEP re-impacted a net positive charge on the proteins and resulted in lower yields. Additionally, the low pH could lead to substantial hydrolysis of the soluble protein into smaller peptide fragments and amino acids that are not detected by the Bradford assay effectively [48]. The precipitation was highest at a wide IEP pH range of 3.0-5.0, suggesting the presence of proteins other than Rubisco, or partial dissociation of Rubisco into the individual

subunits under these conditions. At this point, it is not clear what pH in that range leads to the optimal preservation of functional capabilities of IEP recovered Atriplex proteins, and this point also warrants further investigation.

This study also compared the recovery efficiency of three commonly used food-grade acids — citric, acetic, and hydrochloric acid — in the protein IEP precipitation step. While all three acids showed similar iso-electric point recovery peaks, their efficiency and peak extraction rates varied slightly (Figure 6).

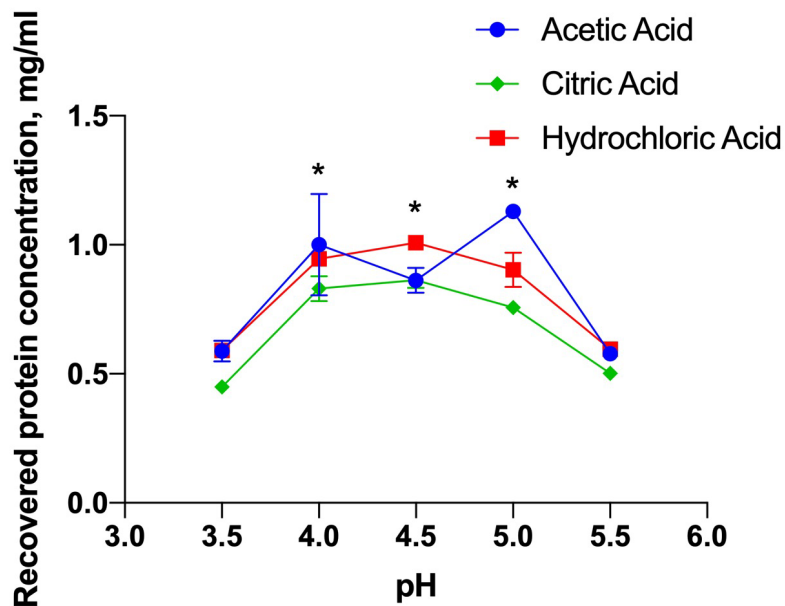


Figure 6. IEP precipitation of Atriplex leaf protein over the working pH range of 3.0-6.0 with commonly used food-grade acids. Data were analyzed using one-way ANOVA followed by Dunnett’s multiple comparisons, * $p < 0.05$ versus pH 5.5 control.

Notably, acetic acid was the only one to display a double peak, similar to the results with the original extraction (Figure 5), suggesting that the type of acid used might influence the

dissociation of protein subunits. In contrast, hydrochloric and citric acids exhibit more rounded extraction curves, with peak extraction occurring at pH 4.5. To maximize the yield of protein recovered from *Atriplex* leaf biomass, hydrochloric acid showed a clear peak in protein recovery, making it advantageous for not selectively prioritizing specific Rubisco subunits in the final product.

Salting out is a commonly used alternative for bulk protein recovery, particularly for high-value proteins such as pharmaceuticals and bio-actives [49]. While it is often effective in isolating proteins gently to preserve their structure and function [50], this method is costly, requiring large amounts of salt—up to 50-70 times the amount of recovered protein [51]. Additionally, it necessitates extensive downstream processing, such as dialysis or stripping columns, to remove the salt from the protein [52]. Membrane filtration is another viable option for the food-grade protein purification and leaf protein isolation that produces high-quality protein concentrates and isolates while preserving functional attributes like gelling, emulsifying, and foaming [33]. However, membrane extraction requires sophisticated infrastructure, increasing initial setup costs, and is prone to degradation and fouling due to the binding of secondary metabolites and cellular debris that needs to be resolved on case by case basis [53]. Iso-electric precipitation however still offers a cost-effective and widely used alternative for protein recovery in commercial food industries that is unlikely to change in the near future [54].

5. Conclusions

This study provides the early insights into isolation and characterization of green leaf protein from garden orache (*Atriplex hortensis* L.), a resource-tolerant species that can be commonly grown as an agricultural or forage crop. The optimal extraction of soluble proteins from *Atriplex* leafy biomass was heavily influenced by pH and temperature, with sodium hydroxide

proving to be an effective and cost-efficient alkali reagent for enhancing protein recovery. While the best results were observed using 0.75 N sodium hydroxide at 95°C for 10 minutes, yielding a 72.6% protein recovery rate, a unique area of enhanced protein solubilization at 0.25 N sodium hydroxide with or without heat treatment suggested a complex interaction that warrants further investigation. This study also found that soluble Atriplex leaf proteins exhibited peak IEPs between pH 4.4 and 5.0, and that hydrochloric acid is most effective for maximizing protein recovery in this range. Overall, the study showed how Atriplex greens can be used to produce a protein concentrate with potential applications to food and dietary supplementation industries.

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Chapter 3. Functional assessment of alfalfa (*Medicago sativa* L.) green leaf protein across the spectrum of dietary proteins

Abstract

Plant sources contribute up to 60% of global protein intake, with plant seed proteins being the primary contributors. While seed proteins have been widely utilized, green leaf biomass remains an underexplored resource for sustainable protein production. In this study, we investigated the protein content, solubility, functional properties, and antioxidant activity of various alfalfa protein powders to assess their potential as viable alternative protein sources in food processing, contextualized by their direct comparison to common animal and pulse protein concentrates. Alfalfa leaf proteins showed variable protein content, and each functional attribute was impacted by their respective extraction strategies, with at least one leaf protein powder being competitive if not excelling in all aspects of functional characterization. Antioxidant activities, assessed using ABTS, FRAP, and DPPH assays, revealed that alfalfa leaf proteins, particularly the complete acid and green fractions, exhibited substantial antioxidant capabilities, that could be attributed in part to their chlorophyll and carotenoid content. These results underscore the potential of alfalfa green leaf biomass in diversifying and enhancing the global protein supply.

1. Introduction

The recommended dietary allowance (RDA) for protein in healthy adults ranges from 0.8 to 1.6 g/kg body weight per day, depending on physical activity, with intake above 2 g/kg/day generally not advised [1]. Globally, most dietary protein comes from plants (57%), though this ratio shifts in favor of animal protein (55-60%) in regions like Oceania, Europe, and the Americas [2]. While animal proteins generally score higher in terms of content, digestibility, and biological

value, additional processing techniques may enhance the digestibility and flavor of plant proteins to more closely match those of animal sources in the future [3].

The growing global demand for sustainable and diverse protein sources based on socioeconomic and environmental demands has therefore led to significant research into plant-based alternatives [4], including proteins from leafy greens. Leafy greens offer a unique opportunity due to their high nutritional value, widespread abundance in mass-cultivated species [3], abundant underutilized waste streams or discards associated with modern agricultural food systems [5], and recent advancements in scalable leaf protein extraction systems [6]. The term "leaf protein" encompasses the thousands of proteins found in the photosynthetic tissue of plants [7], which vary in number and function depending on the species and environmental conditions [8] and are generally categorized into soluble and insoluble fractions.

Insoluble proteins often make up the majority of proteins in leafy tissues, especially in C4 plants, though the ratio between soluble and insoluble proteins can approach 50/50 depending on growth stage and plant type [9]. These proteins are frequently complexed with insoluble cell components such as thylakoid membranes, cross-linked polysaccharides, and polyphenol networks which can make extraction and purification challenging due to potentially irreversible bonding [10]. When extracted, these proteins form what is known as the "green" fraction, associated with color-containing pigments and structures, leading to variability across different plants and complicating standardization efforts. The presence of secondary compounds in the green fraction, while nutritionally valuable, can negatively impact the functional and sensory characteristics of these protein extracts, making them difficult to incorporate into food products without additional processing or flavor masking [11].

Soluble leaf proteins, primarily found in the cytoplasm and stroma, play key roles in metabolic processes such as photosynthesis, respiration, and nutrient assimilation. The most prominent of these is ribulose 1,5-bisphosphate carboxylase/oxygenase, commonly known as rubisco, which can constitute up to 50% of a leaf's total protein content [9]. Rubisco's large, complex structure, consisting of 16 subunits, imparts it with unique functional properties in food systems, such as increasing viscosity, promoting gelation, and stabilizing emulsions and foams by adsorbing at interfaces [12]. These characteristics allow rubisco to outperform many other animal and plant proteins in forming stable textures by trapping water, oil, and air where they would otherwise be immiscible [13].

Leaf protein recovery varies significantly across different plants and extraction techniques, with typical yields ranging from 1% to 10% of protein relative to fresh leaf biomass and up to 1% for purified rubisco [14]. Thus, protein content in leaves on a dry basis can range from about 9% to 30% for the majority of agricultural crops and edible plant species, with spirulina (46-63% dry weight) [15] and duckweed (28-34% dry weight) [16] often reported at the upper range of these values. Among the most notable examples, moringa at 29% dry weight [17], alfalfa (lucerne) at 20-26% dry weight [18], cauliflower at 22% dry weight [19], and sugar beets at 19-24% dry weight [20] have been reported recently, with the extraction yield heavily dependent on the plant species and the method used. Methods like size exclusion, pH adjustment, and thermal denaturation are employed to separate soluble white proteins from insoluble green proteins, with purification yields varying widely even within the same species. Leaf protein extraction is thus highly variable and must be tailored to the specific plant and the desired application, whether it be for food, feed, or other uses. This variability also poses challenges for standardizing processes and optimizing yield across different species [10]. At the same time, this flexibility may allow for targeted extraction of

specific protein functionalities based on their intended application. For instance, extracting mostly soluble proteins is ideal for products needing high solubility and emulsification properties, like beverages and dressings, while a mix of soluble and insoluble proteins is better for creating textures in meat analogs, where gelation and water-binding are key [21].

A systematic comparison of green leaf proteins with commercial animal or plant protein sources is essential to assess their potential as viable alternatives in the food industry. Such comparisons provide insights into the nutritional value, functionality, and sustainability of leaf proteins relative to conventional sources, as it was shown previously for sugar beets leaf protein versus whey and soy protein isolates [22]. This understanding is critical for determining whether leaf proteins can meet industry standards for quality and performance in various applications. Additionally, comparing these proteins helps identify opportunities for improving extraction processes and optimizing their use in diverse food systems [6].

This study therefore aims to compare different fractions of alfalfa leaf protein to evaluate how various extraction strategies influence the functional properties and potential applications of the resulting protein fractions. By analyzing the effects of various extraction methodologies on protein solubility, emulsification, and foaming abilities, we seek to identify the most effective protocols for enhancing specific functional characteristics. This comprehensive evaluation can be useful in providing insights into how extraction methods can be fine-tuned to produce leaf protein isolates and concentrates with tailored properties, facilitating their integration into a variety of food products and contributing to more sustainable and diverse protein sources for the food industry.

2. Materials and Methods

2.1. Commercial protein powders

Two animal-based protein isolates were a 90% whey protein isolate with sunflower lecithin (Bulk Supplements, Henderson, NV, USA) and a 100% dried egg white protein isolate powder that contained 90% egg protein (NutriFoods, Owatonna, MN, USA).

Three plant seed storage protein sources were from pulses including a 90% soy protein isolate (Bulk Supplements, Henderson, NV, USA), an 80% pea protein isolate (It's Just! Foods, Claremont, CA, USA), an 85% fava bean protein isolate (Green Boy Products, Los Angeles, CA, USA), and an 80% chickpea protein isolate (Green Boy Products, Los Angeles, CA, USA). Additionally, one cereal seed storage protein source was a 75% wheat gluten protein isolate (Kate Naturals, Irvine, CA, USA).

Two green leaf protein sources were a 50% AlfaPro alfalfa protein powder (Bioriginal, Saskatoon, SK, Canada) and a 20% protein alfalfa grass powder (XPRS Nutra, South Jordan, UT, USA).

2.2. Fractionated alfalfa protein powders

Alfalfa crop was continuously grown and collected with a sickle herb cutter on a weekly schedule using standard agricultural practices at a farm immediately adjacent to The Leaf Protein Co manufacturing facility (Queensland, Australia). Herbage was harvested when leaf was young and pre-bolt (production of flowering stalk). The proximity of growing and manufacturing locations ensured that fresh-cut green material was processed within 30 min of collection without the need for additional stabilization or preservation steps during the transportation of green leaf materials.

Fresh green leaf tissues were then mechanically treated with a twin-screw press to disrupt plant tissues and expel green leaf juice. Four different alfalfa protein powders were produced from the green leaf juice using the following manufacturing strategies. Complete heat protein was

extracted using two tandem heat treatments to precipitate both green and white protein in a single sample (Leaf Complete Heat Protein, LCHP). Complete acid protein was extracted using isoelectric precipitation with hydrochloric acid to produce a combined green and white protein in a single sample (Leaf Complete Acid Protein, LCAP). Green protein was extracted using a single heat treatment step (Leaf Green Heat Protein, LGHP), and white protein was isolated through thermal treatment separately and subsequently to LGHP (Leaf White Heat Protein, LWHP). The supernatants were decanted, and the wet pellets were collected. Wet protein cakes were diluted with distilled water and spray dried, powder was collected as samples.

2.3. Crude protein determination

Sample moisture content was determined using an Ohaus MB-23 moisture analyzer (Parsippany, NJ, USA). Uniform 20 mg powder samples were heated to 160 °C and the moisture content was recorded automatically when the analyzer detected the weight loss has ended. Next, 300 mg powder samples were loaded in triplicate in an Elementar Rapid N Exceed nitrogen analyzer (Langenselbold, Germany) and the nitrogen content in samples was calculated based on the Dumas combustion method. The equipment was calibrated with an aspartic acid standard, and an N-prot conversion factor of 6.25 was used to obtain the protein content of the samples as described previously [23].

2.4. Quantification of soluble proteins

Bradford protein assay was performed with 5 µl of protein samples, 5 µl of distilled water, and 300 µl of the Pierce Bradford Plus Protein Assay reagent (Thermo Fisher Scientific, Pittsburg, PA) in a 96-well microplate gently shaken for 1 min and incubated for additional 10 min at room temperature, and protein concentrations were determined spectrophotometrically at 595 nm against

the appropriate blank using a BioTek Synergy H1 spectrophotometer (Winooski, VT, USA). Serial dilution of Pierce Bovine Serum Albumin was used to generate a standard calibration curve.

2.5. Solubility

Protein powders were suspended in distilled water at 7.5% w:v (15 g protein powder in 200 ml water) and mixed with a magnetic stirrer at 500 rpm for 5 min. The sample was divided into equal batches of 25 ml, and each batch was adjusted to the respective pH in the range of pH 4-8 with 1M hydrochloric acid or 1M sodium hydroxide, as appropriate. The samples were centrifuged at 3,000 g for 10 min, diluted 10x with distilled water, and 1 ml aliquots were subjected to soluble protein quantification in duplicate using the Bradford assay.

2.6. Foam stability and foam capacity

Working solution was prepared by suspending protein powders in distilled water. 1% w:v of protein (0.25 g protein powder in 25 ml water) was created for each protein, with an original volume of 25ml, and subjected to high-shear homogenization at 11,000 rpm for 2 min using the HQ-2509 High Speed Dispersion Homogenizer (Mxbaoheng, Mountain View, CA, USA). Foam capacity was determined by volume increase (%) immediately after homogenization by the formula $(V1 - V0)/V0 \times 100$, where V1 was the volume of protein solution after homogenization and V0 was the volume of the solution before homogenization. The foam stability was evaluated as the remaining foam volume after 60 min of incubation (V60), and expressed as percentage of the foam volume immediately after whipping (V1) by the formula $(V1 - V60)/V1 \times 100$ as described previously [24].

2.7. Emulsification properties

Protein powders were suspended in distilled water at 1% w:v (0.15 g protein powder in 15 ml water and 5 ml vegetable (corn) oil) and homogenized at 20,000 rpm for 3 min using the HQ-2509 Homogenizer (Mxbaoheng). The 100 µl emulsion aliquots were mixed with 10 ml of 0.1% SDS by gentle inverting, immediately after homogenization (T0) and 10 min later (T10). The 250 µl aliquots of the resulting mixtures were transferred to a 96 well microplate and absorbance was recorded spectrophotometrically at 500 nm against the 0.1% SDS blank using a BioTek Synergy H1 spectrophotometer (Winooski, VT, USA). The emulsifying activity index (EAI, m²/g) was calculated as $(2 \times 2.303 \times A_0)/(0.25 \times \text{protein weight})$. The emulsion stability index (ESI, min) was calculated as $(A_0 \times (T_{10} - T_0)) / (A_0 - A_{10})$ as described previously [25].

2.8. Free radical scavenging (ABTS/TEAC)

The ABTS•⁺ radical was generated by mixing 34.4 mg ABTS (7.4 mM) and 6.6 mg potassium persulfate (2.6 mM) in 10 ml of distilled water and stored overnight in the dark. The ABTS•⁺ radical working solution was prepared by diluting this stock to 49 ml methanol. Trolox standard was prepared as a series dilution of 15.75-1,000 µM. In a 96-well microplate, 190 µl of ABTS•⁺ solution and 10 µl of blanks, standards, or samples were added in triplicate. The plate was kept in the dark for 30 min, and absorbance at 745 nm against the appropriate blank was recorded using the Synergy H1 spectrophotometer (BioTek, Winooski, VT, USA). The results were expressed as µM of Trolox equivalents per 1 g of dry weight (µM TE/1 g DW) as described previously [26].

2.9. DPPH antioxidant assay

The solution of DPPH in methanol (0.07 mM) was prepared fresh daily. Trolox standard was prepared as a series dilution of 15.75-1,000 µM. In a 96-well microplate, 270 µl of DPPH•

solution and 30 μ l of blanks, standards, or samples were added in triplicate. The plate was kept in the dark for 15 min, and absorbance at 515 nm against the appropriate blank was recorded using the Synergy H1 spectrophotometer (BioTek). The results were expressed as μ M of Trolox equivalents per 1 g of dry weight (μ M TE/1 g DW) as described previously [27].

2.10. Ferric reducing antioxidant power (FRAP)

The FRAP reagent was prepared by mixing 10 volumes of 300 mM acetate buffer (pH 3.6) with 1 volume of 10 mM TPTZ in 40 mM HCl, and with 1 volume of 20 mM ferric chloride. Trolox standard was prepared as a series dilution of 15.75-1,000 μ M. In a 96-well microplate, 240 μ l of FRAP solution and 10 μ l of blanks, standards, or samples were added in triplicate. The plate was kept at 37 °C for 5 min, and absorbance at 620 nm against the appropriate blank was recorded using the Synergy H1 spectrophotometer (BioTek). The results were expressed as μ M of Trolox equivalents per 1 g of dry weight (μ M TE/1 g DW) as described previously [28].

2.11. Chlorophyll and carotenoid quantification

The 100 mg aliquots of leaf protein powders were extracted with 1.5 ml methanol or dimethylsulphoxide (DMSO). The samples were vortexed for 1 min centrifuged at 10,000 g for 15 min at 4 °C (methanol, DEE) or room temperature (DMSO). The resulting supernatants were diluted 10 \times (0.5 ml into 4.5 ml of the respective solvent), and chlorophyll a (Cha), chlorophyll b (Chb), and carotenoids x+c (Cx+c) content was quantified spectrophotometrically using a Synergy H1 spectrophotometer (BioTek) as described previously [29]. The corresponding equations are listed in Table 1.

Table 1. Equations for calculating chlorophyll and carotenoid content in different solvents.

Solvent	Chlorophyll and Carotenoid Calculation Equations
Diethylether (DMSO)	$\text{Cha} = 12.47 \times \text{A665.1} - 3.62 \times \text{A649.1}$ $\text{Chb} = 25.06 \times \text{A649.1} - 6.5 \times \text{A665.2}$ $\text{C}_{x+c} = (1000 \times \text{A480} - 1.29 \times \text{Cha} - 53.78 \times \text{Chb})/220$
Methanol	$\text{Cha} = 16.72 \times \text{A665.2} - 9.16 \times \text{A652.4}$ $\text{Chb} = 34.09 \times \text{A652.4} - 15.28 \times \text{A665.2}$ $\text{C}_{x+c} = (1000 \times \text{A470} - 1.63 \times \text{Cha} - 104.96 \times \text{Chb})/221$

2.12. Statistical analysis

Data was analyzed by one-way ANOVA followed by Dunnett's multiple-range tests using Prism 8.0 (GraphPad Software, San Diego, CA). All data was presented as means \pm SEM. Significant differences were accepted when the p-value was <0.05 .

3. Results and Discussion

Vegetal sources dominate the global protein supply, accounting for up to 60% of the protein consumed worldwide [2]. Most of this protein comes from plant seeds, which are integral to the omnivorous human diet, with storage proteins accumulating in the cotyledons and embryos of dicots (pulses) or the endosperm of monocots (cereals). These proteins can be enriched through mechanical fractionation techniques such as milling, air classification, and steeping [30] that

accounts in part for lower processing costs and higher protein content of the resulting protein powders. Additionally, oilseeds provide protein-enriched press cakes (meals) following the initial dehulling and extraction of vegetable oils [31]. These waste products can be upscaled to produce bulk dietary protein as shown for rapeseed, sunflower, and hemp crops, among others, however the associated manufacturing costs are typically higher due to additional processing steps necessary to generate the protein powders of sufficient quality.

Green leaf biomass is an underutilized resource in the spectrum of sustainable protein sources [3]. Traditionally, these materials have been used primarily as forage for ruminants or in animal feed. However, the realization that many green leaf agricultural crops express enhanced protein and essential amino acid profiles, including those suited for challenging soils and different climatic environments, presents a significant untapped opportunity that can be further explored. Additionally, byproducts from the agricultural green leaf biomass that offer improved digestibility or nutritional quality could be leveraged to create new, sustainable plant-based protein ingredients for both human and animal consumption. This unrealized potential could help diversify and strengthen the global protein supply, addressing both environmental and nutritional challenges.

3.1. Protein content

The protein powders used in this study covered a wide range of common animal proteins and plant seed proteins used in food processing and dietary supplementation strategies (Table 2). These proteins were compared to alfa leaf proteins available on the market (Alfapro, Alfalfa Grass powder), as well as four alfalfa protein powders manufactured by The Leaf Protein Co (Table 2).

Table 2. Protein powders used in this study and their sources.

Protein powder	Source	Protein content ¹	Soluble protein limit, mg/ml ²
Animal protein			
Egg	NutriFoods	90%	67.5
Whey	Bulk Supplements	90%	67.5
Plant seed protein			
Chickpea	Green Boy Products	80%	60.0
Fava	Green Boy Products	85%	63.8
Pea	It'sJust! Foods	80%	60.0
Soy	Bulk Supplements	90%	67.5
Wheat	Kate Naturals	75%	56.3
Alfalfa leaf protein			
AlfaPro	Bioriginal	50%	37.5
Grass Powder	XPRS Nutra	20%	15.0
Complete heat (LCHP)	The Leaf Protein Co	55.0%	41.3
Complete acid (LCAP)	The Leaf Protein Co	48.6%	36.5
Green (LGHP)	The Leaf Protein Co	51.6%	38.7
White (LWHP)	The Leaf Protein Co	77.2%	57.9

¹ Protein content as declared by the manufacturer or based on 6.25× nitrogen (The Leaf Protein Co samples).

² Assuming maximum protein solubility (100%) when dissolved at 75 mg/ml.

In the 1970s, the USDA Tropical Agriculture Research Station in Puerto Rico conducted a large-scale screening of 500 plant species to evaluate their green leaf protein content. The study found that green leaf dry matter ranged from 10.2-34.9%, with crude protein content varying between 10.8-35.7% of dry weight [32]. When crudely processed, green leaves yielded a mass balance of 50% leaf juice, 45% leaf fiber, and 5% leaf protein concentrate, with the latter two samples reported at 55% moisture [33]. Taken together, these numbers suggest that leaf protein concentrate can be extracted on average at 2.5% of green biomass on fresh weight basis, or 25 g crude protein for each 1 kg of fresh plant material used in the extraction process.

Alfalfa (*Medicago sativa* L.) is a highly productive C3 perennial forage legume known for its robust growth and adaptability to various climates, making it an excellent crop for leaf protein production [34]. It grows in dense stands, with deep roots that allow it to thrive in both temperate and arid regions, often yielding multiple and even continuous harvests per year. In colder areas, alfalfa is collected at 4-6 tons hay per acre with 4 cuttings, and this increases to 10-11 tons of hay per acre with 8 cuttings a year in the irrigated warmer climates, with the daily temperature higher than 25 °C being most contributing to the alfalfa biomass accumulation [35]. Alfalfa's high leaf-to-stem ratio ensures a significant biomass yield, with leaves containing 20-26% protein on a dry basis as summarized elsewhere [14]. Alfalfa leaf protein can be further fractionated into two main types: green protein with the heat coagulation temperature of about 55 °C and white protein with the heat coagulation temperature of approximately 85 °C; protein yield can be increased by the repeated use of a twin-screw press to remove proteinaceous juice from fiber [36]. Within the recovered protein, white protein, primarily Rubisco, is desirable for its functional properties like gelation and emulsification, while green protein is less desirable due to its association with chloroplast fragments and hydrophobic proteins that bind to polyphenols and fibrous

polysaccharides [22]. For The Leaf Protein Co samples isolated using the basic protocol based on total heat-based precipitation, total acid-based precipitation, as well as heat-based precipitation of the green fraction resulted in protein concentrates with similar protein content in the range of 48-55% that agrees well with previously reported data [37]. Additional steps to enhance recovery of alfalfa leaf proteins by ultrafiltration [37], sonication after blanching [38], and electroporation [39] have been also been recently proposed.

3.2. Protein solubility

Protein solubility is a crucial factor in food science, influencing the functionality and application of proteins in various food products. It directly impacts texture, stability, and overall quality, with highly soluble proteins enhancing emulsifying, foaming, and sensory properties [40]. Solubility is particularly important in ensuring proteins can be effectively utilized in diverse food systems, meeting both nutritional and functional requirements. pH is also a significant environmental factor affecting protein solubility, as it alters the net surface charge of protein molecules, thereby influencing their ability to aggregate or remain dispersed [41].

At pH 7, protein powders typically exhibit neutral or native state solubility since there is no influence on their surface charge. Generally, protein solubility decreases in acidic environments and increases in basic ones, largely due to the acidic isoelectric points (pI) of many proteins, influenced by the abundance of acidic amino acids like aspartic and glutamic acid [42]. Above their pI, proteins carry a net negative charge, enhancing solubility through electrostatic repulsion. Conversely, proteins rich in basic amino acids such as lysine, arginine, and histidine have higher pI values and show minimal solubility around these points, but increased solubility in more acidic or basic conditions [43].

Figure 1 compares the solubility of animal and seed proteins together, and alfalfa leaf derived proteins together, across pH levels 4 to 8. All legume seed storage proteins are predominantly globulins and show similar solubility patterns due to their structural composition. The solubility of all proteins except for wheat increased with pH, with soy protein showing a notable rise from 6.25 mg/ml at pH 4 to 15.72 mg/ml at pH 8, reflecting its low pI due to acidic amino acids like aspartic and glutamic acid [44]. Pea protein exhibited a higher solubility than other seed proteins, particularly at acidic pHs, likely due to its higher content of soluble fractions and fewer hydrophobic components, though factors like extraction methods and harvesting conditions also play a significant role as described for lentils [45]. Chickpea and fava proteins showed steady increases in solubility, with chickpea rising from 4.99 mg/ml at pH 4 to 11 mg/ml at pH 8, and fava protein from 4.03 mg/ml at pH 4 to 10.58 mg/ml at pH 8 in agreement with previously reported data [46].

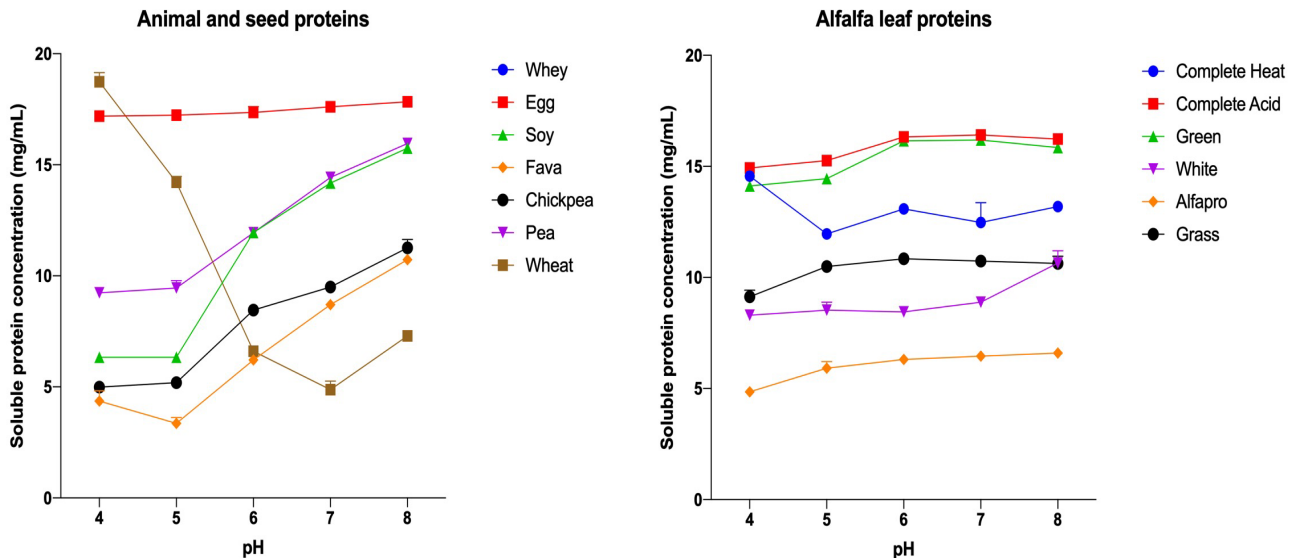


Figure 1. Protein solubility of commercial animal and seed protein concentrates, as well as four alfalfa leaf proteins manufactured by The Leaf Protein Co (complete heat, complete acid, green

and white fractions), Bioriginal (AlfaPro), and XPRS Nutra (Grass) across the range of pH 4-8. Whey protein showed superior solubility twice that of other protein powders and remained off the graph due to the scaling factor. All proteins were dispersed in water at 7.5% (75 mg/ml). Maximal theoretical solubility values for all sources that account for their protein content are listed in Table 2. Data were collected in duplicate and presented as mean \pm SD.

Wheat gluten protein exhibited an unusual solubility profile, with high solubility at pH 4 (around 19 mg/mL), a sharp decrease to about 5 mg/mL at pH 6, and a slight increase at pH 8. This pattern suggests that wheat gluten proteins, particularly glutenin and gliadin, are more soluble in acidic conditions and less so in neutral to basic environments, a trend consistent with other studies showing that hydrolyzation treatment can significantly improve solubility across all pH levels [47].

Whey protein maintained high solubility twice that of egg protein (not shown due the graph scale), and much higher than any sample used in this study. Egg protein maintained high and stable solubility around 17-18 mg/mL across all pH levels, a consistency attributed to its albumin content, which remains soluble over a broad pH range as supported by other studies [48].

Among the Leaf Protein Co samples, green and complete acid proteins exhibited the highest solubility, maintaining levels above 15 mg/mL across all pH values, with a slight increase from pH 4 to 8. Both fractions contain "green protein," which is closely associated with chloroplast structures, cell walls, and other cellular components, resulting in a distinct green color and poor functionality. Thermal precipitation used in their manufacturing potentially explains the comparable solubility of both fractions.

Isoelectric precipitation at a protein's pI point can isolate proteins from solution, but unlike thermal precipitation, it cannot selectively target soluble or insoluble protein fractions like two-stage thermal coagulation can. As a result, the complete acid protein fraction likely contains a mix

of both soluble and insoluble proteins, as observed in other acid-recovered alfalfa leaf protein extracts [49]. Complete heat protein showed a similar trend with slightly lower solubility values, with the highest solubility at pH 4 (14.9 mg/mL), which decreased to 11.9 mg/mL at pH 5, followed by a slight increase at pH 6 (13.2 mg/mL) before dropping again to 12.3 mg/mL, potentially due to the specific thermal processing it underwent which may have exposed hydrophobic peptide motifs.

White protein solubility increased gradually from approximately 8 mg/mL at pH 4 to around 11 mg/mL at pH 8, however was consistently low. This low solubility may be due to the dissociation of protein complexes or structural unfolding that exposes hydrophobic regions, reducing solubility compared to native state proteins [50]. This unfolding, when white protein is recovered through thermal denaturation, can cause irreversible changes in protein structure, particularly in secondary and tertiary structures, lowering solubility across all pH ranges.

Alfapro displayed the lowest solubility, starting at about 4.8 mg/mL at pH 4 and gradually increasing to around 5.6 mg/mL at pH 8. The unknown recovery method likely involved thorough or complete protein denaturation or the isolation of insoluble protein fractions. In contrast, a blended grass powder exhibited more stable solubility across the pH range, starting at 9 mg/mL at pH 4 and holding steady around 10 mg/mL in subsequent pH environments. These findings underscore the significant impact of processing conditions and protein composition on the solubility of leaf proteins, which is crucial for their application in food systems.

3.3. Foam stability and capacity

Many functional properties are interlinked, with high solubility strongly correlating with the ability to foam in large volumes and maintain high stability [51]. Compared to animal-based proteins, especially those from dairy, plant proteins often exhibit significantly lower solubility, as

demonstrated in this paper's studies on soy, fava, chickpea, and leaf protein powders. The extraction processes can further reduce plant protein solubility by causing denaturation or aggregation, which limits their functionality as foaming agents [52]. This underscores the importance of optimizing extraction methods to preserve the native state of plant proteins and exploring plant-based proteins with higher solubility potential than those currently available (Figure 2).

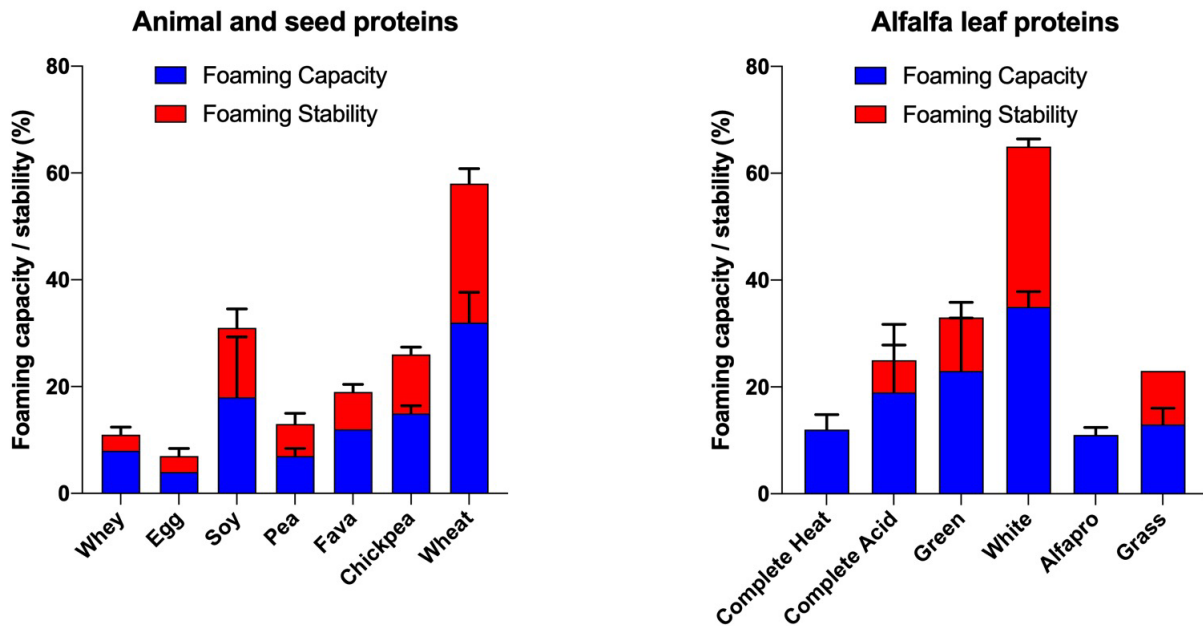


Figure 2. Foam capacity and stability of commercial animal and seed protein concentrates, as well as four alfalfa leaf proteins manufactured by The Leaf Protein Co (complete heat, complete acid, green and white fractions), Bioriginal (AlfaPro), and XPRS Nutra (Grass). All proteins were dispersed in water at 1%. Data were collected in duplicate and presented as mean \pm SD.

Foaming properties, including capacity and stability, are crucial functional characteristics of proteins in the food industry. These properties impact the texture, mouthfeel, and appearance of

products like mousses, whipped toppings, and bakery items, enhancing consumer sensory preference [53]. Food foams are two-phase systems consisting of air cells separated by a thin liquid layer, known as the lamellar phase, and are complex mixtures of gases, liquids, solids, and surfactants [51]. Foams are thermodynamically unstable and tend to collapse over time due to gravitational drainage, bubble coalescence, and disproportionation, where larger bubbles grow at the expense of smaller ones [54]. Proteins help stabilize foams by adsorbing to the air-liquid interface and forming a viscoelastic film, which mitigates collapse and enhances foam stability [41].

The foaming capacity and stability of 13 different protein powders were assessed to evaluate their commercial potential as foaming agents in food applications. The results, shown in Figure 3, indicate significant variability among the protein sources, influenced by their extraction methods and composition. Animal-based protein powders, such as egg white powder and whey protein isolate, exhibited relatively low foaming capacities and stabilities, both below 10%. The lower foaming ability of egg protein, despite its common use in foamed products like meringues and souffles, can be attributed to the high-temperature drying process through which the powder was recovered, which denatures proteins and reduces their surface-active properties in this sample. On the other hand, whey proteins low foaming capacity is likely less influenced by extraction technique (with commercial production centered around ultrafiltration and ion exchange purification) and more around final formulation and structural differences. This particular whey isolate contained sunflower lecithin as an additive, which has been shown to reduce foaming in various processes [84]. Whey protein isolate itself can exhibit moderate to high foamability due to its ability to adsorb at air-water interfaces from its amphipathic lactoglobulin structure [85] which

can be further improved through post processing techniques such high-pressure processing to aid in unfolding and subsequent protein flexibility [86].

Seed proteins are primarily globulins, which range from 70-80% in content and include 7S and 11-12S globulins, with their proportions likely affecting functional foaming profiles [55]. Soy protein demonstrated the highest foaming capacity and stability due to its refined nature and higher proportion of soluble proteins, particularly glycinin and β -conglycinin. Conversely, pea protein showed the lowest foaming capacity and stability due to the weak interface formed by its globulins [56], whereas chickpea protein properties were similar to soy protein but less effective due to possibly its high vicilin content lacking disulfide bonds [57]. Wheat protein outperformed all other proteins with superior foaming properties, possibly attributed to its prolamins and glutelin's [58], which contribute to stable interfacial films and robust foam structures.

Among alfalfa proteins, Alfapro had a foaming capacity of about 10% but lacked foam stability, collapsing before data collection, likely due to thermal processing and the presence of both white and green protein fractions. This was further supported by results of complete heat protein which also demonstrated a similar foaming capacity of around 13% that had zero foam stability, indicating significant variability in foaming properties across alfalfa proteins. Green protein exhibited a higher foaming capacity of 23% and stability of 10%, suggesting that it could have included both green and white proteins, with the latter potentially retaining its superior foaming characteristics. Grass powder and complete acid protein showed similar foaming capacities and stabilities, at 13% and 19% respectively, but latter showed lower foam stability, possibly due to less effective reversion of protein functionality. White protein extracted from alfalfa displayed the highest foaming capacity of tested samples of 35% and stability of 30%, attributed to the presence of hydrophobic amino acids that enhance foam formation despite thermal

denaturation, demonstrating the potential to optimize processing for a functional foaming ingredient from leaf biomass.

3.4. Emulsification properties

Emulsions are thermodynamically unstable systems consisting of homogenized oil and water phases with reduced oil droplet surface tension, commonly found in food products like sausages, ice cream, and salad dressing. The Emulsification Activity Index (EAI) measures a protein's ability to adsorb at the oil-water interface, reducing surface tension and forming stable emulsions. The Emulsification Stability Index (ESI) indicates the long-term stability of these emulsions, which is crucial for preventing phase separation and maintaining the shelf-life and texture of products like mayonnaise and ice cream. At a homogenization speed of 20,000 rpm, whey protein isolate exhibited a moderate EAI of 60 m²/g and a relatively low ESI below 50 min, likely affected by sunflower lecithin's anti-foaming properties [59]. Egg white powder showed an EAI of 35 m²/g and an ESI of around 20 min, with its emulsifying properties potentially impacted by protein denaturation during the drying process [60] (Figure 3).

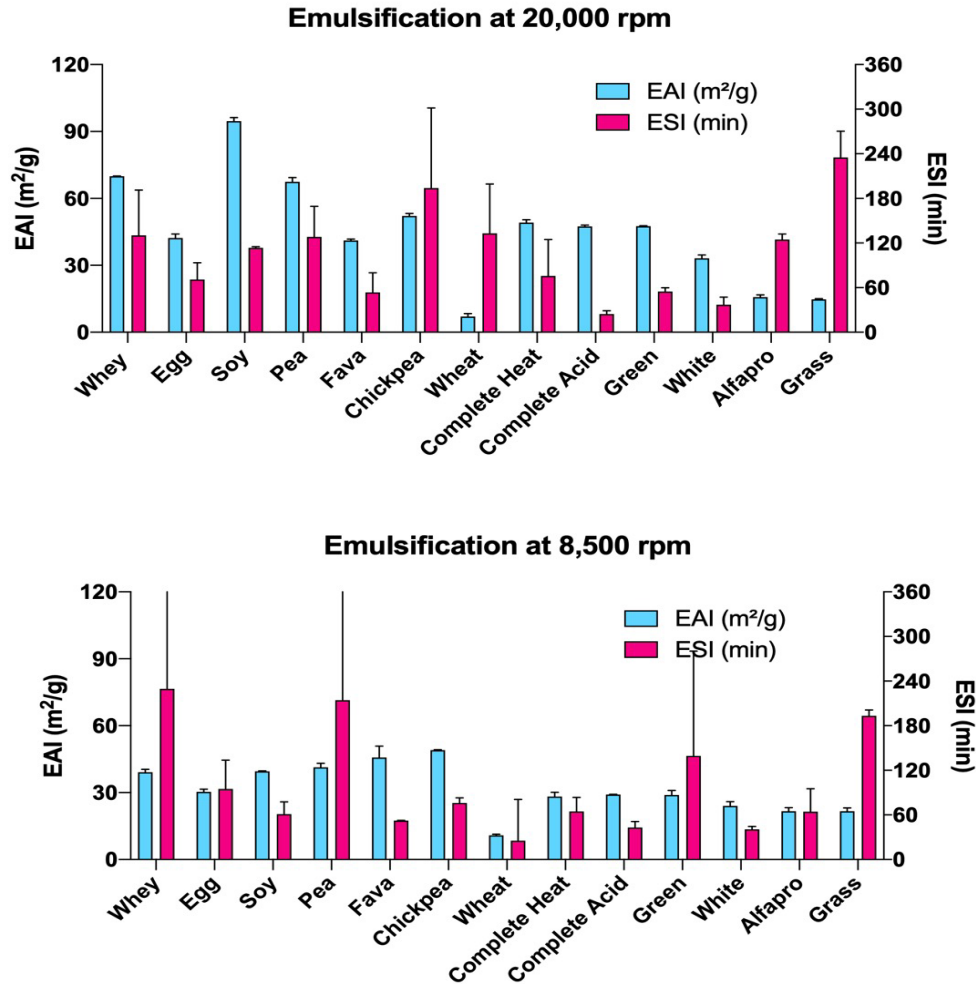


Figure 3. Emulsification properties of the study proteins at 20,000 and 8,500 rpm. All proteins were dispersed at 1% in 3:1 water-oil mixture. Data were collected in duplicate and presented as mean \pm SD.

Soy protein isolate exhibited the highest EAI at 90 m²/g and a moderate ESI of 45 min, possibly attributed to its high protein purity and solubility. Pea protein concentrate had an EAI of 60 m²/g and an ESI of 30 min, with a possibility that its globulins form weak interfaces that impact emulsifying performance [56]. Fava protein concentrate showed an EAI of 75 m²/g but a low ESI below 20 min, indicating its emulsions may lack stability as suggested earlier [61]. Chickpea

protein displayed an EAI of 55 m²/g and a low ESI below 20 min, which may be attributed to lower amount of cysteine residues in this protein affecting its emulsion stability. Wheat gluten protein had an EAI of 50 m²/g and an ESI below 20 min, reflecting the strong viscoelastic properties of gluten proteins [47].

Alfalfa-derived proteins showed varied emulsifying properties. Alfapro had a low EAI below 20 m²/g and negligible ESI, likely due to thermal recovery and drying processes reducing protein functionality [62]. Complete Heat and Complete Acid proteins showed moderate EAIs of 40 m²/g, with both having low ESIs, reflecting limited emulsifying properties as noted earlier [63]. This competitive EAI and ESI of green containing proteins could be due to the retention of thylakoid membrane fragments which contain lipids and can help stabilize oil interfaces. White protein had a moderate EAI of 50 m²/g and a low ESI of 20 min; it is possible that additional exposed hydrophobic residues enhanced its emulsifying properties despite potential denaturation [50]. Interestingly, Grass powder showed a high EAI of 90 m²/g and an ESI above 250 minutes, indicating excellent emulsifying properties, which was rather unexpected and warrants further investigation.

At a lower speed of 8,500 rpm, all protein powders showed reduced EAI and ESI, emphasizing the role of processing conditions. Overall, alfalfa-derived proteins, particularly the white and green fractions, demonstrated notable emulsifying activity, indicating potential for broader applications if processing conditions are optimized.

3.5. Antioxidant properties

Antioxidants play a vital role in human health by donating electrons to neutralize reactive oxygen species (ROS), which can otherwise cause oxidative damage to cellular components. This protective action helps in preventing chronic diseases, including cardiovascular diseases and

neurodegenerative disorders like Alzheimer's and Parkinson's, where oxidative stress is a key factor [64]. Antioxidants are also essential in food preservation, where they prevent oxidative rancidity, thereby extending shelf life and maintaining flavor and nutritional quality [65]. The rising awareness of these health benefits has led to increased demand for antioxidant-rich products across the food, cosmetic, and pharmaceutical industries, with the global market expected to grow significantly in the coming years.

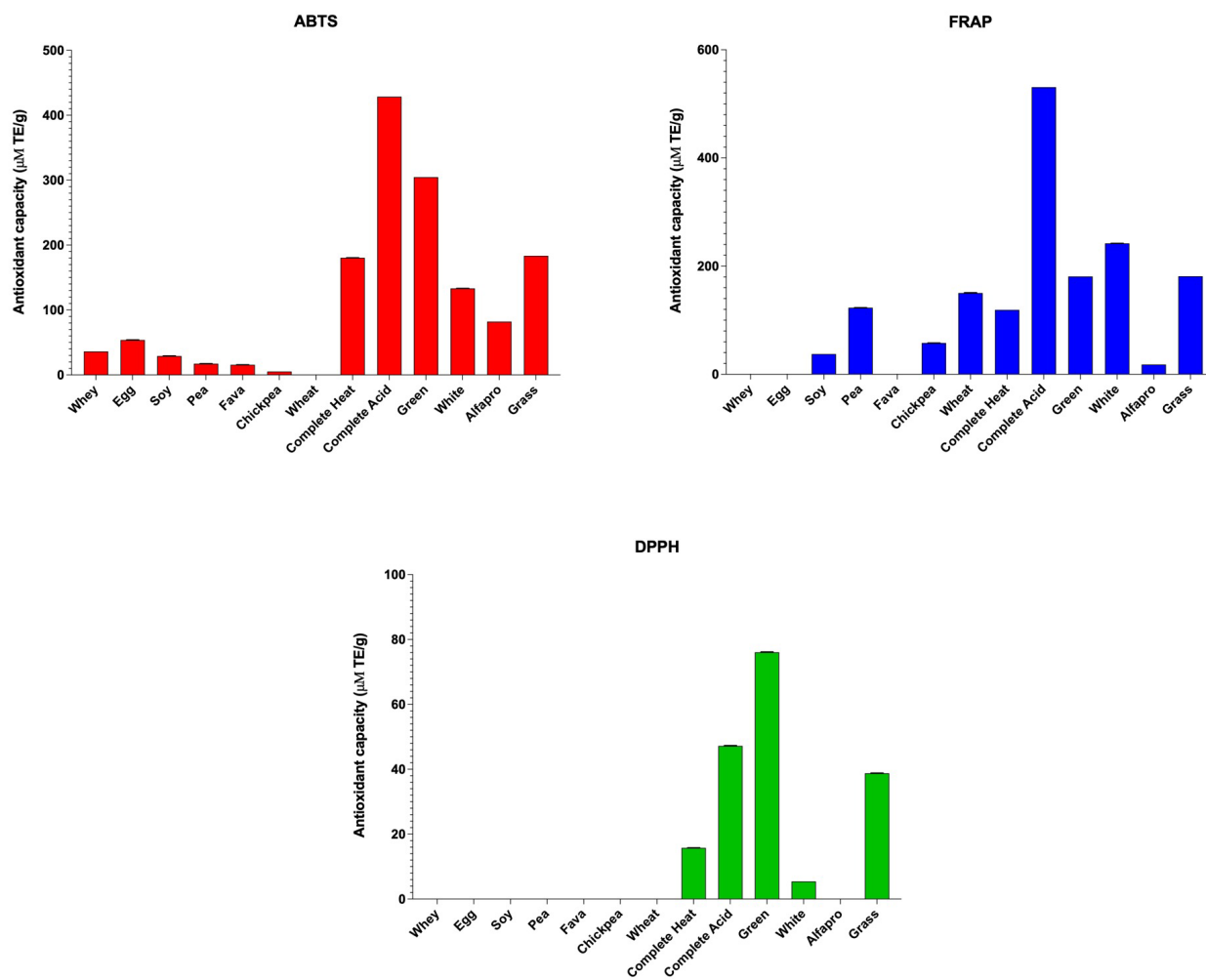


Figure 4. Antioxidant properties of the study proteins evaluated with three different methods based on the source of ROS and free radicals generated with 2,2'-azino-bis(3-ethylbenzothiazoline-6-

sulfonic acid) (ABTS), ferric-tripyridyltriazine complex (FRAP), and 2,2-diphenyl-1-picrylhydrazyl (DPPH). All proteins were dispersed in water at 1%. Data were collected in duplicate and presented as mean \pm SD.

To capture the full spectrum of antioxidant activities, this study utilized three distinct quantification techniques. The ABTS assay measures antioxidant capacity by monitoring the reduction of the ABTS radical cation (ABTS⁺) to its neutral form through electron donation, making it versatile for both hydrophilic and lipophilic antioxidants [66]. The FRAP assay, on the other hand, specifically evaluates the electron-donating capacity of antioxidants by reducing ferric ions (Fe³⁺) to ferrous ions (Fe²⁺) [67]. The DPPH assay assesses the ability of antioxidants to scavenge the stable DPPH free radical by donating hydrogen atoms only. Therefore, while the DPPH assay provides valuable information on the hydrogen-donating ability of antioxidants, when paired with ABTS and FRAP, a more comprehensive overview of antioxidant capacity is obtained [66].

Whey and egg proteins showed no measurable activity in the FRAP and DPPH assays but had some activity in the ABTS assay, suggesting very limited radical scavenging ability of highly purified protein concentrates, and similar observations were recorded for plant seed proteins including soy, pea, chickpea, and fava beans.

Complete leaf acidified protein demonstrated the highest antioxidant activities, indicating robust reducing power, effective radical scavenging, and significant hydrogen atom donation capacity, most likely attributed to the preservation of secondary metabolites during acid-assisted precipitation or even the ability to concentrate them through isoelectric protein removal. Complete leaf heat protein had moderate antioxidant activity, with some reducing power and substantial

radical scavenging ability, but lower hydrogen donation capacity possibly due to the partial degradation of secondary metabolites during heat treatment. Green leaf protein exhibited significant antioxidant activities across all assays, often on par with the complete leaf heat protein, indicating its effectiveness in scavenging free radicals and reducing oxidative stress. As discussed previously, the phenolic content of alfalfa, which is a major contributor to its antioxidant capacity, can be significantly reduced during the extraction and drying process, with pasteurization treatment and drying being highly correlated to decreased phenolic and antioxidant compound loss [68]. White leaf protein that has higher protein content and lower amounts of secondary metabolites showed a significant reduction in the total antioxidant capacity of the protein concentrate, thus also supporting these conclusions. White leaf proteins exhibition of antioxidant capacity could be due to bio-active peptide fragments rather than secondary metabolites.

Alfalfa grass powder exhibited significant antioxidant activity across multiple assays, with high FRAP, ABTS, and DPPH values, likely attributed to its rich content of bioactive compounds like phenolic acids, flavonoids, and peptides [69]. In contrast, Alfapro showed low antioxidant activity, with limited reducing power and some radical scavenging ability, probably due to the degradation of antioxidants during thermal processing. The substantial difference in antioxidant capacity among different alfalfa samples underscores the impact of processing methods on preserving or diminishing of their antioxidant properties.

3.6. Chlorophyll and carotenoid content of alfalfa protein concentrates

Chlorophylls and carotenoids are crucial pigments in leafy material, contributing not only to their vibrant colors but also to their nutritional and health benefits. With strong exhibition of antioxidant properties [71] and chemoprevention [72], the retention of these pigments in processed material confers beneficial traits to products they're incorporated into. However, being color

containing compounds, the need for their removal from neutral color/flavor systems also necessitates development of purification techniques. This study sought out to quantify the chlorophyll A (Ch-A), chlorophyll B (Ch-B), and total carotenoid content (Cx +C) of six samples produced from leafy material containing these pigments, and assess how processing impacts their final concentration. Two different extraction solvents were chosen to quantify pigments, which exhibit differential affinity based on polarity and molecular structure [73] thus displaying a more comprehensive understanding of actual pigment content. Methanol is a proven extraction solvent for chlorophylls and carotenoids, particularly from recalcitrant vascular plant tissue [74] whereas DMSO has shown promise in pigment quantification when content is low [75]. Through solvent extraction and spectrophotometric measurement of the absorption capacity conferred by each pigments unique structure, a quantitative analysis of Ch-A, Ch-B, and Cx + C was obtained for each of the samples originating from leaf matter.

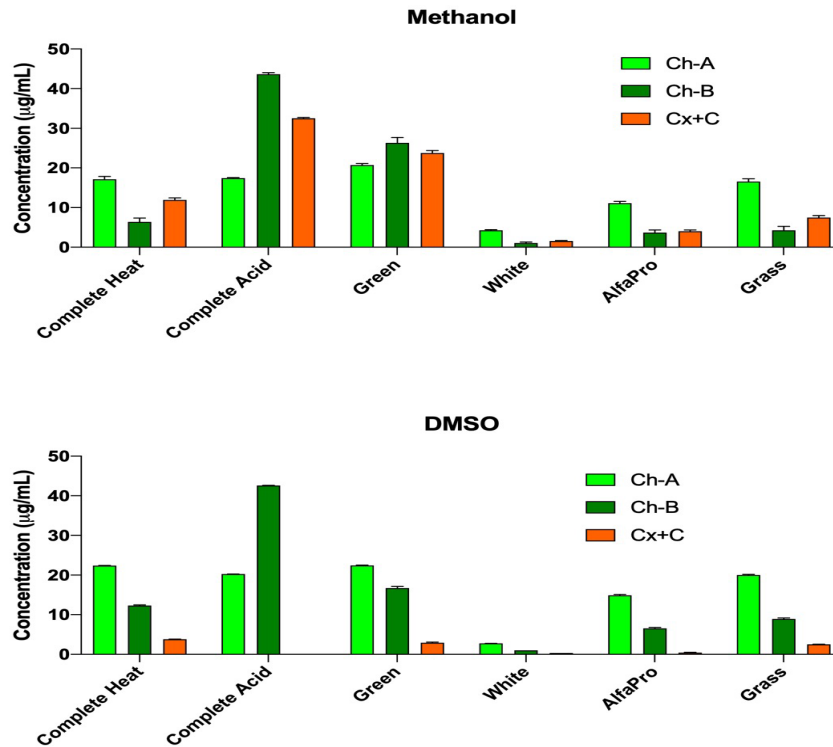


Figure 5. Chlorophyll and carotenoid content of the alfalfa leaf proteins evaluated in two different solvent systems, methanol and DMSO. Proteins were extracted with the corresponding solvents at 0.67% concentration. Data were collected in duplicate and presented as mean \pm SD.

Complete leaf heat protein showed moderate concentrations of all three pigments, in a quantity and ratio that was incredibly similar to alfalfa grass powder. This suggests that the extraction processes of complete heat, while it concentrates the protein, does not concentrate the pigments. This also demonstrates that despite the higher temperature associated with complete leaf protein recovery, the pigments remained stable and were not degraded. Heat recovered leaf proteins have exhibited strong pigmentation in similar studies, establishing that thermal coagulation of proteins is an effective method of binding to and extracting the chlorophyll and carotenoids from the input material [76]. AlfaPro displayed a similar concentration ratio of pigments to both grass and complete leaf heat, however its total concentration was markedly

lower. Given the age of the sample and theorized drying conditions of heat-based tunnel drying (as literature cites similar drying methods are responsible for dark color [77]) of Alfapro, this could explain a uniform degradation of pigments, although biomass source could also account for this difference as well. Interestingly, the green leaf protein exhibited higher concentration of pigments in comparison to the complete leaf heat protein, with nearly a three-fold increase in Ch-B and two-fold increase in Cx+C in methanol quantification. The affinity for insoluble leaf proteins to bind to and remove color containing compounds is well documented [78] as many of these proteins are already bound to pigments such as chlorophyll a/b binding proteins, or membrane proteins imbedded in the cell wall [79]. Additional pigments may be extracted during the green leaf heat recovery processes as the hydrophobic regions of proteins are made accessible due to thermal denaturation, binding to the hydrophobic porphyrin ring, phytol tails, and long conjugated double bonds of these pigments which naturally anchor them to lipid bilayers [80]. Complete leaf acidified protein demonstrated the highest pigment content of the analyzed sample, showing not only retention of native quantity but the concentration of additional pigments as well. Interestingly, whereas other samples show at least some Cx+C content exhibited in DMSO compared to methanol, their content was non-existent in complete leaf acidified protein. This could possibly be due to interactions between DMSO and residual acid ions, When DMSO (CH_3SOCH_3) is mixed with HCl, the DMSO can undergo protonation, leading to the formation of a protonated DMSO species ($\text{CH}_3\text{SOCH}_3\text{-H}^+$) along with chloride ions (Cl^-). This protonated species can further react to form methanesulfonic acid ($\text{CH}_3\text{SO}_2\text{H}$) and formaldehyde (CH_2O) as intermediates, which can facilitate the generation of ROS, such as hydroxyl radicals, oxidizing and degrading sensitive compounds like carotenoids [81]. Studies looking at protein-xanthophyll co-extraction have observed an increased retention with lower

pH, attributed not only to protein-pigment bonding, but the lowering of the solubility of the pigments, enabling efficient removal during the density-based protein extraction process, and yielding a final powder that has high pigment concentration [82]. White leaf protein displayed the lowest levels of pigment concentration across all samples, consistent with conventional understanding of pigment removal through pre-heat treatment and green leaf protein extraction [83].

4. Conclusions

Traditional plant sources, such as seeds and oilseeds, provide essential proteins, but green leaf proteins, particularly from alfalfa, present an opportunity for diversification. Protein content and solubility varied widely among different sources, with alfalfa leaf proteins showing a range of solubility and functional properties depending on the extraction methods used. Notably, alfalfa white protein exhibited the highest foaming capacity and stability, while its antioxidant properties were influenced by the processing methods. These findings underscore the importance of optimizing extraction and processing techniques to enhance the functional properties and nutritional benefits of leaf proteins. Future studies should focus on refining these methods to fully harness the potential of green leaf proteins for both human and animal consumption.

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Chapter 4. Concluding Thoughts and Future Directions

1. Future Directions

Leaf protein's role in the modern food system is complex, and while much of its implementation relies on the optimization of extraction and product application, much more remains to be explored before it can be as integrated as conventional protein sources. Chapter 1 outlined the broad scope of literature pertaining to the field of leaf protein extraction, comparing both existing protein sources and previous work that had been dedicated to leaf protein extraction and its uses. Strengths of leaf protein were seen through its ubiquitous presence in nature, and strong nutritional capabilities, yet questions remained regarding its application and optimal extraction methods. Thus, Chapter 2 set to outline the methods of not only extracting leaf protein from a resource tolerant species, *Atriplex hortensis*, but to combine cost effective food grade extraction techniques to highlight the role that each variable plays. Results showed that a synergistic combination of heat, alkaline, and acid yielded the highest amount of soluble protein. This process for elucidating extraction parameters of *Atriplex hortensis* can then be applied to other species, operating as a diagnostic tool for optimal variables through its stepwise approach. However, the question remained as to what attributes the proteins had, as highest soluble protein recovery is not always seen as the most desirable aspect in a product. Chapter 3 explored the functional classification of extracts from *Medicago sativa* against other industry standard proteins, as well as nutritional aspects of antioxidant potential and pigment content. The results varied in functional properties, ultimately highlighting that the functional characteristics of leaf protein were heavily influenced on the extraction methodology. This understanding allows for the conceptualization of targeted protein functionality, using the same source starting material for multi-product creation by tailoring the

extraction parameters to the needs of downstream users. Furthermore, the retention of beneficial antioxidant capacity was retained by the proteins, albeit impacted by extraction as well, outperforming the recorded values for all other non-leaf protein sources. However, much more work needs to be done to achieve cost and size parity to conventional protein sources, and as follows are potential future research directions and considerations.

1.1 Refinement of Processing Techniques

Proteins are prone to degradation during the extraction processes, which can greatly impact the yield and quality of the recovered product. The creation of extraction buffers has been explored in fields ranging from food science to pharmaceuticals, as process additives that stabilize protein structure through ionic salts, the use of antioxidants to protect amino acids from oxidative damage, or enzyme inhibitors to enzymatic degradation [1,2]. These buffers may prove a vital resource in the optimization for future leaf protein extraction and can already be seen in relevant patents and literature [3]. To further increase protein recovery, novel mechanical disruption techniques can be implemented, with cellular disruption being directly related to total recovery of leaf protein [4]. Techniques such as ultrasound and high-pressure processing offer a pathway to enhance cell wall breakdown, thereby increasing protein yields and process efficiency [5]. Given the diversity of leaf through which proteins can be extracted, degree of cell lysis prior to extraction will become an important parameter needed for optimization. The use of post processing modification could greatly address functional limitations of leaf protein. Great progress has been made in increasing solubility of otherwise insoluble proteins such as lupin [6] and shows promise in applications for leaf protein as well [7]. Moreover, the ‘cleaning’ of these proteins could be accomplished through new technology such as supercritical fluid extraction, removing many of the unwanted color and flavor containing compounds which can target

enhancing the purity and functionality of the final product [8]. With much demand for protein hydrolysates due to their range of nutritional benefits [9], enzyme-assisted extraction is another area ripe for exploration. Not only can this extraction system be used to break down plant cell walls more effectively, releasing proteins that are otherwise difficult to extract, but could effectively modify or hydrolyze the protein into bioactive peptide fragments as well, adding additional value to extracted leaf proteins [10].

1.2 Exploration and Modification of New Plant Sources

To date, many species have been used as a starting point for leaf protein extraction, including beets [11], alfalfa [12], duckweed [13] and olive leaf [14] to name just a few. Older research details comprehensive side by side comparison of protein extractability of various leafy tissues, but given the advances in extraction methodology and equipment, should be revisited [15]. Selective breeding and genetic engineering could further enhance the protein content and extractability of these plants, with work already being done on increasing rubisco content in plants through upregulation of genes involved in synthesis and assembly of proteins, greatly aiding the overall protein yield from input leafy biomass [16]. To this point, other aspects of the plant could be retooled to aid leaf protein extraction, such as the reduction of chlorophyll through down regulation of chlorophyll synthase [17].

1.3 Pre-Processing Logistics and Partnerships

While this thesis was devoted to exploring the parameters in which leaf protein could be extracted, pre-processing of the input material has a large impact on downstream extraction. Pre-processing may encompass the sourcing, collection, transport, and initial handling of the biomass. Additionally, pre-processing is often tasked with ensuring consistency and quality of

the raw material, assigning trackable parameters that allow lot rejection and acceptance. Much exploration is needed in discovering how protein quantity and quality is impacted by these pre-process logistics, although the common understanding allows us to confidently state that both quality and quantity decrease as the time between harvest and processing increases [18]. Post harvest safety will be crucial for the commercial implementation of an agriculturally based ingredient, and the adoption of pre-process product screening and even wash steps can minimize the presence of harmful substances and ensure product consistency [19]. In terms of biomass sourcing, establishing partnerships with farmers and/or processing centers can be seen as advantageous; much expertise has been developed in the best practices for growing and harvesting protein-rich crops, saving commercial leaf protein entities from ‘re-inventing the wheel’ as it were. By establishing partnerships with growers, capital expenditure to cultivate, grow, and harvest the input material is pre-existing and can be leveraged, quickly accelerating the path to commercialization. Partnerships like these have been observed already in New Zealand, as commercial leaf protein partners with alfalfa used for dairy cattle [20], and in the Netherlands with beet leaf [21]. Further partnerships can be considered in respect to green crop waste utilization, valorizing reject material like carrot tops and sweet potato leaves as an input source for protein extraction. Green-crop processing centers may be a promising avenue to explore, as many accumulate large amounts of green waste that could be suitable input for leaf protein production, as seen in cruciferous vegetable processing which collects and discards kilotons of leafy material prior to shipment to retailers [22].

1.4 Biomass Growth and Harvest

In terms of species adoption, much more progress has been made on incorporating leaf protein production into conventional agricultural systems than unconventional. This is likely due

to the scaled harvest systems that already exist around traditional foliage crops such as alfalfa, barely, and wheatgrass, something that would be needed for the quick commercial implementation of a new ingredient. However, to use conventional crops for an unconventional ingredient may require the retooling of the understood methods of harvest. For instance, traditional agriculture often focuses on optimizing crop yields for specific growth stages, but the production of leaf proteins will prioritize different aspects, such as young leaf biomass over older fruit or grain production. Additionally, many crops are left on field to dry completely before harvest, nullifying the ability to harvest them for leaf protein production. While mechanized harvesting techniques can be employed to enhance efficiency and reduce labor costs, fresh leaves have yet to be seen as a value component for many of the proposed species, thus new mechanized harvesters would likely need to be created, further dependent on plant species, age of harvest, and method of recovery. Additional care must be taken to avoid damaging the plant material, which could lead to protein degradation. As soon as the plant is cut, oxidative and degradative pressures begin to act on the biomass which can lead to the browning of the material, protein loss, and the formation of undesirable complexes that reduce protein quality [23]. Additionally, exposure to environmental factors like heat, moisture, and microbial contamination during and after harvesting can further compromise the integrity of the proteins or lead to products that cannot be deemed food grade [24].

1.5 Cost-Effectiveness Analysis

Leaf protein production at scale is not new, in fact, several companies have created large scale production platforms in USA, France, and Canada for many years now [25] however its refinement and economic optimization is something that can be continually improved. Cost benefit analysis in tandem with mass balance will be needed to validate new technologies and

extraction methods at scale; many 'cost effective' processes such as tunnel drying and heat coagulation are very cheap in operational expenditure, but yield products that have little application in the human market due to their dark color and bitter flavor profile [26] while many processes that produce hyper functional isolates such as membrane filtration and supercritical CO₂ are incredibly costly both in capital and operational expenditure [27]. Thus, in conversation with downstream consumers who dictate a price point, a mass balance of each protein's recovery should be determined to evaluate whether the processing cost and operational expenditure can justify the means to which the protein is produced at scale. While optimization of the extraction process for protein recovery and quality will inevitably translate into better economic performance, the fact remains that a minority of the input material, as little as 1-4% by wet weight [28] will be utilized in the final product. Therefore, valorization of byproducts not only offers another avenue for improving the cost-effectiveness of leaf protein production but could be necessitated in certain cost models. The extraction process generates significant amounts of fiber biomass, predominantly cellulose and hemi-cellulose, that could be repurposed into value-added products such as biofuels, bioplastics, fertilizer, animal feed [29].

1.6 Integration into Existing Food Value Chains

While leaf protein may be a direct-to-consumer product, functional and nutritional attributes of the protein concentrate and isolates also give it validity as an inclusionary ingredient within food products. Thus, integration relies on dialogue and product development between those producing leaf protein, and those that are using it. Through partnerships with companies that already have extensive experience in processing and marketing plant-based or better-for-you products, leaf protein producers can leverage existing expertise while also fine-tuning product creation based on communicated needs of downstream products. This also opens the opportunity

for co-manufacturing spaces for leaf protein extraction, as many existing facilities such as pulse and dairy protein share similar processing steps such as maceration, thermal treatment.

1.7 Consumer Perceptions of Leaf Proteins

Even if leaf protein extraction is cost effective, sustainable, and nutritious, its success and large-scale adoption is predicated on consumer acceptance. While the aforementioned attributes can help bolster image, a far larger segment of the target market will be driven by sensorial traits. Taste, texture, color, smell, all of these will play a vital role in its adoption. Taste and texture are primary determinants of consumer acceptance when it comes to alternative proteins, and while the white cytoplasmic leaf protein isolates have been described to have a neutral flavor profile [28], green concentrates have a notable grassy and vegetal flavor which can be off putting to consumers and difficult to formulate [8]. Advancements in flavor maskers can help improve the sensorial properties and thus increase consumer acceptability but can also have an adverse effect of longer e-numbers or defeating naturalistic claims made by products.

1.8 Regulatory Frameworks

As with any new ingredient, leaf protein will be subject to the regulatory framework surrounding both the extraction process and the final product. This is further expanded based on the countries to which the product will be launched, with the European Union for instance, exhibiting a much more rigorous approval process for food safety acceptance, taking a minimum of a year and a half if no additional documentation is requested [30]. Due to this, many novel products are first launched in countries with more lax regulations, such as Singapore, which while still stringent, offers a much faster pathway to the commercial market [31]. The momentum of pre-existing regulatory compliance can help expedite federal approval, and leaf

proteins have already been receiving ‘letters of no objection’ from the United States FDA with leaf protein concentrates being produced from duckweed. While the generally recognized as safe (GRAS) filing indicated revisitation for each plant species the protein extraction is applied on, this initial ruling is a large step forward in regulatory compliance of leaf proteins.

2. Conclusion

Leaf protein is unique. Its abundance in nature, its physicochemical structure, its functional attributes and nutritional benefits, all contribute to a plant-based protein that could have incredible impact on sustainable nutrition. For many years the idea of leaf protein extraction has been investigated and iterated upon, with advances in tangential fields of food product development and technological innovation making the concept more feasible than ever. Paired with shifting consumer demand and product visibility, it is no surprise that the literature being created around leaf protein extraction is regaining momentum and visibility. While much more is left to be done, from idea, to lab, to pilot, to commercial implementation, the means through which leaf protein production can be applied are as diverse as the plants themselves.

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