ABSTRACT

GUTIERREZ CASTRO, JANELLE. Potential for the Valorization of Green Leaf and Oilseed Byproducts. (Under the direction of Dr. Slavko Komarnytsky).

The global agricultural industry generates significant by-products, many of which are discarded, contributing to economic losses and environmental pollution. Despite their potential, secondary biomass such as seeds, leaves, roots, oil cakes, brans, and peels remain underutilized, though they are rich in proteins, carbohydrates, lipids, dietary fibers, and bioactive compounds. Oilseed cakes, the protein-rich by-products of oil extraction, and green vegetable matter from processed crops offer promising alternative protein sources. Repurposing these agricultural waste streams for food functional ingredients can reduce waste, lower environmental impact, and contribute to economic growth. The increasing demand for sustainable protein sources underscores the need for innovative food solutions. Oilseed cakes provide high-quality protein alongside beneficial phytochemicals and healthy fats, making them a viable option for plant-based protein production. Similarly, protein extraction from green vegetable biomass offers an eco-friendly approach to improving the nutritional value of animal feed and human food products. Furthermore, expanding the variety of locally grown crops enhances agricultural resilience against climate change while optimizing the nutritional profiles of food products. Beyond food applications, agricultural by-products are valuable in the cosmetic industry, where demand for sustainable, plant-derived ingredients is growing. For instance, cottonseed oil from *Gossypium hirsutum* has a unique fatty acid composition, making it an excellent candidate for skin, hair, and cleansing formulations. Agricultural history also offers insights into sustainable crop use. Before maize became dominant in North American agriculture, indigenous communities cultivated native plants as part of the Eastern Agricultural Complex, including marshelder (*Iva annua* L.). Recent research in the southeastern U.S. has identified promising

agronomic traits in marshelder, such as stable genome size and increased seed yields. Genetic and morphological analyses reveal significant diversity among wild populations, with farm-grown plants maintaining seed size while achieving higher yields. However, the potential nutritional value of marshelder remains largely unexplored, necessitating further investigation into its micronutrient content, bioactive compounds, and fatty acid profile. Evaluating its protein digestibility and amino acid composition could confirm its viability as a functional food ingredient. Additionally, research into marshelder's processing methods and sensory properties could improve consumer acceptance. Comparing marshelder with other crops and oilseeds may provide further insights into its potential applications. Reintroducing marshelder as a cultivated crop could enhance biodiversity, support food security, and contribute to sustainable agriculture. More broadly, utilizing agricultural by-products for food, cosmetics, and sustainable farming practices can reduce waste, promote environmental sustainability, and drive economic growth. Future research should focus on optimizing extraction processes, improving product formulations, and assessing consumer acceptance to fully unlock the potential of these agricultural by-products.

Potential for the Valorization of Green Leaf and Oilseed Byproducts

by
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DEDICATION

To God and the Virgin Mary for giving me discernment and wisdom on this journey.

To my wonderful parents, Victor, and Elsie, who have taught me that in life you should never give up and that with God, anything is possible. To my siblings, Isa, Vic, Victor, and Tomás, for their unconditional love.

BIOGRAPHY

Janelle Fernanda Gutiérrez Castro was born in the land of lakes and volcanoes, Nicaragua, and developed a deep passion for food science early on. She pursued her bachelor's degree in Food Technology in Costa Rica, where she gained a strong foundation in the field. In 2021, she embarked on her MBA journey, further expanding her expertise in business and quality management. Driven by her enthusiasm for research and innovation, she joined North Carolina State University for an internship at Plant for Human Health Institute in Kannapolis, where she explored advanced topics in food science at Dr. Komarnytsky's lab. This experience solidified her commitment to the field, leading her to pursue a master's degree in Food Science at NC State in 2023. Through her academic and professional journey, Janelle continues to contribute to the advancement of food science with a focus on research and innovation.

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CHAPTER 1

PLANT PROTEIN FROM SPENT OILSEEDS AND GREEN LEAF BIOMASS AS A VALUABLE BYPRODUCT OF AGRICULTURAL CROP PRODUCTION

Abstract

The global agricultural industry generates large amounts of by-products annually from the processing a diverse set of agricultural commodities. However, these by-products are wasted, contributing to economic losses and environmental contamination. Despite their potential, by-products like seeds, leaves, roots, oil cakes, brans, and peels are often discarded. Much of this secondary biomass is rich in proteins, carbohydrates, lipids, dietary fibers, and bioactive compounds, offering substantial nutritional and functional properties. Two major agricultural waste streams with significant protein content are oilseed cakes, generated after oil extraction, and green vegetable matter, including discarded aerial parts and by-products from processed crops. Oilseeds are rich in proteins, phytochemicals, and healthy fats, with their residual cakes presenting a promising alternative protein source to help meet the growing demand for sustainable protein. Protein extraction from green vegetable biomass, also offers a sustainable way to extract proteins, providing an eco-friendly solution to reduce waste and enhance the nutritional value of animal feed. Utilizing these agricultural waste streams for recovery of food functional ingredients not only reduces waste and environmental impact but also creates valuable, sustainable protein sources, thereby contributing to both economic growth and environmental sustainability.

Keywords: agricultural by-products, protein concentrates, sustainable protein sources, waste utilization, oilseed cakes, green vegetable biomass, protein extraction, environmental sustainability

1.1 Introduction

Global interest in sustainable and alternative protein sources is rising as consumers become increasingly aware of the health, environmental, and ethical implications of conventional protein production (Tso et al., 2020). The rising cost of meat and dairy products has made animal protein less accessible for many populations, particularly in low-income regions (Lewis et al., 2023). As a result, there is a growing need for affordable, high-quality plant-based protein sources that can help meet global nutritional demands while reducing reliance on resource-intensive animal agriculture. With the global population expected to reach 9.8 billion by 2050, primarily driven by growth in emerging countries across Africa and Asia, ensuring access to alternative proteins will be essential for meeting future food security challenges (Wood and Tavan, 2022).

At the same time, growing awareness of the environmental footprint of livestock farming, including greenhouse gas emissions, deforestation, and water scarcity, has led consumers in the developed world to seek plant-based alternatives that are both nutritionally beneficial and ecologically sustainable (Springmann, 2024).

These two trends have coincided, creating a global push toward diversifying protein sources beyond traditional animal-based options. The global alternative protein sector has then expanded rapidly, reaching approximately USD 4.7 billion in 2019 (Cho et al., 2022) and is projected to grow to at least USD 290 billion by 2035 as estimated by BCG Global (Witte et al., 2021). A study of high-income countries identified key factors driving this surge in demand. These include the potential health benefits of plant-based diets, such as increased dietary fiber intake, and growing concerns over the negative health effects of traditional animal protein consumption, including high saturated fat intake. Additionally, rising consumer preferences for

environmentally sustainable and ethically produced food have further accelerated the shift toward alternative protein sources (Sadowski et al., 2024).

Comparing plant and animal proteins highlights the distinct nutritional advantages of plant-based sources (Langyan et al., 2022), but not necessarily the ultra-processed plant protein ingredients (Metz et al., 2023). Plant proteins can support higher indispensable amino acid intake, as many whole-food plant sources are less calorie-dense than animal proteins, encouraging greater overall food consumption to meet energy needs (Hertzler et al., 2020). While animal proteins are often considered optimal for meeting protein requirements (Tso et al., 2020), concerns about food safety, environmental impact, and the risk of zoonotic diseases have also driven a shift toward plant-based alternatives. The potential impact of novel plant proteins is significant, particularly given the projected growth of the global protein ingredient market (Devaux et al., 2020). Ongoing research continues to explore new, cost-effective plant protein sources, including those derived from agricultural by-products, paving the way for a transformation in protein production. Extracted plant proteins, available in various forms and compositions, offer opportunities to align with consumer preferences while fulfilling both functional and nutritional needs (Kearney, 2010). Additionally, plant-based proteins serve as valuable additives in food formulations, acting as thickening and gelling agents, emulsion and foam stabilizers, and fat and water binders. Some also exhibit bioactive properties, such as antioxidant and antibacterial effects, further enhancing their potential applications (Nikbakht Nasrabadi et al., 2021).

Agricultural wastes, such as green offcuts and by-product seeds, represent affordable yet underutilized protein sources, despite being naturally produced as part of existing crop cultivation (Putra et al., 2023). These by-products, including leafy residues from vegetable

harvesting and protein-rich seeds, often go to waste or are used inefficiently as animal feed or compost. Exploring these underutilized sources, optimizing extraction methods, and understanding the factors influencing consumer acceptance and demand are essential for advancing sustainable protein solutions (Balfany et al., 2023). This review focuses on green leaf biomass and other promising sources, highlighting their potential to contribute to a more sustainable and nutritious future in the global alternative protein market.

1.2 Conventional plant proteins in food chains

Conventional plant proteins commonly used in food chains include legume seeds (soy, pea) and cereal grains (wheat, rice) proteins, which serve as primary ingredients in meat alternatives, dairy substitutes, and protein-enriched foods (Bedsaul-Fryer et al., 2023). Soy protein, widely recognized for its high-quality amino acid profile and functional versatility, dominates the market in products such as tofu, soy milk, and textured vegetable protein (van den Berg et al., 2022). Pea protein has gained popularity due to its allergen-free status and excellent emulsifying properties, making it a key component in plant-based meat and dairy alternatives (Shanthakumar et al., 2022). While these proteins are well-established in the food industry, emerging legume proteins such as faba bean and lentil are gaining attention for their functional and nutritional benefits.

1.2.1 Advantages of mechanical (dry) fractionation

Isolation and manufacturing of protein products from seeds including soybeans, pulses, cereal grains, and oilseeds offer several advantages, making them valuable for both human nutrition and food formulation (Sá et al., 2020b). Legume seed provide high-quality protein with

essential amino acids, are rich in dietary fiber, and have a low glycemic index, supporting metabolic health (Semba et al., 2021). Grain proteins (e.g., wheat, rice, and oats) contribute to texture and elasticity in food applications, with wheat gluten being particularly useful for meat alternatives and baked goods (Kumar et al., 2023a). Oilseed proteins (e.g., soybean, sunflower, canola, and hemp) are by-products of oil extraction, offering cost-effective, sustainable protein sources (Mondor and Hernández-Álvarez, 2022)

The functional properties of seed proteins vary depending on their source and processing methods that rely on different protein content of each seed class. Oilseeds show macronutrient profiles dominated by oils (10-50%) and proteins (20-40%), with soybeans found at the lower range of oil (15-20%) and higher range of protein content (35-40%). Pulses are characterized by a higher range of crude proteins (20-25%) and low oil content (less than 5%). In contrast, cereal grains mostly accumulate carbohydrates in the endosperm (70-80%) and lower amounts of proteins (5-15%) concentrated in germs (Mondor and Hernández-Álvarez, 2022). Seed protein profiles in oilseeds and pulses are dominated by storage globulins (60-80%) soluble in salt solutions and water-soluble albumins (15-30%) that contain metabolic proteins and enzymes, while prolamins and glutelins are generally low (5-10%). This profile is inverted in cereal grains that are dominated by prolamins (30-50%) soluble in aqueous alcohol solutions or glutelins (30-60%) that require alkaline solutions for extraction, depending on the grain source. By nature of the storage proteins, they require specialized processing to improve their solubility and functionality in food applications (Day, 2013).

Dry seeds with low oil content can be dehulled and milled into flour using hammer, pin, roller, and stone mills (Mondor and Hernández-Álvarez, 2022). Fractured dry particles produced by impact and shearing are expelled through screens using air flow to modify the particle size

distribution into the light or fine fraction (high protein) and the heavy or coarse fraction (high starch) (Rempel et al., 2019). Additionally, electrostatic separation can be used to classify milled seed cakes and other flours by dry separating lignin contaminants (Kdidi et al., 2019). These methods generate no effluent and eliminate the final thermal drying step, thus increasing cost efficiency and sustainability (Pulivarthi et al., 2023). The dry protein enrichment step in these materials generally translates to higher protein purity in the resulting protein concentrates and isolates (Etzbach et al., 2024). The process, however, fails to separate major off-flavors and lipids from the resulting protein fractions, and often requires thermal debittering to maintain the food quality profile (Saffarionpour, 2024).

1.2.2 Wet extraction to obtain higher purity concentrates

Seed flakes, pressed cakes, flours, or dry fractionated powders (all less than 50% protein) are next used to produce protein concentrates (50-80%) or isolates (80-95%) using the widely varying starting raw material composition and extraction parameters, resulting in significant variability of the final products on the market (Etzbach et al., 2024). The process generally includes an agitation and alkaline extraction step (pH 9-11) at a set solvent ratio (1:5-1:20) and temperature (50-60 °C) for 30-180 min, followed by a centrifugation step and isoelectric precipitation at or near pH 4.5, the average isoelectric point of many plant proteins (**Figure 1.1**). The precipitated proteins are solubilized at pH 7 and dried to a final protein product (Mondor and Hernández-Álvarez, 2022). Increased protein functionality often requires extraction at pH < 10 and reduced salt content (ash) after neutralization (Boye et al., 2010)

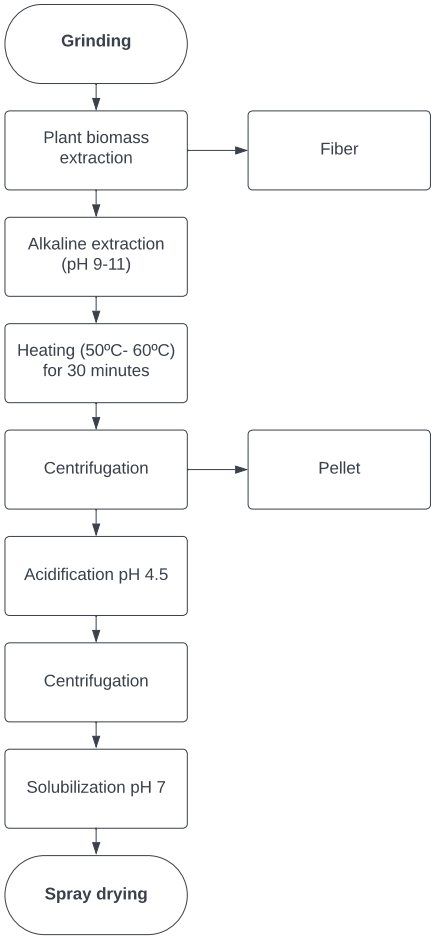

Figure 1.1 Green leaf protein extraction process. The process outlines essential stages such as plant material grinding, extraction, centrifugation, and protein isolation by acidification. Each process guarantees maximal yield and purity for subsequent applications.

1.2.2.1 Soybeans

Soybeans *Glycine max* (L.) Merr. were the first plant-based protein source developed for human consumption. Defatted soybean flakes are a frequent byproduct of oil extraction, which is the most frequently used component of oilseeds. Soybeans are crushed in a roller mill, and the oil is extracted with a solvent, often hexane (Preece et al., 2017). Residual hexane is removed by either indirect heating with steam sparging or direct contact with superheated hexane.

Desolventized soy is heat-processed, ground, and segregated to create the required particle-size distribution, which defines whether the finished product is flour, grits, or meal. The fundamental difference between these items is in their particle size range (Porter and Jones, 2003).

A traditional soy protein isolate (SPI) production process is shown in **Figure 1.2** The basic steps include: solubilizing the protein in ground white flakes, at a 1:10 to 20 solids to solvent ratio, in 60°C (140°F) water adjusted to pH 9 to 11 with sodium hydroxide; removing the insoluble fiber by centrifuging; precipitating the protein at pH 4.2 to 4.5 by acidifying with hydrochloric acid; centrifuging to separate the protein curd from the whey containing the soluble sugars; washing the curd with water and re-concentrating by centrifuging; neutralizing to pH 6.8 with sodium or calcium hydroxide; and spray-drying the washed, neutralized protein curd at 157°C (315°F) inlet air temperature and 86°C (187°F) outlet (Deak et al., 2008).

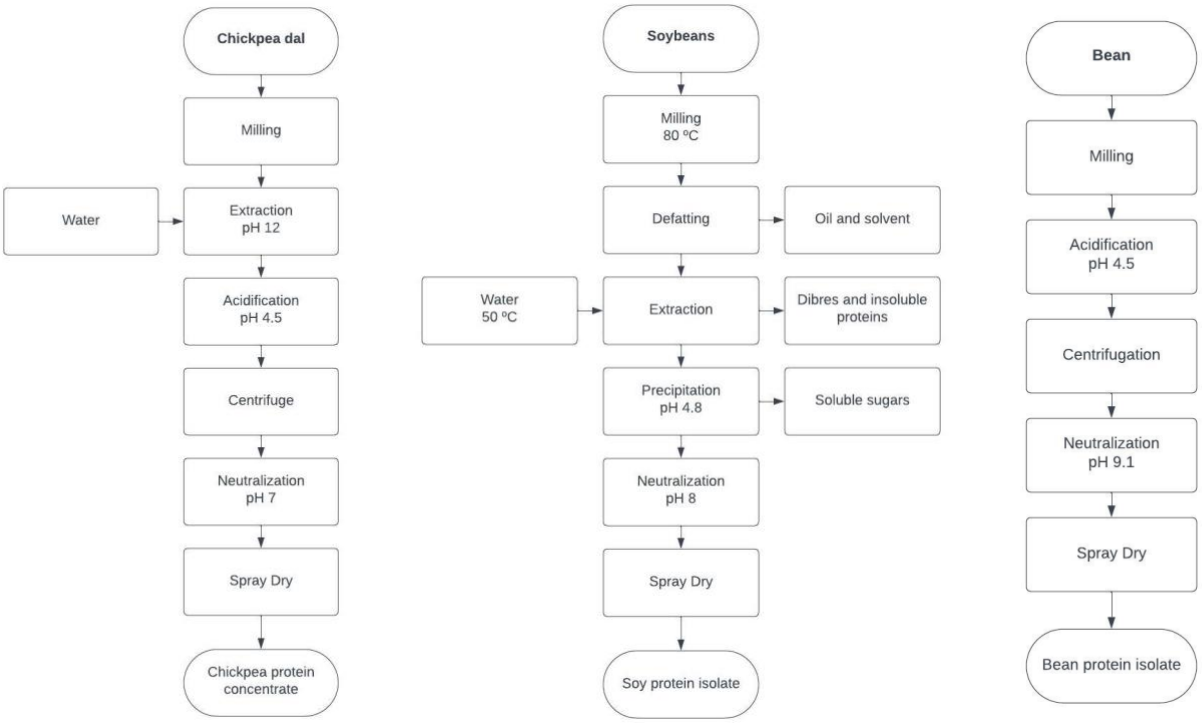

Figure 1.2 Protein extraction for legumes. Diagrams illustrate the protein extraction process for soybean, chickpea, and beans, showcasing key steps such as milling, extraction and acidification. Highlights include differences in processing techniques and protein yield optimization for each legume.

1.2.2.2 Peas

Peas *Pisum sativum* L. it's the second most common protein in the market. Pea protein extraction uses the AEIEP method, which involves alkaline solubilization followed by centrifugation to remove insoluble material. Pea flour is mixed with water (1:10 ratio), adjusted to pH 7.5 with NaOH, and stirred overnight at 4°C. After pH readjustment, centrifugation separates the supernatant. Acid precipitation, induced by fermentation with bacterial starters (S, SL, SLB), is used to obtain globulin-rich and albumin-rich fractions. Fermentation is conducted at 37°C with pH monitored until reaching 4.8. The resulting suspensions need to be centrifuged, yielding pellet samples (globulin-rich) and supernatants (albumin-rich). Globulin fractions were further purified by resuspension, pH adjustment, and centrifugation. (Emkani et al., 2021)

1.2.2.3 Fava beans

Fava beans *Vicia faba* L. is one of the oldest cultivated crops and ranks as the third most significant leguminous feed grain, following soybean and cowpea. It possesses characteristics comparable to soybean, containing approximately 27% protein and a reduced lipid content of 1%, in contrast to soybean's 18% lipid content. The wet extraction process for faba bean protein begins with commercially milled faba bean flour. Dehulling and milling, using equipment like centrifugal, hammer, ball, roller, or disc attrition mills, help remove fiber and cellulosic material,

facilitating protein extraction. Since faba beans have low lipid content, defatting is unnecessary. Protein extraction is typically done using aqueous methods like alkaline extraction. Alkaline extraction is most common, as proteins dissolve well in high pH. The mixture is stirred, and insoluble materials are removed, with careful pH and temperature monitoring. After solubilization, the pH is adjusted to the isoelectric point to precipitate proteins, followed by centrifugation, washing, neutralizing, and drying (Dangi et al., 2022).

1.2.2.4 Common beans

Beans *Phaseolus vulgaris L*. protein separation uses solvent acids to deposition proteins at isoelectric pH, which is crucial for protein crystallization. The choice of an acidic atmosphere during extraction is due to the minimal interaction between amino acid residues, increasing solubility. The material is dissolved in HCl solution, hydrolyzed, and centrifuged to form deposits. The deposits are neutralized with NaOH, filtered, dried, and analyzed for protein isolates. The difference in isoelectric points in proteins is based on the constituent acids' characteristics, with low pH resulting in positively charged amino acids and high pH negatively charged ones (Kusumah et al., 2020).

1.2.2.5 Chickpeas

Chickpeas *Cicer arietinum L*. protein is extracted using a conventional method for protein extraction, utilizing alkaline solvents like potassium hydroxide or sodium hydroxide to adjust the pH of the extraction solution. This process disrupts disulfide bonds within proteins, enhancing their solubility and facilitating their release and retrieval. Protein precipitation is initiated by introducing an acid, such as hydrochloric acid, to lower the pH, causing proteins to

reach their isoelectric point and precipitate. Separation of insoluble constituents, primarily fibers and starch, from the settled protein fraction can be achieved through centrifugation, filtration, or sieving techniques. Optionally, a purification step involving rinsing with water or an acidic solution, followed by re-dissolving at a neutral pH with an alkaline solution, may be incorporated to enhance protein quality. Alkaline extraction is favored for its elevated digestibility and bioavailability of isolated proteins, with studies showing high protein content and purity compared to air classification (Patil et al., 2024).

1.3 Plant proteins from agricultural by-products and wastes

The global agricultural industry generates approximately 190 million tons of by-products annually from the processing of foods such as beer, wine, sugarcane, coffee grounds, oilseeds, vegetables, and fruits. However, these by-products are often discarded as waste, contributing to environmental contamination (Hadidi et al., 2024a). The environmental impact of agro-waste, including the loss of valuable nutrients and increased pollution, remains a significant financial, ethical, and sustainability concern. As a result, reducing food waste has become a key focus of sustainable management, offering both economic and environmental benefits (El-Ramady et al., 2022)

Despite their potential value, by-products such as husks, seeds, leaves, roots, oil cakes, brans, and peels are often discarded by the food and agriculture industries. However, many of these secondary products can be repurposed to generate economic benefits while reducing waste. These by-products are rich in proteins, carbohydrates, lipids, dietary fibers, and bioactive compounds such as phenolics, alkaloids, and pigments, which offer significant nutritional and functional properties. Extracting valuable molecules from agro-waste can enhance global food

sustainability, support environmental conservation, and create new economic opportunities (Hadidi et al., 2024a).

Two major agricultural waste streams that contain significant amount of protein include oilseed cakes generated after extraction of oil (i.e. sunflower, canola, hemp, cottonseed, linseed, flaxseed, and pumpkin), and green vegetable matter in the form of discarded aerial parts, offcuts, tops, peels, or pulp after the target crop is collected and processed.

1.3.1 Oilseeds and pressed oilseed cakes

Oilseeds are versatile food ingredients rich in beneficial compounds, including oleochemicals, phytochemicals, proteins, fats, fibers, vitamins, minerals, and antinutritional factors (Vichare and Morya, 2024). They are also the primary source of vegetable oil, with the residual press cakes and meals remaining after oil extraction containing high protein content. With global oilseed production reaching 600.47 million metric tons in 2018/2019, significant quantities of these by-products are readily available. As a result, oilseed cakes and meals present a promising alternative protein source to help meet the rising global demand for sustainable protein (Arrutia et al., 2020).

Oilseed cakes are a rich source of protein isolates with diverse functional properties, including emulsification, creaming stability, and water- and oil-holding capacities, making them valuable for various food industry applications. The protein quality proteins derived from oilseed cakes such as hemp, canola, sunflower, and palm kernel is comparable to that of soy protein isolates (Singh et al., 2022). Several studies have demonstrated that protein can be effectively isolated from oilseed cakes using an alkaline extraction process, followed by precipitation at the protein's isoelectric point. This widely recognized method efficiently extracts valuable proteins,

contributing to the development of protein-rich ingredients for food products, animal feed, and industrial applications. Additionally, utilizing proteins from oilseed cakes enhances nutritional value while promoting the sustainable use of agricultural by-products (Teh and Bekhit, 2015).

1.3.1.1 Sunflower seed

Sunflower *Helianthus annuus* L. meal is used to extract protein isolates through two main phases: extraction and separation. In the extraction phase, proteins are solubilized using aqueous solutions with varying pH levels and ionic compositions, while the separation phase involves isolating proteins from other meal constituents. Sunflower proteins exhibit low extractability, around 20%, within a pH range of 4–6, due to their low net charge near their isoelectric point. However, moving away from this point increases protein charge and solubility. The effectiveness of protein extraction is influenced by several factors, including pH, ionic strength, temperature, solid-to-liquid ratio, and extraction duration. Acidic conditions generally enhance extraction yield, while basic pH values significantly improve it. Additionally, the presence of salts like NaCl can further enhance protein extractability (Hadidi et al., 2024b).

1.3.1.2 Pumpkin seed

Pumpkin *Cucurbita pepo* L. seed meal is used to extract under varying conditions of temperature (30–60 °C), extraction time (10–30 min), and pH (7–10). The extraction takes place using an alkaline sodium hydroxide solution in a water bath with continuous stirring. After the required extraction time (10–30 min), the solution is centrifuged at 1000 rpm for 15 min at 4 °C. The supernatant is then filtered through filter paper, and the soluble protein content is determined using Lowry's method (Wani et al., 2008). Protein yield (percentage) is calculated as

the amount of extracted protein relative to the total protein in the pumpkin seed meal, which is estimated by multiplying the nitrogen content by a factor of 6.25. Protein isolates are obtained by adjusting the extract's pH to 5 using 0.1 N hydrochloric acid, followed by washing with water. The proteins are then collected by centrifugation (6500 rpm, 30 min, 4 °C) and subsequently dried (Lalnunthari et al., 2019).

1.3.1.3 Canola seed (rapeseed)

Canola *Brassica napus* L., the third-most abundant oil crop globally, has a vast value chain, with its residual proteins gaining increasing attention for applications in the chemical sector (Fetzer et al., 2020). Rapeseed meal, a by-product of oil extraction and thermal processing, is ground and sieved to obtain uniform particles. To improve its usability, the meal undergoes ethanol treatment to reduce phenol and glucosinolate levels, after which the residue is collected, dried, and stored for protein extraction. Protein-rich ingredients (PI) are produced by dispersing 5% meal in NaOH, followed by protein precipitation with HCl at pH 4.5. The precipitated protein (PI) is then collected and dried via lyophilization, while the supernatant, containing acid-soluble proteins, is also lyophilized to obtain acid-soluble protein (ASP). The crude protein content of both PI and ASP is determined using the Kjeldahl method with a conversion factor of 6.25 (Kalaydzhiev et al., 2019)

1.3.2 Green vegetable biomass

Extracting proteins from discarded green leaf biomass represents a significant advancement in sustainable agriculture, transforming agricultural waste into valuable protein sources (Nynäs et al., 2024). This process not only reduces waste but also enhances resource efficiency by utilizing the nutritional potential of leaves that are often discarded during

harvesting. Green protein concentrates, derived from fresh biomass, retain much of the chlorophyll and essential nutrients, making them rich in amino acids and bioactive compounds (Sherwood, 2020). However, their distinct green color and grassy taste may limit their application in certain food products, though they remain well-suited for animal feed and specialized food formulations.

To expand their usability, beige protein concentrates undergo additional processing to remove chlorophyll and other pigments, resulting in a more neutral color and flavor, making them more versatile for food applications. Processing techniques such as solvent extraction, membrane filtration, and enzymatic treatments help isolate and purify proteins while eliminating undesirable components (Kumar et al., 2023b). This approach not only improves the functional properties of leaf proteins but also supports the development of sustainable and alternative protein sources for both human and animal nutrition.

Plant leaf proteins can be classified based on their solubility into two main groups: soluble white proteins and insoluble green proteins. The soluble white fraction accounts for 37.6% of the total protein, while the insoluble green fraction makes up 31.3% (Anoop et al., 2023). The insoluble fraction consists of integral membrane proteins embedded in the photosynthetic complex, bound to pigments, lipids, and other molecules within the thylakoid membrane. In contrast, the soluble fraction mainly comprises cytoplasmic proteins, with Rubisco as the dominant protein, representing up to 50% of the total soluble protein.

Rubisco, a key enzyme in the stroma and one of the most abundant proteins in nature, plays a crucial role in photosynthesis (Møller et al., 2021). Its nutritional and functional properties rival those of many animal proteins, offering high digestibility, essential nutrients, and excellent foaming, gelling, and emulsifying properties, making it valuable for food applications.

Commercial-scale extraction requires efficient processing technologies capable of handling large plant volumes while ensuring food-safe extraction. Research highlights promising opportunities for large-scale Rubisco extraction, but further studies are needed to assess practical feasibility (Pearce and Brunke, 2023). Optimizing extraction techniques improves yield and purity, directly impacting energy efficiency and sustainability. Additionally, maximizing co-products from the extraction process enhances economic viability while reducing waste, contributing to a more sustainable protein supply.

Transforming green leaf biomass from various crops into protein concentrates represents a holistic approach to sustainable agriculture. By utilizing all plant components efficiently, this strategy reduces waste while contributing to food and feed security (Bala et al., 2023). Integrating traditional agricultural practices with modern sustainability principles strengthens the resilience and efficiency of food systems. The incorporation of leaf proteins into the global protein supply chain offers a diverse and resource-efficient solution to meeting the rising demand for high-quality, sustainable proteins.

1.3.2.1 Sugar beets (wasted green aerial parts)

Sugar beets *Beta vulgaris* L. provide a valuable source of green biomass. The leaves and other by-products of sugar beet processing, often discarded, can be repurposed into protein concentrates. This approach not only enhances the sustainability of sugar production by reducing waste but also creates an additional revenue stream. Rich in nutrients and bioactive compounds, sugar beet leaves are a promising raw material for protein extraction (Tamayo Tenorio et al., 2017). Integrating protein extraction into existing sugar beet processing facilities can improve overall efficiency and economic viability. Protein concentrates derived from sugar beet leaves

can be used in animal feed, reducing dependence on traditional protein sources such as soybeans and fish meal, thereby promoting more sustainable livestock farming (Ebrahimi et al., 2022).

The process includes thawing sugar beet leaves at room temperature and mixing with a 0.02 M carbonate-bicarbonate buffer (pH 9.2) at a 1:8 (w/v) ratio. The mixture is then homogenized at 10,000 rpm for 4 min. The solution is heated to 50 °C and shaken at 80 rpm for 30 min in a water bath. After filtration, the solution is centrifuged at 6,000 rpm for 20 min. For isoelectric point precipitation, the pH of the supernatant is adjusted to 3.5 using 3 N HCl and stirred at 300 rpm for 30 min. The resulting suspension is then centrifuged at 6,000 rpm for 30 min, and the pellet is collected and freeze-dried (Goktayoglu et al., 2023).

1.3.2.2 Cabbage (wasted green offcuts)

Cabbage *Brassica oleracea* L. generates a significant amount of wasted green biomass in the form of unprocessed leaves and the offcuts. These nutrient-rich tissues can be converted into protein concentrates, offering a sustainable source of high-quality protein while reducing agricultural waste. Additionally, incorporating cabbage leaf protein into animal feed can enhance livestock nutrition and reduce the environmental footprint of animal farming by lowering dependence on conventional feed ingredients (Shinali et al., 2024).

The process of obtaining cabbage protein isolate begins with extracting liquid from the leaves to obtain the juice. To separate the green and white protein fractions, the juice is heated to 55 ºC for 25 min. It is then centrifuged at 32,000 rpm for 10 min. Acid is added to precipitate the protein at pH 4.5. The sample is then agitated at room temperature for 30 min and subjected to mild centrifugation at 260 g for 5 min at 4 ºC to remove insoluble particles. Finally, the collected pellet is freeze-dried (Nynäs et al., 2021).

1.3.2.3 Potato (wasted pulp after starch extraction)

Potato *Solanum tuberosum* L. farming generates substantial residual biomass, including soluble proteins in potato mash as a byproduct of starch manufacturing. The extracted proteins can be incorporated into various food applications, such as fortifying processed foods, thereby enhancing the nutritional value of potato-based products and contributing to improved dietary quality (Herreman et al., 2024).

Several factors influence the coagulation of proteins in potato juice. One method involves heating the juice to temperatures up to 120 °C at the isoelectric point of most potato proteins, typically between pH 4.5 and 5.2. Alternatively, lowering the pH to 2-4 can induce coagulation of patatin proteins at lower temperatures. Adjusting the pH can also affect the ionic strength of the juice, with pH levels of 4 and 6–7, combined with temperatures between 50–80 °C, influencing protein coagulation. Salting out proteins using ferric chloride salt enhances protein precipitation and improves recovery efficiency to over 86%, due to its strong affinity for potato proteins (Pęksa and Miedzianka, 2021).

1.3.2.4 Duckweed (growing on agricultural liquid waste streams)

Duckweed *Lemna* spp. is a good candidate for sustainable protein production due to its rapid growth and high protein content. Its ability to thrive in diverse aquatic environments makes it particularly advantageous, as it can be cultivated on water bodies unsuitable for traditional crops or supplied by liquid agricultural wastes, avoiding competition with arable land. Converting duckweed biomass into protein concentrates unlocks an underutilized resource, reducing waste while providing a high-quality protein source for both food and animal feed (Sosa et al., 2024).

Duckweed is mechanically treated to break open the cells, allowing protein solubilization. It is then pressed using a slow juicer, separating a juice fraction. The juice fraction is heated to 70 °C and maintained at this temperature for 1 h to denature and precipitate the proteins. The material is then centrifuged at 16,500 g for 15 min, after which the supernatant is decanted and the pellet collected. The collected pellet is spray-dried to concentrate the leaf protein (Nieuwland et al., 2021).

1.3.2.5 Alfalfa

Alfalfa *Medicago sativa* L. is valued for its high nutritional content and harvested multiple times per season on marginal lands, with cutting height significantly influencing both biomass yield and quality. Repurposing alfalfa biomass for protein concentrate production presents a sustainable solution of generating both green protein concentrates that retain chlorophyll and are rich in essential amino acids, as well as beige concentrates that generate edible protein with high functionality (Hojilla-Evangelista et al., 2017).

Numerous processing techniques have been explored for extracting protein from alfalfa. The process begins with fractionating the alfalfa leaves to yield a fibrous pulp and a protein-rich juice using a double screw press (Nissen et al., 2022). The juice is preheated by hot water steam and its pH is adjusted to 8.0 through alkalinization. Once solubilized, the mixture's pH is adjusted to 3.5 to promote protein precipitation, as this is close to the isoelectric point of the proteins. Excessive heat can lead to protein denaturation, negatively affecting protein functionality. Therefore, optimizing the temperature is essential to maintain protein integrity throughout the extraction (Hadidi et al., 2023)

1.4 Quality and functional properties of protein concentrates

Protein concentrates are evaluated based on their amino acid profile, digestibility, and nutritional value, which directly influence their suitability for various applications in food and feed. The functional properties of these concentrates, such as solubility, emulsifying capacity, and gel formation, are key indicators of their versatility in different formulations. High-quality protein concentrates not only offer essential nutrients but also contribute to the texture, stability, and sensory attributes of the final product.

1.4.1 Solubility and viscosity

Protein solubility refers to the ability of a protein solute to dissolve in a liquid solvent and produce a uniform solution, based on its physicochemical characteristics. It means the degree to which protein remains in the liquid portion after being spun at specified time and force settings during centrifugation. Solubility can be understood at a molecular level as a dynamic equilibrium process, where dissolving and solid phase creation occur simultaneously (Gao et al., 2024). Solubility is key because it determines how well a protein can dissolve in water, which is crucial for a wide range of food applications. For example, proteins from soy and peas vary in solubility based on their processing and environmental conditions (Grossmann and McClements, 2023). If you're making a plant-based milk, you need the protein to dissolve well to create a smooth, uniform drink. Factors like pH and temperature can significantly impact this. Soy protein, for instance, dissolves better in alkaline conditions, which can be beneficial for some products (Sajib et al., 2023).

On the other hand, the viscosity of protein solutions emerges from various interactions between proteins molecules, providing insights on the fluid's thickness and flowability (Hong et

al., 2018). Understanding the elements that influence plant protein viscosity and gelation performance, as well as how to control them, can assist food scientists in optimizing formulations for specific products, resulting in new plant-based food innovations, and to prevent viscosity from affecting the quality attributes of food (Zhu et al., 2024). The viscosity of protein systems is closely correlated with protein content, pH, particle size, and the morphology of protein aggregation (Qi et al., 2023).

1.4.2 Emulsifying activity and stability

A stable emulsion is one that exhibits a low rate of undergoing the processes that lead to the separation of oil and water phases. These processes encompass creaming, flocculation, and coalescence (Pearce and Kinsella, 1978). Plant proteins can act as emulsifiers, meaning they help keep these mixtures stable. However, their application as emulsifiers is not widespread due to their weaker emulsifying characteristics compared to animal proteins. The emulsification properties of proteins are inherently linked to their structural characteristics. An essential characteristic of the protein, to be used as an emulsifier, is its immediate or inherent amphiphilicity. The arrangement of the individual amino acids along the polypeptide chain dictates the configuration and location of the hydrophobic and hydrophilic regions within the structure, hence establishing the amphiphilic nature (Lima et al., 2023).

Therefore, the emulsifying capacity of proteins is influenced by their molecular properties, with key factors including molar mass, hydrophobicity, conformation stability, charge, and physicochemical factors such as pH, ionic strength, and temperature. The solubility of proteins is crucial, as proteins that are excessively insoluble do not function well as emulsifiers and can lead to coalescence. The emulsifying capabilities have a strong link with the

presence of hydrophobic residues on the protein surface, which are not stable at the oil-water interface. Emulsion stability is affected by the presence of salts and the pH value. Denaturation can enhance the emulsifying characteristics of proteins by increasing their hydrophobic surface area and flexibility (Moure et al., 2006)

1.4.3 Foaming capacity and stability

Proteins play a crucial role in creating stable foams, which are essential in food production. Foam is a two-phase system consisting of air cells separated by a lamellar phase. The size distribution of air bubbles in foam affects its appearance and textural properties. Proteins contribute to the uniform distribution of fine air cells in food structure, allowing volatilization of flavors and enhanced palatability. Common protein foaming agents include egg white, gelatins, casein, milk proteins, soy proteins, and gluten. Protein foaming properties vary significantly, with serum albumin being an excellent foaming agent and purified ovalbumin being poor. Protein foaming agents should stabilize foams rapidly and effectively at low concentrations, perform well over various food pH ranges, and work effectively with foam inhibitors (Zayas, 1997).

Hence, foam research employs several techniques to create and analyze foam, such as shaking, whipping, high shear mixing, and gas sparging. Foam creation and ageing are described using several parameters. The words overrun, foamability, and foam capacity are frequently used interchangeably, where the resulting foam volume is commonly proportional to the starting liquid volume of the sample. The language used to describe foam aging has been standardized, with "foam stability" referring to the variations in foam and liquid volume that occur over time, which is also commonly referred to as drainage. Foam destabilization is primarily caused by the

process of bubble coalescence and coarsening. Foam capacity (FC) is utilized to express the increase in volume, whilst foam stability (FS) pertains to the enduring volume of foam, unless stated otherwise (Amagliani et al., 2021).

1.4.4 Water holding capacity

The water holding capacity (WHC) of proteins refers to their ability to physically and physicochemically retain water against the force of gravity. The primary functional features of proteins are closely linked to their interaction with water, influencing their functional characteristics in food like water binding, swelling, solubility, emulsifying qualities, viscosity, gelation, and syneresis. WHC is a significant factor in determining the texture of food, particularly in goods made from ground beef and dough that is baked. The water retention properties of plant proteins, when used as additions in different foods, affect the qualitative qualities of the final food items (Zayas, 1997).

The most common methods to determine these parameters involve dispersing a known mass of protein in distilled water or vegetable oil followed by vigorous mixing, then centrifuging to remove excess water or oil. The WHC values of plant protein isolates generally increase with increasing protein content, but the extent of the increase is typically different. Plant proteins with good water and oil holding capacities are often used as meat extenders or in plant-based meat analogs. For example, adding 2.5% bean flour as an extender improved the water holding capacity of beef sausage. The addition of chickpea and pea flour to low-fat pork bologna resulted in higher cooking yield and reduced purge loss (Ma et al., 2022).

At the same time, water retention plays a crucial role in formulated foods and can impact the sequence in which dry components are added to the formula. The water retention rate can be

utilized to determine whether a protein should be supplied in powder form or rehydrated prior to its inclusion to the mixture (Xiao et al., 2024). The study of protein powder-moisture interaction is used to identify and create suitable packing materials that can effectively preserve the desired moisture level in the product. There is still a significant amount of knowledge to be gained on the interactions between proteins and water, which play a crucial role in determining protein structure and functionality (Zayas, 1997).

1.4.5 Oil absorption capacity

The oil absorption capacity (OAC) is another significant functional characteristic in food processing for pulse flours and proteins. This is about how well the protein can bind and retain oil when the hydrocarbon chains of oil bond to the nonpolar side chains of amino acids, which affects the flavor and mouthfeel of the product, it also impacts the consistency, and the amount of product obtained. OAC is thought to derive from the trapping of oil within proteins and the presence of noncovalent connections, such as hydrophobic, electrostatic, and hydrogen bonding, which play a role in the interactions between lipids and proteins (Wang et al., 2020).

As for plant proteins, the absorption of oil is influenced by the concentration of proteins, the quantity of nonpolar sites, and the interactions between proteins, lipids, and carbohydrates (Zayas, 1997).

1.4.6 Protein digestibility

Proteins are natural polymers made up of amino acids held together by peptide bonds. The composition, quantity, and arrangement of amino acids along a polypeptide chain dictate the inherent configuration of a protein molecule, which impacts its breakdown and uptake in the

human gastrointestinal system. Furthermore, the process of breaking down and assimilating proteins is influenced by various elements, including their state of denaturation, aggregation, and the effects of the dietary matrix. Protein digestion commences in the stomach as a result of the presence of pepsin in the gastric juices but mostly takes place in the small intestine due to the existence of a sequence of proteases, such as trypsin and chymotrypsin. The rate and extent of protein digestion are determined by the accessibility of the proteases to the peptide bonds within the polypeptide chains (Shaghaghian et al., 2022).

Digestibility refers to the amount of food or nutrients that can be absorbed into the bloodstream or body after being consumed. Additionally, it is employed to characterize the degradability of proteins, specifically referring to the amount of unbroken protein that remains, the consequent level of hydrolysis, or the fraction of small peptides that are formed following the activity of digestive enzymes (Rivera del Rio et al., 2022).

Food protein digestibility refers to the extent to which a protein may be broken down by proteolysis. It is influenced by factors such as the protein's structure, the intensity of thermal processing, and the presence of certain substances known as antinutritional factors that can hinder protein digestion. The reduction or elimination of these compounds is important to improve the biological utilization of plant proteins, which are a bit harder to digest compared to animal proteins because of anti-nutritional factors like trypsin inhibitors and phytates. Additionally, plant proteins present lower enzyme accessibility due to rigid cell walls and seed coats. But, through methods like fermentation and enzyme treatment, can improve their digestibility, making plant-based foods a viable and healthy protein source (Sá et al., 2020a).

1.4.7 Color, texture, and taste

The nutritional value of plant proteins is significant, but as significant is their utility, which allows them to be used as components in food composition to provide appearance, flavor, color, odor, texture, and even structure to food products. These are crucial for getting consumers on board with plant-based products. The color of plant proteins can impact the look of the final product. For instance, the greenish tint of pea protein might not be ideal for all foods, whereas soy protein's neutral color is often preferred (Akharume et al., 2021). Generally, plant proteins possess a darker color than dairy protein, although it varies depending on the plant source (Tang et al., 2023).

Prior studies have demonstrated that the protein chemistry of plants and their interactions with water and other physicochemical factors can impact the texture of the result. Plant proteins can create a range of textures, from creamy to chewy, depending on how they're processed (Flory et al., 2023). For example, by treating wheat protein to processing and extrusion techniques, it is possible to mimic the texture of meat. This is made possible due to the viscoelastic and cost-effective qualities of wheat gluten. Therefore, wheat gluten is specially included in the composition of meat mimics, distinguishing it from other cereals. Products containing wheat gluten may include the constituents of textured vegetable protein. These materials can be employed as additives to increase the volume of meat and as substitutes for meat (Siddiqui et al., 2024).

Although plant protein concentrates and hydrolysates are gaining increasing attention for food applications, their use still results in low consumer acceptance primarily because of their "green", "grassy", or "beany" off-odor as well as long-lasting bitter and/or astringent off-taste, which limits palatability in human consumption (Mittermeier-Kleßinger et al., 2021). These

flavors can be associated to the production of characteristic off-flavor volatiles, like aldehydes, alcohols, ketones, pyrazines, furans and non-volatiles from various plant derived foods (Leonard et al., 2023). The quality and functional properties of plant proteins are vital for creating successful plant-based foods. By understanding and optimizing these properties, we can develop nutritious, sustainable, and delicious alternatives that appeal to consumers.

1.5 The need for new crops to maintain diversity of food supply chains

In view of the current climatic and social changes, it is necessary to identify environmentally viable alternative sources of food proteins and oils. These novel crops should not only possess a wide range of nutritional characteristics but also bring the distinct functional attributes that can be utilized for various purposes (Singh et al., 2023).

For example, soy protein and wheat gluten are currently the predominant plant-based protein sources used as substitutes for animal protein. Nevertheless, numerous people refrain from consuming these ingredients because of gluten sensitivity, celiac disease, or the allergenic properties of soy proteins (Arora et al., 2023). There is a requirement for novel sources of protein derived from plants. Unexplored legume pulses and oil crops are particularly well-suited as new sources of plant-based food ingredients because of their diverse characteristics and nutritional properties (Rajpurohit and Li, 2023).

Another example is hemp (an oilseed crop) that attracts significant attention due to its versatile applicability, rapid production cycle, and minimal cost for growth, as well as its potential as a carbon-negative material. Hemp and its industrial products have the potential to play a crucial role in the development of sustainable and resilient food systems (Ahmed et al.,

2022). Hemp seeds exhibit a high-quality nutritional profile similar to that of soybeans (Yano and Fu, 2023).

For future plant-based food ingredients, developers should account for the selection of the nutrient sources, supporting crop diversity, using the right ingredients, creating healthy formulations, and targeting high consumer acceptance (He et al., 2019). Supporting crop diversity is crucial for soil fertility and combating declining yields and nutritional quality caused by climate change. Blending different plant-based raw materials improves nutritional and technological quality of final food products (Fu et al., 2023).

1.6 Conclusions

Providing a wide range of palatable and convenient plant-based foods with a healthy nutritional pattern can help steer consumer food choices towards healthier and more sustainable products. Researchers and organizations are actively investigating novel food sources that go beyond common alternatives. This exploration can lead to the development of nutritional solutions that are more inclusive and can accommodate those with dietary restrictions and allergies. By utilizing existing and novel crops, and other innovative uses of agricultural waste streams, the food sector can meet the increasing customer need for healthier and more sustainable choices. Additionally, this can help protect the environment by reducing resource use and carbon emissions.

Furthermore, the significance of choosing a variety of crops that are cultivated in the local area cannot be emphasized enough, as it improves both the nutritious composition of food items and the ability of agricultural systems to withstand the impacts of climate change. By including a diverse range of plant-based raw materials, the finished products are enhanced in

terms of their quality and functioning, as they contain a well-rounded combination of important nutrients, amino acids, and bioactive substances. Likewise, the utilization of by-products from plant-based food production contributes to environmental sustainability by reducing waste and optimizing the utilization of existing resources. This comprehensive approach to food development not only emphasizes the importance of nutritional value and sustainability but also fulfills consumer demands for taste and convenience, which are essential for universal acceptance.

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CHAPTER 2

COTTONSEED OIL AS A BYPRODUCT OF COTTON FIBER PRODUCTION AND ITS APPLICATION TO SKIN HEALTH AND PERSONAL CARE

Abstract

The historical use of oils for beauty and hygiene dates back to ancient civilizations. While mineral oil and its derivatives dominated the personal care industry in the twentieth century due to chemical stability and low cost, the environmental impact and sustainability concerns have driven a resurgence in the use of vegetable oils. Cottonseed oil derived from *Gossypium hirsutum L.* (*Malvaceae*) has been often overlooked in favor of other plant oils, likely due to cotton's primary use as a fiber crop. Yet cottonseed oil stands out in cosmetics for its beneficial linoleic to oleic acid ratio, which supports skin barrier function, and its rich profile of phytosterols and tocopherols that provide higher oxidative stability and extended shelf life. Cottonseed oil is also adaptable for use in a variety of formulations, offering a lightweight, non-greasy emollient base with potential applications in skin care, hair, and cleansing products. This review highlights cottonseed oil as a potentially underutilized ingredient in the personal care sector and emphasizes the need for further research and development to fully exploit its properties.

Keywords: fatty acid profile; carrier oil; skin barrier function; skin conditioning; sebum; emulsions; formulations; cosmetic applications; edible cosmetics

2.1 Introduction

Oil is a common ingredient found in many cosmetic and personal care products. In the past, oils were extracted from natural sources including animal and vegetable fats based on their geographical distribution, accessibility, and changes in contemporary agricultural practices associated with animal and crop domestication (Komarnytsky et al., 2022). While these oils may not have been processed on a large scale like in later periods, they still played an essential role in personal care and practices (Moore et al., 2020). Among the earliest records of using natural oils for skin care, and in balms and ointments by the Mesopotamian and Mediterranean cultures, are olive (*Olea europaea L.*), sesame (*Sesamum indicum L.*), castor (*Ricinus communis L.*), laurel (*Laurus nobilis L.*), and argan (*Argania spinosa L.*) oils. The lesser-known oils of pre-Columbian Americas include avocado (*Persea americana Mill.*), sunflower (*Helianthus annuus L.*), cotton (*Gossypium hirsutum L.*), as well as jojoba (*Simmondsia chinensis* (Link) C. K.) liquid wax esters.

This trend has changed abruptly when mineral oil was discovered as a byproduct of refined crude oil in the mid-19th century. Its low cost, chemical stability and inertness rapidly established it as an ideal base ingredient in skincare formulations. Refined mineral oils are colorless, odorless, and tasteless lipids have been safely used in cosmetics for many decades to achieve occlusive moisturizer (non-absorbent) and emollient (skin softening) outcomes (Rawlings and Lombard, 2012). Common ingredients that are mineral oil-based are petrolatum, paraffin, microcrystalline cera and wax, ceresin, and ozokerite. While these promote healthier skin, their major downsides such as environmental contamination of water and soil, lack of biodegradability, and limited sustainability revitalized the search for alternative ingredients that have similar skin benefits (Juliano and Magrini, 2017). In the 21st century, consumers demanded

better, longer-lasting, and safer personal care products that are cruelty-free and environmentally friendly (Amberg and Fogarassy, 2019). In response, the industry has once again increased the use of vegetable and seed oils in their formulations (Bom et al., 2019).

Allotetraploid *Gossypium hirsutum L*. (tribe *Gossypiae*, family *Malvaceae*), commonly known as upland cotton native to Mesoamerica, emerged within the last 1 to 2 million years, resulting from the unlikely transoceanic dispersal of an A-genome species to the New World, where it hybridized with a native D-genome diploid (Wendel and Grover, 2015). It was brought into cultivation as early as 3,500 BC in the Tehuacan Valley of Mexico (Smith and Stephens, 1971), and further developed into a vigorous cotton industry in the lowland Veracruz by 300 AD (Stark et al., 1998). Botanical remains of cotton seeds and embryos were found in three huts at the Ceren site in El Salvador, which was buried by volcanic ash from the nearby Loma Caldero volcano (585-600 AD). These remains, especially those found on a metate surface in hut 4, strongly suggest that the Ceren inhabitants were grinding cotton seeds to extract oil (Lentz et al., 1996). Cottonseed DNA was also detected in the Huecoid coprolite sample from Vieques, Puerto Rico dated to 500 AD, although it is not clear whether it was consumed or introduced via saliva during spinning of cotton fibers (Reynoso-García et al., 2023). A more recent use of the crushed cotton seeds (*taman*) by Yucatecan Maya to manage tenesmus may have provided an alternative medicinal explanation to these findings (Roys, 1931). Scalp diseases, ulcers, and other conditions were also routinely treated with boiled and crushed cotton leaves and flowers in this region (Roys, 1931). Elsewhere, Pima Indians from American Southwest consumed ground or parched cotton (*taki*) seeds (Russell, 1908). Although cotton could only be cultivated in the limited ecological zones defined by a narrow range of warm temperatures (11-25 °C) and a rainy period followed by dry weather, extensive trade networks ensured that cotton (ichcatl) was accessible to

elites across most regions of the Americas during the Aztec empire in the early 16th century (Berdan, 1987). Other species of cotton were developed in South Asia (*Gossypium arboreum L*.) and Northeast Africa (*Gossypium herbaceum L.)* (Fuller et al., 2024), but historical records of cottonseed oil use in these regions prior to modern times are virtually unknown, much like with soybeans.

Cotton seeds contain a significant amount of oil with a high-quality fatty acid composition (Riaz et al., 2023a). Rich in linoleic, palmitic, and oleic fatty acid, sterols, tocopherols, and other beneficial compounds, cottonseed oil has moisturizing, antioxidant, and anti-inflammatory properties that can benefit personal care (Egbuta et al., 2017). Historically, cottonseed oil has not received the same recognition as other plant-based oils, likely due to cotton's primary role as a fiber crop rather than an oilseed, and its potential in the cosmetic industry remains largely untapped. Cottonseed oil is a readily available, inexpensive agricultural byproduct that is regulated under strict food-grade standards, making it an eco-friendly alternative to more expensive oils. In this review, we aim to highlight the underutilized potential of cottonseed oil in skin and personal care product development, and accentuate the opportunities it presents as a sustainable, eco-friendly ingredient. The analysis emphasizes distinctive balance of linoleic and oleic acids, oxidative stability, and formulation versatility of cottonseed oil, shifting the focus from its traditional role in agriculture to its overlooked cosmetic potential.

2.2 Cottonseed oil extraction and refining

The cottonseed oil available on the market is mostly refined; both solvent and mechanically expressed crude oils can be used in the refining process (Riaz et al., 2023b). Refining also accomplishes the removal of a naturally present terpenoid aldehyde gossypol and

cyclopropenoids such as sterculic (CPE 19:1) and malvalic (CPE 18:1) fatty acids. Both components are considered undesirable for nutritional and health purposes (Yang et al., 2021). This is similar to rapeseed oil that was naturally high in erucic acid until it was cross bred to produce canola cultivars with a very low erucic acid trait (G et al., 2008). The refined cottonseed oil also contains up to 2% of a variety of nonoil substances (Andersen, 2001), while several volatile substances end up in the deodorizer distillate byproduct (Hussain Sherazi et al., 2016) (**Figure 2.1**).

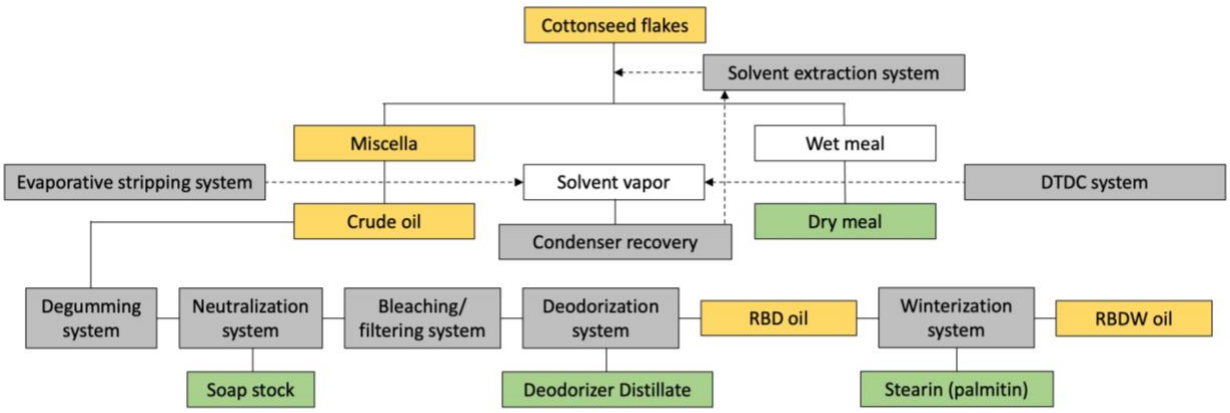

Figure 2.1 Cottonseed oil manufacturing flowchart. Oil-containing materials are highlighted in yellow as crude, RBD (refined, bleached, deodorized), and RBDW (RBD winterized) oils. The byproducts remaining after the processing steps are highlighted in green.

Refined cottonseed oil can be further modified through winterization, a fractional crystallization process that slowly cools oil and removes the precipitating higher melting stearin (palmitin) byproduct. Winterization produces oil that remains clear at lower temperatures, with a pleasant flavor and increased stability (Khan et al., 2021a). The winterization process is very successful in cottonseed oil because it contains only trace amounts of the linolenic acid in contrast to canola and soybean oils. The cottonseed oil can be also modified through full

hydrogenation or interesterification to harden it without generating trans-fatty acids (Imran and Nadeem, 2015). Multiple types of the cottonseed oils are therefore available to the manufacturers and consumers to tailor the physiochemical and functional properties of their formulations (**Table 2.1**).

Table 2.1 General differences among vegetable oils based on extraction and refining methods applied.

Cold-pressed[1,2]	**Virgin**[1,2]	**Crude (whole)**[1]	**Refined**[1]	**Winterized**[1]	**Hydrogenated**[1]
					
Mechanical below 30 °C	Mechanical with heat	Solvent extraction	All oils can be refined	Chilled	Processed
High flavor, phytochemicals	Moderate	Moderate	Neutral	Neutral	Solid at RT
High compositional variability	Moderate	Low	Uniform	No stearin	No M/PUFAs
Most susceptible to oxidation	More susceptible	Less susceptible	High stability	Improved	Highly stable

[1] All vegetable oils can be produced at a pharmaceutical (USP, NF), food (FCC), cosmetic, feed (AAFCO), or technical grades.
[2] Cottonseed oil is typically not manufactured as a cold-pressed or virgin oil on a large scale.

Cotton varieties with an ultra-low seed gossypol trait have been recently approved for human use by the FDA (Rathore et al., 2020), and there are some early indications that breeding low cyclopropenoid fatty acid cotton without negatively influencing other compositional factors of cottonseeds may be possible (Dowd et al., 2010a). When successful, these developments hold the potential to establish additional use of cold-pressed or virgin cottonseed oils in cosmetic formulations. Cold-pressing of cottonseed oil can be also facilitated with multi-enzyme applications (Latif et al., 2007).

2.3 Cottonseed oil component that support skin health

The cottonseed oil is classified as a liquid seed oil similar to avocado, canola, soybean, hemp, and sunflower oils and in contrast to palm, coconut, and cacao bean oils that remain solid under the normal storage conditions. The cottonseed oil is also a semi-drying oil, similar to canola and unlike non-drying (olive, almond) or drying botanical oils (soybean, hemp, sunflower). Semi-drying oils slowly react with oxygen in the air to form soft elastic films in contrast to dying oils that harden to solid rigid films, and non-drying oils that remain as sticky liquids (Orlova et al., 2021). All vegetable oils are available as a natural mix of triglycerides with different ratios of fatty acid esters, and the latter define the bulk of their physicochemical properties (**Figure 2.2**).

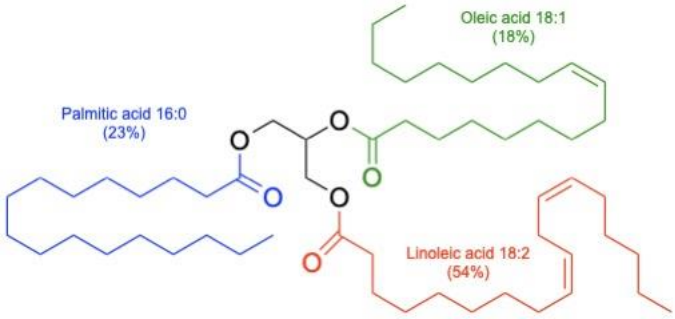

Figure 2.2 Schematic triacylgliceride of cottonseed oil. Different ratios of fatty acid esters are indicated for the three major fatty acids found in cottonseed oil.

2.3.1 Fatty acids

Cottonseed oil has a different fatty acid composition compared to other vegetable oils widely used in cosmetic and personal care formulations. This oil is dominated by the linoleic acid similarly to the spectrum of high-linoleic vegetable oils that include sunflower, hemp, soybean, and rosehip oils, among others. However, cottonseed oil harbors a saturated palmitic acid as the second most abundant fatty acid ester. This is a rather uncommon feature among the

linoleic acid-dominated vegetable oils, and a similar high amount of palmitic acid can be found only in avocado, which is a high-oleic oil (**Table 2.2**). Both naturally-occurring (Shockey et al., 2017) and transgenic (Chapman et al., 2001) high oleic acid cotton cultivars have been also reported, but not grown widely.

Table 2.2 Cottonseed oil among the selected vegetable oils based on their major fatty acid profiles (%).

Botanical source	**Almond kernel** *Prunus dulcis*	**Olive drupe** *Olea europaea*	**Rapeseed seed** *Brassica napus*	**Avocado fruit** *Persea gratissima*	**Argan kernel** *Argania spinosa*	**Rosehip fruit** *Rosa canina*	**Soybean seed** *Glycine max*	**Cotton seed** *Gossypium hirsutum*	**Hemp seed** *Cannabis sativa*	**Sunflower seed** *Helianthus annuus*
Fatty acid profile[1], %										
Palmitic acid 16:0	4-9	8-20	3-6	5-25	10-15	2-5	7-14	17-28	4-13	4-9
Palmitoleic acid 16:1 n-7	1	3	trace	1-13	trace	trace	trace	1-2	1	1
Stearic acid 18:0	2-3	1-5	2-3	2-3	4-7	2-3	1-6	1-4	1-4	1-7
Oleic acid 18:1 n-9	62-86	56-85	50-67	50-74	43-50	13-18	17-30	13-25	6-21	14-40
Linoleic acid 18:2 n-6	7-30	4-20	16-30	6-20	29-37	35-50	44-62	45-63	46-66	48-74
α-Linolenic acid 18:3 n-3	1	1	6-14	3	trace	22-38	4-11	trace	14-30	trace
γ- Linolenic acid 18:3 n-3	0	0	0	0	0	0	0	0	3-4	0
Saturation ratio[2]	8:71:21	17:69:14	6:61:32	22:66:12	19:46:35	6:15:79	18:24:58	26:18:54	8:12:82	11:16:71
Physicochemical properties										
Color	Yell	Yell Grn	Yell Brn	Drk Grn	Yell Orn	Orn Red	Yell Brn	Lt Yell	Drk Grn	Drk Yell
Refractive index	1.469	1.469	1.473	1.469	1.471	1.480	1.475	1.473	1.479	1.474
Density, g/cm^3	0.915	0.913	0.917	0.916	0.915	0.920-950	0.922	0.918	0.926	0.921
Melting point, °C	-18	-6	-10	-2	1	-24	-17	-1	-8	-11
Iodine value[3], g/100g	90-100	75-88	94-106	75-95	91-105	180-190	128-135	103-111	150-167	118-144
SAP value[4], mg/g	188	190	174	186	191	187	191	194	193	189
Unsaponifiable frac., %	0.9	1.5	1.5	2.0	1.5	1.5	1.5	2.0	1.5	1.5
Absorption speed	3	3	2	4	3	1	3	3	3	3
Comedogenic value	2	2	1	3	0	1	5	3	0	1
Soapmaking qualities										
Hardness	7	17	6	22	15	6	16	26	8	11
Conditioning	89	82	91	70	81	89	82	71	90	87
Cleansing	0	0	0	2	1	0	0	0	0	0
Creaminess	7	17	6	22	14	6	16	26	8	11
Bubbliness	0	0	0	0	1	0	0	0	0	0
INS[5]	97	105	56	99	95	10	61	89	39	63

[1] Fatty acid composition (influences skin feel and absorption).
[2] Ratio of saturated (SFA), monounsaturated (MUFA), and polyunsaturated (PUFA) fatty acid esters.
[3] Amount of iodine (g) absorbed by 100 g of oil (the degree of unsaturation, affecting stability and drying time).
[4] Amount of potassium hydroxide (mg) required to saponify 1 g of oil (the average molecular weight of fatty acids).

[5] Calculated number derived by subtracting the iodine value from SAP (hardness, lather, and conditioning).

Several physiochemical characteristics collectively influence the oil's texture, skin feel, penetration ability, and stability in cosmetic formulations. Most vegetable oils are produced with an acid value of 2–4 mg KOH/g and a peroxide value of 10–20 meq O_2/kg. These parameters indicate free fatty acid content and oxidative rancidity, serving as markers for oil freshness, stability, and shelf life. Refined cottonseed oil typically exhibits improved quality parameters (acid value: 0.5 mg KOH/g; peroxide value: 5–10 meq O_2/kg), resulting in higher oxidative stability and an extended shelf life when used in formulations (Taghvaei et al., 2014).

Another important property influenced by the composition of major fatty acid esters is the iodine value, which serves as a key indicator of hydrothermal and oxidative stability. Higher iodine values indicate greater unsaturation and lower stability. Cottonseed oil has the lowest iodine value among high linoleic acid oils, with values only slightly higher than those of almond, canola, and argan oils (**Table 2.2**), which is particularly beneficial in formulations where stability is crucial.

2.3.1.1 Linoleic acid 18:2 (omega-6)

Linoleic acid is an essential fatty acid in the full-thickness human epidermis, making up 22%, followed by oleic acid (15%), palmitic acid (14%), and stearic acid (11%). However, this composition shifts in sebaceous secretions, which contain only trace amounts of linoleic acid but are rich in palmitic acid (22%), sapienic acid (22%), oleic acid (15%), and myristic acid (13%), as summarized previously (Knox and O'Boyle, 2021).

The linoleic acid is selectively utilized by sebaceous cells for β-oxidation, serving as a unique energy source for their function (Pappas et al., 2002). Additionally, linoleic acid

metabolites are released following skin exposure to chemical irritants, suggesting the presence of a natural skin defense mechanism based on linoleic acid (van de Sandt et al., 1995). Within cellular metabolism, linoleic acid acts as a precursor to signaling molecules like arachidonic acid, which can regulate inflammatory responses (Komarnytsky et al., 2021). Lower skin linoleic levels are also associated with psoriasis (Mysliwiec et al., 2019) and acne (Zhou et al., 2018) skin pathologies.

2.3.1.2 Palmitic acid 16:0 (saturated)

Similar to linoleic acid being an important link to cell lipid metabolism that helps maintain skin barrier integrity and resilience, exogenously supplied palmitic acid can be desaturated at an unusual C6 position to produce sapienic acid 16:1(n-10) and undergo elongation to sebaleic acid 18:2 (n-10) in human sebaceous glands (Ge et al., 2003). The synthesis of sapienic acid and wax esters, along with the accumulation of squalene and the presence of very long-chain branched or hydroxylated fatty acids, are unique to sebum and rarely occur in other organs (Pappas, 2009). Due to its occlusive nature, palmitic acid is effective in sealing moisture, which is advantageous for dry skin types. It may be too heavy for oily skin, potentially exacerbating oiliness or clogging pores, especially if used in excess. However, the linoleic acid component may help balance sebum production, so light application or diluted use might still benefit some individuals with oily skin.

Higher levels of palmitic acid in the cottonseed oil are the reason why it is one of the few oils stable in the beta crystal form, which is desirable in most solidified products because it promotes a smooth, workable consistency usually referred to as plasticity which is important in most formulations. At the same time, low levels of polyunsaturated fatty acids found in

cottonseed oil result in a greater oxidative stability of cottonseed oil when compared to other high-linoleic oils (Pazzoti et al., 2018).

2.3.2 Changes in fatty acid composition associated with processing

Individual oil properties can also vary based on the extraction and refining methods used in its preparation. Both refined, winterized, and fully hydrogenated cottonseed oils offer distinct advantages in cosmetic and personal care formulations.

Refining alone does not significantly change the fatty acid profile and physiochemical properties of the cottonseed oil (**Table 2.3**). Both crude and refined cottonseed oils contain measurable amounts of diglycerides (5.8%) and minor monoglycerides (0.3%) that can be further fractionated (deMan et al., 1989). The diglyceride fatty acid composition is dominated by oleic acid (45%), linoleic acid (29%), and palmitic acid (23%) in a striking difference to the triglycerides (deMan et al., 1989). Full hydrogenation results in a spreadable cottonseed fat with melting point of 53 °C that is dominated by stearic (76%) and palmitic (22%) acids (**Table 2.3**).

Table 2.3 Changes in fatty acid profiles (%) during refining of cottonseed oil after (deMan et al., 1989; Dowd et al., 2010b; El-Mallah and El-Shami, 2011; Khan et al., 2021b; Ruzibayev et al., 2021).

Cottonseed oil	Olein (oil)						Stearin (palmitin)		
	Crude	Refined							
	(range)	Neutra-lized	Blea-ched	Deodo-rized	Winte-rized	Hydro-genated	Conven-tional	Solvent extracted	Hydro-genated
Myristic acid 14:0	0.6-1.4	0.8	0.8	0.8	trace	0.7	0.6	0.6	0.8
Palmitic acid 16:0	19.6-27.6	24.7	24.3	24.7	trace	22.3	37.3	52.1	38.3
Stearic acid 18:0	2.0-3.2	2.5	2.5	2.6	trace	76.2	4.8	2.1	56.7
Arachidic acid 20:0	0.2-0.5	0.4	0.4	0.5	trace	0.4	0.3	0.2	0.4
Palmitoleic 16:1 n-7	0.4-0.8	0.9	0.8	0.8	1.2	0	1.3	0.8	0
Oleic 18:1 n-9	12.8-22.2	17.3	17.6	18.1	25.4	trace	25.6	9.1	2.7
Linoleic 18:2 n-6	44.0-59.3	52.6	52.8	52.6	73.7	0	30.0	35.5	1.1

Linolenic 18:3 n-3	0.2-0.3	trace	trace	trace	0.2	0	0.1	0	0
Iodine value, g/100g	103-111	109	110	110	114	4	96	72	5
Melting point, °C	0	-1	-1	-1	-12	53	24	35	59

Winterization of the refined cottonseed oil can be performed using either conventional or solvent-assisted chilling process that separates a solid stearin (palmitin) byproduct dominated by the palmitic acid (38-52%) that can be further fully hydrogenated into a stearic acid (57%) and palmitic acid (38%) saturated cottonseed fat (Table 3). The high melting point makes it stable in solid form at room temperature, ideal for structuring personal care products in warm climates. The stearin also holds the potential to add thickness and a silky texture, thus replacing synthetic thickening agents like carbomers; work as a stabilizing base that improves firmness and melting properties, potentially replacing beeswax or paraffin; and add hardness to soaps without the drying effects of sodium stearate.

The resulting winterized cottonseed oil has a lower melting point (-12 °C), allowing it to remain in a liquid state even in colder temperatures and a low to moderate viscosity, which contributes to its light, non-greasy texture, possibly replacing mineral oil in formulations. Its mild emollient properties make it effective for gentle cleansing, potentially substituting for sunflower or grapeseed oil.

2.3.3 Phytosterols

Cottonseed oil naturally contains a mixture of phytosterols (approx. 1%) dominated by β-sitosterol, campesterol, stigmasterol (also known as beta-stigmasterol or delta-5-stigmasterol), and isofucosterol (delta-5-avenasterol) (El-Mallah and El-Shami, 2011). This is 2-3 times higher than many other vegetable oils, and on par with the rice bran oil known to contain higher levels

of plant phytosterols (Liu et al., 2023). Refining reduces the phytosterol content of cottonseed oil by about 15% (**Table 2.4**), and the remaining phytosterols can be recovered from the deodorizer distillate byproduct where they accumulate at the level of 10-20% (El-Mallah and El-Shami, 2011). This has been successfully performed using molecular distillation (Jafri et al., 2014), and confirmed with the gas chromatography analysis (Verleyen et al., 2001)

Table 2.4 Phytosterol profiles during refining of cottonseed oil, summarized after (El-Mallah and El-Shami, 2011).

Phytosterols	**Cottonseed oil**			
mg/L (ppm)	**Crude**	**Neutralized**	**Bleached**	**Deodorized**
β-Sitosterol	8199	7713	7495	6869
Campesterol	874	855	800	725
Stigmasterol	255	240	224	210
Isofucosterol	171	162	161	144
Avenasterol	58	50	46	38

Under the normal physiological conditions, phytosterols are naturally secreted into the human skin surface lipids together with cholesterol at the rate of 3.2-8.5 mg/day for β-sitosterol and 0.2-0.4 mg/day for campesterol and stigmasterol (Bhattacharyya et al., 1972). In clinical settings, an ointment containing β-sitosterol in a base of beeswax and sesame oil improved management of skin burns, acute dermatitis, and post-operative wound healing in part due to its anti-inflammatory properties (Geara et al., 2018). The anti-inflammatory activity of β-sitosterol, campesterol, and stigmasterol was further confirmed in the activated keratinocyte and macrophages, as well as a preclinical model of psoriatic inflammation when applied topically at 1.4 mg/ml (Chang et al., 2023). Beta-sitosterol also showed moderate inhibition of 5α-reductase

and induction of early hair follicle transition when delivered topically at the concentration of 2.4-3.6 mg/cm2 in a preclinical model of androgen-induced hair loss (Prabahar et al., 2022).

2.3.4 Tocopherols

Tocopherols are natural antioxidants that stop or delay primary oxidation, thus contributing to high oxidative stability of cottonseed oil and its formulations by scavenging lipid peroxyl radicals and protecting the cell membrane integrity (Salimath et al., 2021). Cottonseed oil contains high levels of tocopherols (approx. 0.1%), with γ-tocopherol (55%) and α-tocopherol (45%) in near equal concentrations, and trace amounts of the others. This is 1.5-2 times higher than many other vegetable oils, and on par with soybean oil, although high content of α-tocopherol was a unique feature that classified cottonseed and sunflower oil in the same category (Wen et al., 2020). Similar to sterols, refining of cottonseed oil reduces the tocopherol content of cottonseed oil by about 18% (**Table 2.5**). Cottonseed oil generally does not contain tocotrienols, although the biosynthesis pathway for tocotrienols can be successfully engineered into cotton plants (Salimath et al., 2021).

Table 2.5 Tocopherol profiles during refining of cottonseed oil, summarized after (El-Mallah and El-Shami, 2011).

Tocopherols	**Cottonseed oil**			
mg/L (ppm)	**Crude**	**Neutralize**	**Bleached**	**Deodorized**
γ-Tocopherol (7,8-dimethyl-tocopherol)	489	434	414	377
α-Tocopherol (5,7,8-trimethyl-tocopherol)	470	461	432	411
δ-Tocopherol (8-methyl-tocopherol)	6	5	3	2
β-Tocopherol (5,8-dimethyl-tocopherol)	4	2	1	0

While α-tocopherol is the predominant form of vitamin E in human tissues, other forms of vitamin E such as γ-tocopherol, δ-tocopherol and γ-tocotrienol and their natural carboxychromanol metabolites have stronger anti-oxidative and anti-inflammatory effects (Jiang, 2014). Under the normal physiological conditions, tocopherols are transported via the sebaceous glands and sebum, with the highest levels of vitamin E registered in sebum-rich facial skin (Ekanayake-Mudiyanselage and Thiele, 2006). Tocopherols are known in dermatology for their high UV radiation absorbing properties (Pedrelli et al., 2012). In a preclinical study, oral tocopherols also improved recovery of stitched skin wounds both in normal and diabetic states (Bijo Elsy et al., 2017). Synthetic α-tocopherol acetate increased the stratum corneum hydration when applied topically at 2.5% (Gehring et al., 1998), was substantially absorbed in skin, however no evidence of biotransformation within skin has been reported (Alberts et al., 1996). This can be associated with low incidence of α-tocopherol derivative-induced allergic contact dermatitis (Kosari et al., 2010), and switching to natural tocopherols found in vegetable oils may lead to better skin compatibility.

2.4 Current cosmetic applications of cottonseed oil

Cosmetic and personal care uses of vegetable oils are defined for external parts of the human body, teeth, or mucous membranes of the oral cavity to maintain them in good condition, change in appearance, protect, or correct body odors (EU 2009/1223) or cleansing, beautifying, promoting attractiveness, or altering the appearance (21 U.S.C. § 321(i)). In typical cosmetic formulations, the cottonseed oil is mostly used alone or in blends with the high-end botanical oils (argan, macademia, rosehip) in support for bulk applications where cost is a factor and refining is accepted. However, cottonseed oil also presents other opportunities in personal care as a prime

ingredient, particularly for products focused on skin conditioning and moisturizing, thanks to its rich fatty acid profile, bioactive constituents, and potential for sustainable sourcing.

2.4.1 Carrier oils

The cottonseed oil is easily absorbed and has a moderately low comedogenic value (**Table 2.2**), thus it is often used as a carrier oil for other bioactive ingredients in cosmetic and skin care applications. This oil also contains a significant unsaponifiable fraction dominated by phytosterols and tocopherols that contribute to its greater oxidative stability when compared to other fixed oils (Taghvaei et al., 2014). The extended shelf life (Pazzoti et al., 2018), thermal stability, and absence of the inherent odor and color also make cottonseed oil a choice media in lipophilic botanical macerations. This provides both oxidative stability and a smooth, moisturizing quality without excessive oiliness.

2.4.2 Skin barrier function

Due to its high linoleic content, as well as a higher linoleic acid to oleic acid ratio, cottonseed oil may have a high skin barrier repair potential (Vaughn et al., 2018). Whether this effect is the same for healthy and damaged skin continues to be discussed (Moore et al., 2020; Poljšak and Kočevar Glavač, 2022).

Cottonseed oil also has a beta-type crystal structure similar to that of animal fats, which distinguishes it from many other vegetable oils (Ribeiro et al., 2014). This property makes cottonseed oil useful as a modifier to adjust the physical characteristics of other fats, such as cocoa butter (Ribeiro et al., 2013). This crystal habit is more similar to the structure of lipids naturally found in the skin, which allows cottonseed oil to blend effectively with the native lipid

matrix and to enhance the skin barrier by reducing transepidermal water loss and preventing external irritants from penetrating.

Tolerance of the Johnson's Natural baby lotion in a complex formulation with cottonseed oil and at least 95% naturally derived ingredients was evaluated on a torso, arms and legs of 1-36 months old children and showed no adverse effects in association with 37-48% improvement in stratum corneum hydration (Coret et al., 2014). The cottonseed oil blends were also developed as a topical emollient lotion for improving the skin barrier and reversing microbial dysbiosis (Capone et al., 2022). Other potential uses may include recreation of a creamy and smooth texture in formulations, gentle cleansing and makeup removing, as well as massage slip and skin nourishment.

2.4.3 Hair follicles and sebaceous glands

Cottonseed oil contains significant amounts of palmitic acid, a natural precursor for production of human sebum components (Ge et al., 2003). Sebaceous glands are associated with hair follicles and lubricate both skin and hair to maintain hydration, a physical barrier, and a complex environment for the beneficial microbiome (Swaney et al., 2023). Cottonseed oil was used as a major ingredient (25%) in formulation of artificial sebum based on the chemical similarity to human sebum (Lu et al., 2009). This oil is a popular ingredient in some hair oils (Mannan et al., 2023), where it provides nourishing and conditioning benefits to the hair. High smoking point of cottonseed oil (230 °C) makes it more suitable for heat styling.

2.4.4 UVB photoprotection

Although there are some limited reports indicating that the cottonseed oil blocks out about 20% of ultraviolet radiation (Korać et al., 2011), this translates to a low SPF value of 1-5, similar to other vegetable oils (Kaur and Saraf, 2010). This effect is most likely associated with a higher vitamin E (tocopherol) content of the cottonseed oil (Darr et al., 1996).

2.4.5 Cleaning soaps

Soaps formulated with different vegetable oils and natural herbal extracts are a major segment of the cosmetic and personal healthcare global market (Adigun et al., 2019). High linoleic and palmitic acid content of cottonseed oil defines the physiochemical soapmaking qualities of cottonseed oil (**Table 2.2**) and makes it suitable for manufacturing of soft (semi-liquid) soap bars and liquid soaps (Lynch, 1997). The cottonseed oil lacks a strong aroma, making it versatile for fragrance-free or scented soap products.

2.4.6 Biodegradable films

Biodegradable and edible (food grade) cosmetic formulations that reduce waste and can be consumed directly provide a novel experience and a sustainable alternative to promote environmental responsibility in cosmetics and personal care (Dini, 2024). Cottonseed oil can be successfully used to formulate biocompatible emulsion films that contain 1% glycerol and 3.4% lipids (Prus-Walendziak and Kozlowska, 2021). Since this oil is a byproduct of the cotton industry, it is manufactured at strict food-grade regulations and reduced raw materials costs, creating an economically attractive option for manufacturers focused on affordable food-safe

cosmetics. For example, Lush's biodegradable soap wrappers incorporate plant-derived waxes and oils to create dissolvable, plastic-free alternatives.

2.4.7 Delivery systems

The cottonseed oil is one of the solubilizing excipients often used in oral and injectable formulations. It can solubilize very lipophilic drugs and, following an intramuscular administration, serve as a sustained drug delivery depot for 2-4 weeks as the oils diffuses within the muscle tissue (Strickley, 2004). It also performs well in the microemulsion systems with improved solubility and penetration, outperforming oleic acid and Labrafil M1944 (Ryu et al., 2020). Cottonseed oil is compatible with a wide range of oils and ingredients, allowing it to blend well in complex formulations. For example, cottonseed oil-based oleogels with carnauba wax as a gelling agent have been successfully developed in consistencies from a thickened beverage to yogurt pudding in order to assist pediatric dosing and palliative care (Kirtane et al., 2022). Cottonseed oil showed good permeation enhancer abilities on par with sunflower oil (H. Patel et al., 2018).

2.4.8 Fermentation substrates

Increasing number of conventional cosmetic ingredients change sources from synthetics to the fermentation-based products that support the sustainable growth of the industry (Pérez-Rivero and López-Gómez, 2023). Cottonseed oil was described as an advantageous fermentation substrate in production of natural sophorolipid biosurfactants that promote emulsification, wetting, solubilization, and detergency functions in cosmetic formulations (Qin et al., 2023).

2.4.9 Ethnopharmacological uses

The use of cottonseed oil to extract roots of pale bugloss (*Echium italicum L*.) and prepare an ointment to heal wounds and knife cuts was reported in Turkish herbal tradition (Simsek et al., 2004). Cottonseed oil and other plant parts were also used in various Ayurveda preparations to manage inflammation and wounds (Velmurugan et al., 2012). The cottonseed milk (paruthi paal) is popular in preparation of moisturizing skin and personal care products in the Tamil Nadu state of India (Kumar, 2019). Application of cottonseed oil was also reported to lighten skin spots and freckles (Raheman et al., 2012).

2.4.10 Other potential byproducts and their uses

Gossypol, a naturally present phenolic aldehyde in unrefined cottonseed oil, was shown to specifically inhibit lactic acid dehydrogenase (Judge et al., 2018). Lactic acid decreases skin extracellular pH and activates TGF-β1 signaling to promote the survival of skin myofibroblasts, overproduction of collagen, and fibrotic tissue degeneration. Thus, a possible management approach to skin fibrosis would be application of gossypol to suppress myofibroblasts differentiation and excessive collagen deposition in the skin tissue. Two other byproducts of cottonseed oil production, the hydrolyzed cottonseed protein (Essendoubi et al., 2022) and cottonseed oligosaccharides (Oberto et al., 2005) have been explored for heat protection of human hair fibers and reduction of hair chipping, respectively.

2.5 Regulatory and safety considerations

Cottonseed oil in cosmetics is listed under its INCI name as Gossypium Herbaceum (Cotton) Seed Oil. Regulatory checkpoints described for cottonseed oil are based on its

agricultural background similar to other vegetable oils, levels of gossypol and cyclopropenoid acids that are eliminated during refining, and the need for strict quality control to ensure safety and efficacy in cosmetic formulations.

2.5.1 Raw materials

Cottonseed oil can be manufactured in several grades depending on its intended application. The pharmaceutical USP (US Pharmacopoeia) or NF (National Formulary) grade requires highest purity in excess of 99.9% with no binders, fillers, excipients, dyes, or unknown substances.

Food grade oil is the next highest grade as defined by the international standards for purity and classification established by the FCC (Food Chemical Codex) for cooking and ingestion by humans. Codex Alimentarius Commission (CAC) for fats and oils as a part of the FAO/WHO Food Standards Program also offers a set of internationally adopted standards that contribute to the safety and quality of food grade oils. Although not as frequent for cottonseed oil, adulterations and fraud of vegetable oils remains a significant issue (Green and Wang, 2023). Stricter quality controls, traceability measures, and advanced analytical techniques, such as HPLC and NMR are routinely used to detect adulteration and reinforce consumer confidence (Srivastava et al., 2024).

Cosmetic-grade oils are of lower quality, as the FDA (Food and Drug Administration) allows them to be only 70% pure, with the remaining oil blends and ingredients often undisclosed on the ingredient list. The organic cosmetic label still uses cosmetic grade ingredients. Lowest grade vegetable oils are of feed grade as regulated by the AAFCO

(Association of American Feed Control Officials) or technical grade that do not meet the above criteria and should not be used in food and cosmetic applications.

2.5.2 Safety and sustainability

As a cosmetic ingredient, cottonseed oil was previously reviewed as safe (Cosmetic Ingredient Review Expert Panel, 2001; Raj et al., 2023). Routine tests for mycotoxins (aflatoxin), pesticide residues and heavy metals that can be introduced by the agricultural practices, environmental pollution, processing, and/or transportation are important (Xia et al., 2021). Due to a refining process, cottonseed oil is also generally non-irritating and hypoallergenic, suitable for sensitive skin, which is a major advantage over the cold-pressed or virgin oils that may contain proteins with IgE-binding epitopes, especially when derived from soybeans, wheat, peanuts, tree nuts, and sesame (Yu et al., 2016). Refined oils are also attractive for personal care because they have minimal aroma, reducing conflicts with other fragrances, pose low contamination risk, and generally offer a longer shelf life due to high oxidative stability.

Cottonseed oil is readily available and inexpensive, providing an eco-friendly alternative to more expensive oils. As the agriculture cultivation phase of vegetable oils is the main source of climate change impacts and environmental concerns, cottonseed oils holds an advantage of being a byproduct of a cotton textile industry (Prado et al., 2021).

2.6 Conclusions

The use of oils in cosmetic and personal care products has a rich history rooted in natural sources, shaped by geographic and cultural influences. In recent years, consumer demand for eco-friendly, sustainable options has revitalized interest in plant-based oils, including

underutilized options like cottonseed oil. Distinctive fatty acid profile of cottonseed oil, with its beneficial linoleic to oleic acid ratio and a higher-than-expected level of palmitic acid, contributes to its unique characteristics in terms of stability, texture, and the way it interacts with other lipids. As the cosmetic industry shifts toward more naturally derived ingredients, cottonseed oil holds potential as a versatile, affordable, and safe component suitable for diverse personal care applications. Further research is needed to assess its long-term efficacy, stability in complex formulations, suitability for sensitive skin, and its interaction with other bioactive ingredients.

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CHAPTER 3

REINTRODUCING WILD NATIVE MARSHELDER (*IVA ANNUA* L.) INTO FARM CULTIVATION AS A NOVEL SOURCE OF PROTEIN AND OIL INGREDIENTS

Abstract

Before maize became widespread, indigenous groups in the Eastern United States cultivated a suite of native plants known as the Eastern Agricultural Complex, which included marshelder or sumpweed (*Iva annua* L.). Marshelder appears to have been locally domesticated in the Eastern U.S., with evidence of its cultivation dating back to 4,000 BCE. Over time, as maize agriculture expanded, marshelder largely disappeared from indigenous diets, and its cultivated variety went extinct. This study explores the potential for reintroducing marshelder as a cultivated crop by examining wild populations from the Southeastern U.S. (North Carolina, South Carolina, and Georgia). Seeds from wild populations and farm-grown plants produced similar-sized seeds but with increased yields, suggesting that cultivation may enhance productivity without altering seed morphology. Genome size estimation via flow cytometry confirmed a nuclear DNA content of Iva annua at 15.2 ± 0.2 pg (2C). Chloroplast genome analysis revealed structural similarities with other Asteraceae species, indicating evolutionary conservation. These findings provide a foundation for future research on Iva genetic diversity, domestication traits, and nutritional potential. Ultimately, this study supports efforts to revive marshelder as a sustainable, regionally adapted crop with ecological and agricultural benefits.

Keywords: marshelder; Eastern Agricultural Complex; domestication; genome size; chloroplast DNA; seed morphology; nutritional profile; sustainable agriculture

3.1 Introduction

The beginning of agriculture, a profound transformation in landscapes and roaming cultures that previously relied on hunting wild animals, fishing, and foraging for edible plants to survive is currently estimated to have occurred around 11,700 years ago (9,000 BCE) (Zeder, 2015). The reasons behind the rise of agriculture are still debated, with theories suggesting population pressures or climate change as key drivers (Allaby et al., 2022). The geographic locations where the target resources were most abundant served as the nucleation centers for early domestication and dispersal of agricultural crops (Vavilov et al., 1992). Many "lost" crops, once successfully cultivated, now forgotten, illustrate the complex evolving relationship between humans and local plants (Shelef et al., 2017).

Similar to wheat in the Near East (Arranz-Otaegui et al., 2018) and rice in the South-East Asia (Liu and Reid, 2020), the Americas also developed cultivation of a staple cereal, maize (corn), centered in Southern Mexico around 7,000 BCE (Kistler et al., 2020). It is currently believed that this was an extended event characterized by a nonuniform gene flow between teosinte and the cultivated lineages as maize was dispersed in Mesoamerica and South America (Zeder, 2018). However, maize has not reached US Southwest until 2,300 BCE (Smith, 2017). Moreover, the first northern maize macrobotanical fragments have not been reported until around 100 CE from the Turkey Pen shelter in Colorado (Swarts et al., 2017), 550 CE from the Turpin site in Ohio (Comstock and Cook, 2024), and 900 CE from the Icehouse Bottom site in Tennessee, the Edwin Harness site in Ohio (Simon et al., 2021), and the Holding site in Illinois (Simon, 2017). The eastward dispersion of maize most likely occurred across the Great Plains to the Ozarks and beyond between 100-900 AD, and not via the Gilmore Corridor along the Gulf coastal plains of Texas as discussed elsewhere (Ramos-Madrigal et al., 2025).

In the absence of maize, several native crops were first cultivated in the Eastern US as a part of the Eastern Agricultural Complex (Yarnell, 1978; Asch and Asch, 1985; Smith, 2006). The list is rather extensive and probably includes acorn squash (*Cucurbita pepo ssp. ovifera* L.), sunflower (*Helianthus annuus* L.), marshelder (*Iva annua* L.), pitseed goosefoot (*Chenopodium berlandieri* Moq.), erect knotweed (*Polygonum erectum* L.), carolina maygrass (*Phalaris caroliniana* Walt.), little barley (*Hordeum pusillum* Nutt.), and giant ragweed (*Ambrosia trifida* L.) (Zeanah, 2017). After the introduction of maize, many of these plants mostly vanished from native diets, and it has only been in the last 50 years that archaeologists reidentified the contemporary significance of these lost crops (Asch and Asch, 1977).

Modern marshelder or sumpweed grows wild across much of the Eastern and Central US, though it is likely native to the South-Central US west of the Mississippi, with a center of diversity in or near Texas. Unlike squash and sunflower, no archaeological remains of marshelder have been found in Mexico or the Southwest, supporting the case for a local domestication event within the Eastern Agricultural Complex (Smith, 2006). Marshelder strives in the river floodplains and is nearly absent from mountainous landscapes. The timing and method of its introduction east of the Appalachian Mountains remain unclear, as it now exists there in dispersed populations (Weakley, 2020).

Wild populations of marshelder were foraged as early as 4,000 BCE as evident from the wild size achenes (single seeds enclosed in a dry pericarp) found at the Koster site near Eldred, Illinois (Asch and Asch, 1978). It has been reported that the rate of wild harvesting the uncleaned small seeds was estimated at 1-1.5 kg/h at 3,200 kcal/kg of chenopod seeds (Smith, 1987) and 5,500 kcal/kg of oilseeds (Asch and Asch, 1978).

Collection from the wild riverine sites or reseeding at the exposed sites after recent floods (with an intent to return at the harvest time) was envisioned as a first step towards domestication of this crop (Smith, 2011). Findings of stored and/or transported caches of marshelder seeds in the highland areas also point to its significance as a managed or cultivated food source (Gilmore, 1931; Jones, 1936). Using these and other specimens, an extinct cultivated variety of marshelder was described as *Iva annua var. macrocarpa* (S.F. Blake) R. Jackson (Blake, 1939; Jackson, 1960). Phenotypic variability of marshelder seeds was reviewed recently (Weiland and Gremillion, 2018), in agreement with the previously reported average size of wild marshelder achenes at 2.8 × 2.2 mm, as well as the baseline for the domesticated achene length at 4.0 mm (Smith, 1989). Starting around 400 BCE, the domesticated marshelder achenes were also reported from the US Northeast (Reamer, 2024).

In this study, we present findings from collecting seeds from the wild marshelder populations in the Southeast (North Carolina, South Carolina, and Georgia) and cultivating them in the conventional farm settings to establish benchmarks for their seed size, germination, genome size, chloroplast DNA, and nutritional profiles. This will allow for future research to assess potential domestication traits, genetic diversity, and agricultural viability of marshelder as a reintroduced crop. Ultimately, this research adds to the first steps for the revival of marshelder as a sustainable source of nutrition and biodiversity within modern agricultural systems.

3.2 Materials and Methods

3.2.1 Collection of plant materials

Six populations of wild marshelder were located in three southeastern states including North Carolina, South Carolina, and Georgia outside of the native range reported for this species.

The populations were sourced to provide the wider geographical coverage of the region, but also in alignment with known palaeobotanical records (Minnis and Project Muse, 2016) and archaeological findings (Gibbon and Ames, 1998). Small batches of seeds (5-20 g) were hand collected from wild stands between September and November 2022, winnowed of chaff, and stored dry at room temperature until further use.

3.2.2 Seed measurements and germination

Seed sizes were recorded from digital images that included reference grid lines to ensure accurate measurements, and analyzed using SmartGrain 1.1 software (Tanabata et al., 2012). The largest uniform seeds were selected for germination by placing them between two wet paper towels in a Petri dish, protected from light. Seeds were considered to have germinated upon the emergence of the radicle, after which they were transplanted into 10 cm square pots filled with a potting soil mix (Miracle-Gro, Marysville, OH, USA) and grown under the Aerogarden 45W LED grow lights (Boulder, CO, USA) set to 16 h lights on and 8 h lights off cycle. Plants were fertilized weekly using the complete fertilizer (MasterBlend, Morris, IL, USA).

3.2.3 Farm cultivation

Plants were transferred to a field at the NC State University NCDA&CS Piedmont Research Station (Salisbury, NC, USA) in May 2023 and grown stalked until maturity using single row, drip-irrigated beds covered with white plastic mulch film pierced at 1 m in-row spacing. Mature seeds were collected in October-November 2023 by uprooting the plant to be stripped of seeds onto the surface of a plastic mulch film. Seeds were then air-dried in shallow trays, winnowed of chaff and stored as described above.

3.2.4 Plant nuclei isolation

Fresh young leaves were collected from plants growing in the lab or at the research station and analyzed within 1-2 h after collection following a previously reported protocol with some modifications (Bolin et al., 2018). The 1x1 cm sections of the leaves were rapidly chopped into 1 mm slices using a single edge industrial razor blade (VWR, Radnor, PA, USA) into 2 ml of ice-cold LB01 media in a chilled Petri dish. The LB01 media contained 15 mM Tris (363.4 mg), 2 mM EDTA (148.9 mg), 0.5 mM spermine (34.8 mg), 80 mM KCl (1.193 g), 20 mM NaCl (233.8 mg) and 0.1% Triton-X100 in 200 ml distilled water adjusted to pH 7.5 with 1N HCl (Doležel et al., 1989). One ml of released nuclei was filtered through a single layer of Miracloth (EMD Millipore, Burlington, MA, USA) into a new microcentrifuge tube and stained with 100 µl of propidium iodide (1 mg/ml stock), in the dark for 20 min.

3.2.5 Genome size determination by flow cytometry

Samples were then analyzed on the BD Accuri C6 flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) using FL2 585/40 and FL3 670 LP filters. Runs were set to 10 min or 20,000 events with FSC threshold of 10,000 and FL2 threshold of 50,000. Bivariate data plots were generated using the instrument software by plotting log(FL2) vs log(FL3), gated on a diagonal line, and the univariate histograms were constructed as log(FL2) versus nuclei counts. Mean peak values of propidium iodide-stained nuclei were recorded for each sample and 3 reference standards corn *Zea mays* CE-777 (2C = 5.43 pg DNA), barley *Secale cereale cv. Dankovske* (2C = 16.19 pg DNA), and broad bean *Vicia faba cv. Inovec* (2C = 26.90 pg DNA), kindly provided by Prof. Jaroslav Doležel, Institute of Experimental Botany, Olomouc, Czech

Republic (Doležel et al., 1998). Standard 2C values were plotted as a linear regression, and the samples 2C values were calculated from this equation.

3.2.6 Plant DNA extraction

Fresh young leaves were collected from plants growing in the lab or at the research station, place inside T-Sac tea filter bags (Magic Teafit, Columbus, OH, USA) and rapidly desiccated with the excess amount of Dry&Dry silica gel beads (Orange, CA, USA), in sealed Quart Zipper bags (Glad, Oakland, CA, USA). Genomic DNA was isolated from 20 mg of dry leaves using the DNeasy Plant Mini kit (Qiagen, Hilden, Germany). Leaf tissues were homogenized with steel beads using TissueLyserII bead mill for 2 min at 30 Hz (Qiagen). The eluted DNA was quantified using Qubit 3.0 Fluorometer (Thermo Fisher Scientific, Pittsburg, PA, USA) and checked for quality on 2% agarose gels using 6x TriTrack DNA loading dye and SYBR Green I on ChemiDoc MP Imager (Bio-Rad, Hercules, CA, USA).

3.2.7 Sequencing and chloroplast genome analysis

Fresh DNA cleaning was performed with AMPure XP Beads (Backman Coulter, Brea, CA, USA). DNA shearing (1-20 ng/µl in 100 µl total volume) was done on Bioruptor sonication system (Diagenode, Sparta, NJ, USA) by vortexing the samples for 10 sec, centrifuging for 10 sec, chilling on ice for 10 min, and sonicating for 9 cycles (15/30). DNA libraries were constructed using the Celero DNA-Seq Library Prep (Tecan Genomics, Redwood City, CA, USA) and subjected to Illumina sequencing (San Diego, CA, USA).

Chloroplast DNA (cpDNA) was reconstructed using FastQC and Trimmomatic to remove low-quality reads and adapter sequences, then aligning and extracting reads with Bowtie2. The

collected reads were assembled using SPAdes, resulting in scaffolds that were evaluated and refined to correct mistakes. These were loaded into Geneious Prime software (Boston, MA, USA) for mapping and alignment. The circular cpDNA was annotated and visualized using the GeSeq and OGDraw Chlorobox toolkit (Greiner et al., 2019).

3.2.8 Seed sample preparation and dry matter

Seeds were cleaned to remove debris and grinded into powder using RRH-500 grain grinder mill (Zhejiang Winki Plastic Industry, Jinhua, Zhejiang, China). Water content was recorded on 4TE Water Activity meter (Aqualab, Pullman, WA, USA). Dry seed hulls were estimated at 10% (w:w) based on the literature data available for comparable small oilseeds such as canola and hemp (Bárta et al., 2023).

3.2.9 Total fat content

The oil was extracted from seed powder using 90% (v/w) hexane by sonicating the samples at 50% power (20 kHz, 900 W, 3/4 cycles) for 30 min in the FS-900N Ultrasonicator (Henan Chuanghe LEC, Zhengzhou, Henan, China), with an automatic shutdown enabled if the temperature exceeded 45 °C. The mixture was stirred at 460 rpm for 1 h and centrifuged at 4,200 g for 30 min at 4 °C. The organic layer was decanted and the solvent was evaporated using Rotovapor R210 (Büchi, New Castle, DE, USA). The extracted oil and defatted seed flour were air-dried under a fume hood overnight and stored at -20 °C.

3.2.10 Total protein content

Defatted seed flour (20 mg) was transferred into a new microcentrifuge tube and extracted with 1 ml either 0.3 M NaCl (globulins) or 0.1 N NaOH (albumins and glutelins) by vortexing for 10 sec, sonicating for 30 min, storing at room temperature for 10 min, and centrifuging at 15,000 g for 10 min, as modified from a previously reported protocol (Deans et al., 2018). Samples were neutralized with 11 µL of 5.8 M HCl and proteins were precipitated with 90 µl of trichloroacetic acid on ice for 30 min. The samples were centrifuged at 15,000 g for 10 min at 4 °C, the pellets were washed with 100 µl of -20 °C acetone and quickly dried to prevent dissolution and dissolved in 1 ml of 0.1 N NaOH. Total protein was quantified in 25 µl aliquots using the Pierce Bradford protein assay kit (Thermo Fisher Scientific) and a bovine serum albumin (0-2,000 µg/ml) as a reference control.

3.2.11 Total and digestible carbohydrates

Defatted seed flour (20 mg) was transferred into a new microcentrifuge tube and extracted with 1 ml of 0.1 M H_2SO_4 in a boiling water bath for 1 h as reported previously (Deans et al., 2018). The samples were cooled to room temperature and centrifuged at 15,000 g for 10 min. Sample and standards aliquots (7.5 µl) were mixed with 200 µl water, 200 µl of 5% phenol in water, and 1 ml of concentrated H_2SO_4 for 10 min, vortexed briefly, and incubated for another 30 min. Digested carbohydrates were quantified at 490 nm using D(+)glucose (0-5,000 µg/ml) as a reference control.

3.2.12 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 ± SEM. Significant differences were accepted when the p-value was <0.05.

3.3 Results and discussion

3.3.1 Wild collected seeds

Seeds from wild populations of Iva annua collected in 2022 exhibited notable variation in size and weight across different regions (**Table 3.1**). The heaviest seeds were found in the West South Carolina population, averaging 2.96 mg, while the lightest seeds came from North Georgia, averaging only 1.29 mg. Seed length and width also varied, with the largest individual seed recorded in the East North Carolina population (4.57 mm x 2.96 mm), whereas North Georgia had the smallest seeds overall. These findings highlight regional differences in seed morphology, which may reflect genetic diversity, environmental influences, or both, providing a baseline for future cultivation efforts.

Table 3.1 Seeds from **WILD** populations of *Iva annua* collected in 2022.

ID	Area	Seeds, n	Average seed			Largest seed, length x width	Yield, g (per plant)
			Weight, mg	Length, mm	Width, mm		
1	NC West	104	2.10	2.72 ± 0.23	2.14 ± 0.23	3.90 x 2.25	n/a
4	NC East	176	2.38	2.84 ± 0.48	2.18 ± 0.39	4.57 x 2.96	n/a
6	SC West	139	2.96	2.83 ± 0.48	2.26 ± 0.47	3.98 x 2.67	n/a
10	SC East	289	1.89	2.55 ± 0.47	1.93 ± 0.38	4.06 x 0.38	n/a
14	GA South	228	1.78	2.56 ± 0.26	2.01 ± 0.22	4.84 x 2.40	n/a
31	GA North	85	1.29	2.64 ± 0.30	2.05 ± 0.24	3.33 x 2.64	n/a

3.3.2 Seeds from the same populations grown at the farm

Seeds from farm-grown Iva annua in 2023 showed differences in size, weight, and yield compared to wild populations (**Table 3.2**). The West South Carolina population produced the heaviest seeds (2.77 mg), while the South Georgia population had the lightest (1.50 mg), reflecting some regional variation. Overall, seed size remained similar to wild counterparts, but average yields per plant were higher. These results suggest that cultivation under farm conditions increased overall seed production while maintaining size characteristics comparable to wild populations.

Table 3.2 Seeds from **FARM** samples of *Iva annua* collected in 2023.

ID	Area	Seeds, n	Average seed			Largest seed, length x width	Yield, g (per plant)
			Weight, mg	Length, mm	Width, mm		
1	NC West	401	1.76 ± 0.27	2.84 ± 0.51	2.17 ± 0.44	4.56 x 2.41	48.6 ± 18.1
4	NC East	297	1.77 ± 0.30	2.52 ± 0.29	1.80 ± 0.24	3.55 x 2.71	56.6 ± 14.6
6	SC West	344	2.77 ± 0.68	3.43 ± 0.43	2.67 ± 0.34	4.53 x 3.21	53.3 ± 18.8
10	SC East	358	2.50 ± 0.98	3.18 ± 0.39	2.55 ± 0.31	4.04 x 2.93	63.4 ± 20.6
14	GA South	255	1.50 ± 0.18	2.78 ± 0.31	1.97 ± 0.23	3.49 x 2.37	68.4 ± 35.9
31	GA North	182	1.97 ± 0.15	2.68 ± 0.16	2.22 ± 0.15	3.35 x 2.64	45.5 ± 11.3

3.3.3 Genome size in a model wild population 14 (South Georgia)

Collected leaves displayed notable variation in shape, margin serration, and overall symmetry. These varied between a relatively smooth surface with moderate serrations and a more ovate shape, to a more elongated profile with a slightly less pronounced serration pattern. The most pronounced phenotypes showed deep indentations, giving it a more dissected appearance (**Figure 3.1**).

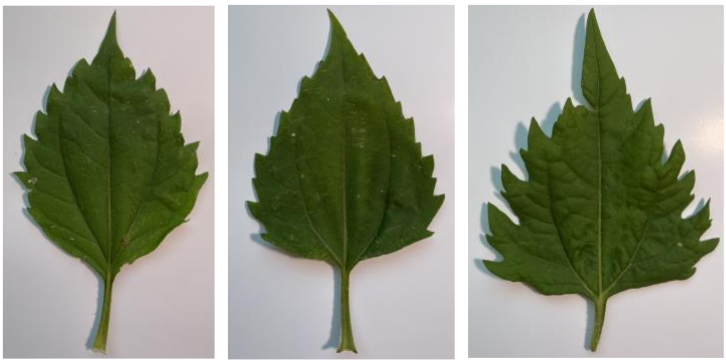

Figure 3.1 Variation in leaf morphology of marshelder plants grown on the farm in 2023.

The flow cytometry results displayed scatter plots (FL2 vs. FL3) alongside histograms (FL2 intensity) for all 6 populations analyzed (**Figure 3.2**). The histograms revealed a single distinct peak in fluorescence intensity for all populations, indicating no genome size variation among samples. The 2C value for the species was calculated in reference to 3 model genomes (corn, barley, and broad beans, **Figure 3.3**) and established an estimate for the total nuclear DNA content of *Iva annua* as 15.2 ± 0.2 pg (1C = 7.6 pg). Using a marshelder population from Austin, TX, a previous report confirmed its chromosome counts (2n = 34) and estimated its genome size (2C) at 15.6 ± 0.2 pg using *Pisum sativum* L. as an internal standard (Paniego et al., 2019)

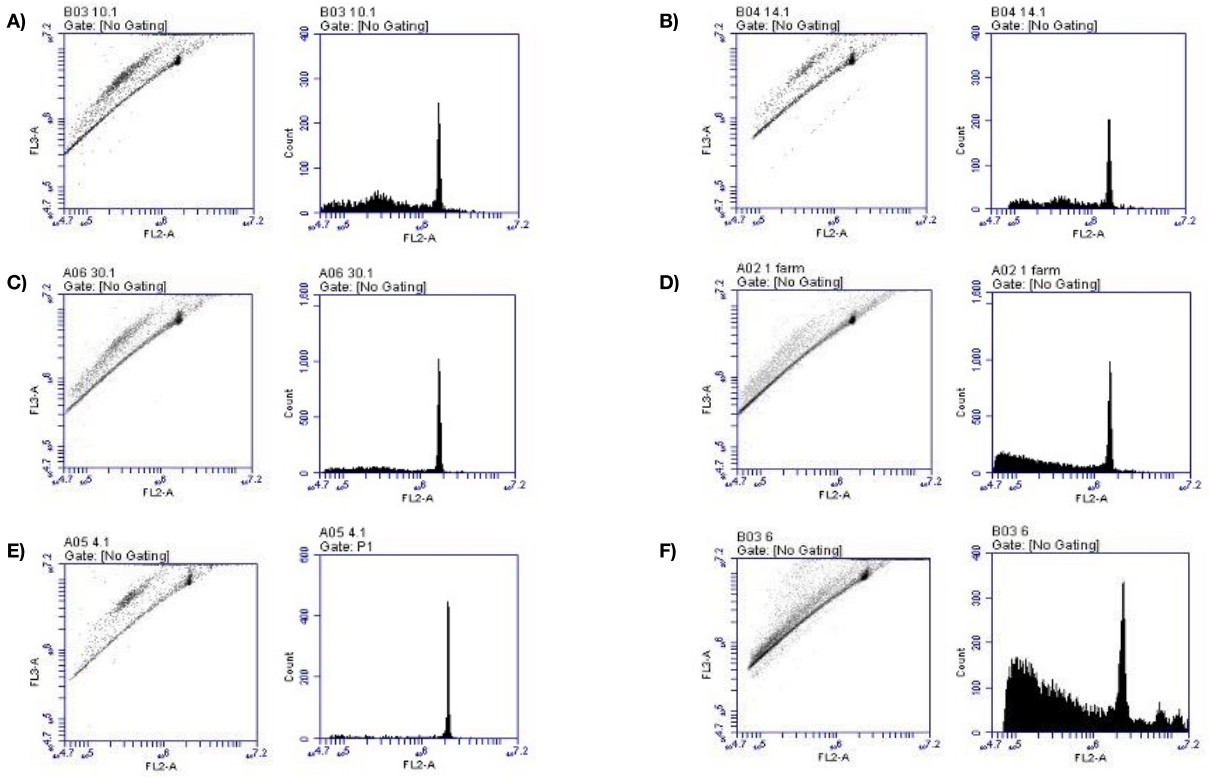

Figure 3.2 Representative flow cytometer scatterplot diagrams (left) and histograms (right) of events collected from marshelder populations. (A) Populations 10 (East South Carolina), (B) 14 (South Georgia), (C) 31 (North Georgia), (D) 1 (West North Carolina), (E) 4 (East North Carolina), and (F) 6 (West South Carolina).

The standard curve for 2C DNA content using 3 reference species by plotting genome size (pg) against fluorescence intensity (FL2) at 488/585 nm is shown on **Figure 3.3 Iva annua** with an estimated genome size of 15.2 pg is positioned immediately next to *Secale cereale* (2C = 16.19 pg). The regression line shows a strong linear correlation ($R^2 = 0.9894$, $p < 0.0001$), indicating the accuracy of the fluorescence-based genome size estimation. High significance confirms that fluorescence intensity reliably reflects genome size across the tested species.

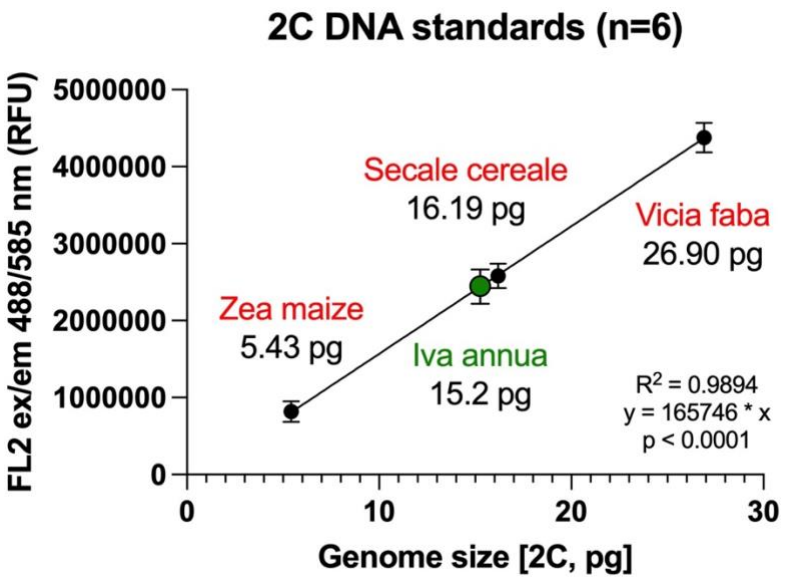

Figure 3.3 An established correlation between fluorescence intensity and genome size across the tested species (R^2 = 0.9894, p < 0.0001).

3.3.4 Chloroplast genome in a model wild population 14 (South Georgia)

This circular map (**Figure 3.4**) represents the complete chloroplast genome of *Iva annua* (wild population 14 from South Georgia), which is 152,125 base pairs in length. The genome is divided into four major regions: a large single-copy (LSC) region, a small single-copy (SSC) region, and two inverted repeat (IR) regions. Genes are color-coded according to their functional categories, including photosystem components, ribosomal RNAs, transfer RNAs, ATP synthase subunits, and ribosomal proteins. Protein-coding genes such as rbcL, psaA, and ndhB are distributed throughout the genome, indicating conserved chloroplast functions. The presence of genes involved in transcription and translation, such as rpoB and rps16, suggests a self-contained protein synthesis system. The inverted repeats contain duplicate copies of genes like rrn16, rrn23, and ycf2, a common feature in angiosperm chloroplast genomes.

The large single-copy region harbors most of the protein-coding genes, while the small single-copy region contains a lower density of genes. Some genes, such as ndhA and clpP, contain introns, reflecting post-transcriptional regulatory mechanisms. The annotation reveals a structure similar to other Asteraceae family members, confirming evolutionary conservation. While the marshelder chloroplast genome has not been described previously, some studies used cpDNA restriction site variation to determine phylogenetic relationships among ten Iva species (Miao et al., 1995). Illumina sequencing of transcriptome (36.72 Mbp, 42.6% full length) from a marshelder population in Granite City, IL among other Asteraceae species was also attempted (Hodgins et al., 2014). The reference chloroplast genomes can be also found in the sequencing projects completed on common and giant ragweed (Ambrosia artemisiifolia L. and Ambrosia trifida L.) (Laforest et al., 2024), chromosome-level assembly in giant ragweed (Yin et al., 2024), as well as more distantly related sunflower Helianthus annuus L. and lettuce Lactuca sativa L. (Timme et al., 2007), including multiple sunflower species (Azarin et al., 2021). Cotton Gossypium hirsutum L. chloroplast genome represents a convenient, phylogenetically distant oilseed reference for future studies and comparisons (Lee et al., 2006).

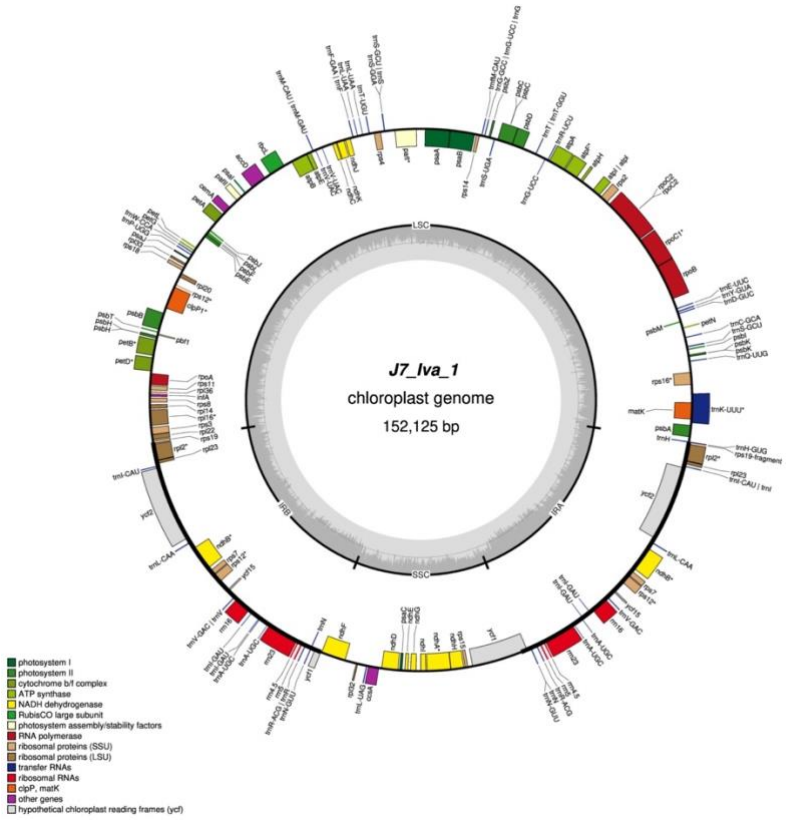

Figure 3.4 The gene map illustrates the *I. annua* chloroplast genome, with genes positioned inside the circle transcribed clockwise. Different functional groups of genes are color-coded. The inner circle visually represents the GC- (darker) and AT- (lighter) rich regions.

3.3.5 Nutritional profile

The preliminary proximate composition of marshelder seeds is shown in **Table 3.3**. For protein determination, two different fractions of protein were extracted, including albumins and glutelins (17.5%) and globulins (2.3%). The amount of crude fat or oil was around 25%, making it a significant oilseed. These results need to be repeated for further validation.

Table 3.3 Preliminary proximate composition of farmed-collected marshelder seeds (mean ± S.D.).

Nutrient (n=3)	Composition, %
Water content	4.13 ± 0.35
Oil (crude fat)	25.00 ± 0.09
Crude protein	19.75 ± 0.35
Carbohydrates, digestible	5.18 ± 1.26
Carbohydrates, undigestible	45.94 ± 0.44
Seed coat, estimated	10.0
Total	100.0

3.4 Conclusions and future directions

The domestication of plants was a transformative event that reshaped human societies and ecosystems. While the exact causes remain debated, factors such as population pressures and climate shifts likely played roles in this transition. In the Americas, maize became the dominant cereal, but its spread into North America was a gradual process. Before maize reached the Eastern Woodlands, indigenous peoples cultivated a variety of native crops, collectively known as the Eastern Agricultural Complex. Among these, marshelder (*Iva annua*) played a significant role as an oilseed crop, though it was largely abandoned following the adoption of maize.

This study assessed genetic, morphological, and agronomic characteristics of wild Iva annua populations from the southeastern U.S. Our findings revealed significant regional variation in seed size and weight, with the largest seeds found in North Carolina and the smallest in Georgia. Seeds from farm-grown plants showed increased yields while retaining the size characteristics of wild populations. Flow cytometry confirmed a stable genome size of 15.2 ± 0.2 pg (2C). Chloroplast genome sequencing of a South Georgia population further confirmed structural similarity with other Asteraceae species, suggesting evolutionary conservation.

These results provide a baseline for future research into the domestication traits and agricultural potential of marshelder. The observed phenotypic diversity indicates genetic variability that could be harnessed for selective breeding. Understanding its agronomic viability could support efforts to reintroduce marshelder as a sustainable crop. Given its historical role in indigenous agriculture, marshelder holds promise as a modern crop with nutritional and ecological benefits. This research contributes to broader efforts aimed at restoring lost crops, improving biodiversity, and enhancing food security in contemporary agricultural systems.

Future studies should focus on conducting compositional analyses to identify and quantify essential micronutrients, bioactive compounds, and fatty acid profiles. Evaluating the amino acid composition of its proteins and investigating their digestibility could further validate the plant as a valuable protein source. To evaluate their possible health advantages, studies can additionally investigate both seeds and plant biomass the presence of phenolic compounds, antioxidants, and secondary metabolites. Comparative studies of other ancient crops and oilseeds would offer helpful comparisons for understanding marshelder nutritional benefits and processing requirements. Developing processing methods meant to lower the inherent bitterness of the seed, such as roasting or enzymatic treatments, should help to enhance its sensory qualities and consumer acceptance.

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