



Valorizing Kale Side-Streams for Sustainable Leaf Protein Production

– A Methodological Assessment of Fractionation Parameters for White Protein Concentrate Recovery from Kale (*Brassica oleracea* var. *sabellica*)

Zeji Lin

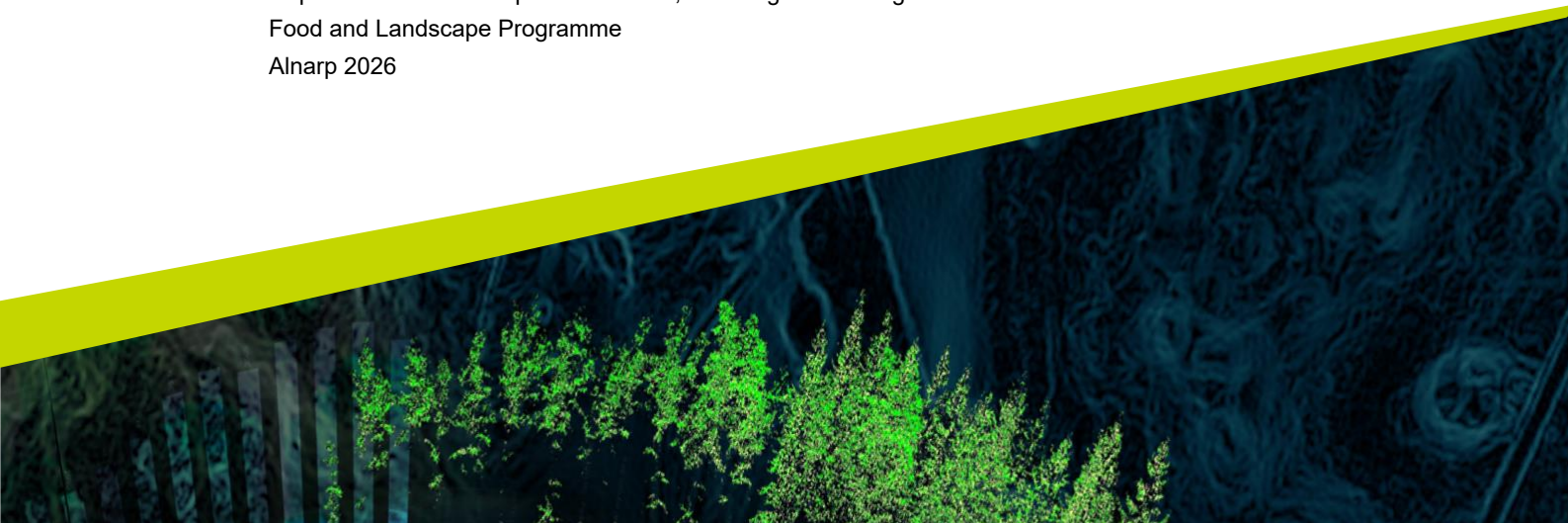
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Abstract

Kale (*Brassica oleracea* var. *sabellica*) side-streams may provide a promising source of leaf protein within a green biorefinery framework, but producing a white protein concentrate (WPC) for food applications remains technically challenging. Valorization of this biomass could support protein diversification and improve the use of underutilized green leafy biomass without requiring additional cropland. This thesis evaluated selected fractionation methods and processing parameters for recovering the white protein fraction from kale leaves. Fresh kale biomass was first mechanically pressed to obtain green juice, followed by thermal treatment, acid precipitation, acid washing, resolubilization, and freeze-drying. Four thermal treatment groups, including water bath heating at 60 °C and steam injection at 40 °C, 50 °C, and 60 °C, were compared. Two non-heated control groups were included to test whether low-temperature acid precipitation and resolubilization improved protein recovery. Protein recovery was assessed using the bicinchoninic acid (BCA) protein assay and mass balance calculations. Final product appearance and color changes during thermal treatment were evaluated by colorimetric analysis in the CIE L*a*b* (CIELAB) color space. Dry matter measurement and chlorophyll determination were used as additional assessments of the methods above.

Thermal treatment changed the color profile of the white juice fraction, especially at higher steam injection temperatures. However, the decolorization in white juice did not result in higher final resolubilized white protein fraction recovery or a brighter freeze-dried product. Final resolubilized protein recovery remained below 9% in all thermally treated groups, while the non-heated controls showed higher recovery but also higher dry mass content. Low-temperature acid precipitation and resolubilization did not significantly improve recovery compared with room-temperature processing under the conditions used. Overall, the results suggest that kale WPC fractionation is limited mainly by acid precipitation, acid washing, and resolubilization rather than thermal treatment alone. Further work should focus on improving protein recovery and characterizing the composition and sensory characteristics of the final WPC product.

Keywords: kale, white protein concentrate, leaf protein concentrate, green biorefinery, protein fractionation, thermal treatment, acid precipitation, CIELAB color analysis.

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Abbreviations

Abbreviation	Description
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
CIELAB	Commission Internationale de l'Éclairage L*a*b* color space
GJ	Crude green juice obtained after primary screw pressing
GJ _{sp}	Green juice obtained from rehydrated pulp after second pressing
GPF	Green protein fraction
LPC	Leaf protein concentrate
P1	Pellet from green juice after centrifugation
P2	Precipitated fraction after thermal treatment
P3	Precipitated fraction after acid precipitation
P4	Insoluble residue after resolubilization
P _{fp}	Freeze-thaw precipitates
PPO	Polyphenol oxidase
P _{wash}	Washed protein precipitate
RuBisCO	Ribulose-1,5-bisphosphate carboxylase/oxygenase
S1	Clarified green juice supernatant after centrifugation
S2	Soluble fraction after thermal treatment
S3	Supernatant after acid precipitation
S4	Resolubilized white protein fraction
S _{fp}	Freeze-thaw clarified S1 supernatant
S _{wash}	Supernatant collected after washing of P3
WPC	White protein concentrate
WPF	White protein fraction

1. Introduction

1.1 Protein transition, circular bioeconomy, and kale side-stream valorization

The global food system is facing increasing pressure to provide sufficient protein for a growing population while remaining within environmental limits. The world population is projected to reach 9.7 billion by 2050, and rising income levels, urbanization, and the homogenization of diets in many regions are expected to increase the demand for protein-rich foods, particularly meat and dairy products (Yano and Fu 2022; Hadidi et al. 2024). This creates a serious challenge for future food security due to the strong dependency on animal-based production in the current protein supply system (Wu et al. 2014).

Although animal proteins have historically played an important role in human nutrition, their production is highly associated with substantial environmental burdens. Compared with plant-based protein production, animal protein production generally requires more land, water, and feed input per unit of protein produced. The extensive use of these resources is closely related to greenhouse gas emissions, deforestation, biodiversity loss, environmental pollution, and animal welfare concerns (Boukid et al. 2022; Hadidi et al. 2024; Furia et al. 2025). Therefore, these pressures have made the diversification of protein sources an important issue in the transition toward more sustainable food systems.

In this context, plant-based proteins have gained increasing attention as alternatives to conventional animal proteins. However, replacing animal proteins with a narrow range of conventional crops, such as soybeans and cereals, would only partially address this sustainability challenge. An intensive plant protein supply may create new pressures on agricultural biodiversity, land use, and allergen management, especially when the food industry relies heavily on a few dominant raw materials (Muthukumar et al. 2020). Therefore, the sustainable protein transition requires not only more plant proteins, but also a broader and more diverse portfolio of protein sources.

Circular bioeconomy offers a useful framework for this diversification. Traditionally, the value chain follows a linear “take-make-dispose” flow of resources, in which agricultural residues are treated as low-value side-streams. In contrast, circular approaches aim to harness and leverage their nutritional and biochemical value within the food system (Bruins & Sanders 2012; Khatami et al. 2024). The valorization of side-streams can reduce waste, improve resource efficiency, and generate new ingredient streams without requiring additional arable land or primary crop production (Famuwagun et al. 2020; Boukid et al. 2022; Hadidi et al. 2023). In this sense, alternative protein development is not merely a question of finding new crops, but also of making better use of biomass already produced within existing agricultural systems.

Green leafy biomass is a particularly relevant but underutilized resource in this context. Leaves are abundant, renewable, and protein-containing plant materials. Yet, they are often left in the field, used as low-value livestock feed, or treated as post-harvest residues. However, green leafy biomass may contain high-quality proteins with essential amino acids, minerals, and vitamins, making it a potential material for food-grade protein ingredients if appropriate extraction and purification technologies are applied (Famuwagun et al. 2020; Kumar et al. 2022). Green biorefinery provides a technological pathway for this purpose by fractionating leafy biomass into multiple value streams, including protein, fibers, and residual fractions for feed, fertilizer, or bioenergy (Nynäs 2022; Quintieri et al. 2023).

Kale (*Brassica oleracea* var. *sabellica*) represents a relevant biomass for this approach. As a Brassica crop, kale is valued for its nutritional profile and market identity, but its production and supply chain can generate side-streams from damaged, downgraded, cosmetically rejected, or otherwise underutilized leaves. According to previous estimations, these residues, which still contain potentially valuable proteins and bioactive compounds, account for up to 50% of the original plant biomass (Fink et al. 1999). Also, previous work has reported that kale leaves can contain approximately 15% protein on a dry matter basis, indicating that kale side-streams might be a promising raw material for protein recovery (Prade et al. 2021). Therefore, valorizing kale side-streams through a green biorefinery could diversify plant protein sources, reduce agricultural waste, and improve biomass utilization within a circular food system.

1.2 Leaf protein concentrates and the white protein fraction as food ingredients

The idea of using green leaves as a source of food protein is not new. Early work on leaf protein separation can be traced back to the eighteenth century, when Rouelle reportedly separated protein material from green juice by heating (Nynäs 2018). In the twentieth century, Pirie further developed the concept of leaf protein concentrate (LPC) as a potential human food resource, particularly in response to food shortage and protein deficiency (Pirie 1942; Nynäs 2018; Boukid et al. 2022; Furia et al. 2025). During the 1970s, this concept entered a more industrial phase through the development of the Pro-Xan process at the Western Regional Research Center of the United States Department of Agriculture. The original Pro-Xan product was mainly designed as a livestock feed, while later developments such as Pro-Xan II and Welpro explored the separation of a visually lighter, soluble white protein fraction for human food applications (Spencer et al. 1971; Edwards et al. 1975; Nynäs 2018; Prade et al. 2021). These historical developments show that leaf protein extraction technology has long been recognized.

The recent renewed interest in leaf proteins is driven by the search for sustainable, nutritious, and underutilized protein sources. Leaf proteins have strong nutritional and functional potential. The major soluble protein in green leaves is ribulose-1,5-

bisphosphate carboxylase/oxygenase, commonly known as RuBisCO. RuBisCO is one of the most abundant proteins on Earth and can account for a major proportion of soluble protein in C3 plant leaves (Ellis 1979). From a nutritional perspective, leaf proteins are generally rich in essential amino acids and may be comparable with several conventional plant protein sources (Aletor et al. 2002; Santamaría-Fernández & Lübeck 2020; Balfany et al. 2023). In addition, RuBisCO has been reported to show promising functional properties, including solubility, foaming, emulsifying, and gel-forming capacities. These characteristics make leaf proteins relevant as functional ingredients for plant-based foods, such as beverages, bakery products, meat analogues, and dairy alternatives.

Another potential advantage of leaf proteins is their market positioning compared with several major plant protein sources. Soy and wheat proteins are widely used in plant-based foods, but they are also associated with allergen labeling and consumer concerns. Leaf proteins may provide an alternative ingredient category for food formulations avoiding soy and gluten, depending on the biomass source and processing method (Akyüz et al. 2024).

Despite these advantages, crude green LPC is not suitable for human food applications. Early crude LPC products, such as Pro-Xan, contain chlorophyll, carotenoids, polyphenols, membrane-associated proteins, and fibrous residues. These components can result in dark green or brown colors, grassy or earthy flavors, bitterness, astringency, reduced solubility, and lower sensory acceptability (Furia et al. 2025). Such characteristics may only have minor impact on animal feed products, but they strongly restrict the use of LPC in human food formulations where color, odor, taste, and texture are critical. Therefore, the challenge is not only to extract protein from leaves, but to obtain a protein fraction with suitable sensory and functional quality for food use.

This distinction explains why the white protein fraction is the main target of the present thesis. In the extracted leaf juice, leaf proteins are commonly divided into a green protein fraction (GPF) and a white protein fraction (WPF). The green fraction is associated with chloroplast structures, pigments, membrane proteins, and hydrophobic components, while the white fraction is enriched in soluble proteins such as RuBisCO (Nynäs 2018; Hadidi et al. 2024). Compared with the GPF, the WPF and its product, white protein concentrate (WPC), are expected to contain less pigment-associated material, fewer color-active compounds, and a milder sensory profile (Nynäs 2022). For human food applications, WPC fractionation should therefore aim not only to recover protein, but also to reduce the green color, off-flavor, and non-target solids while maintaining sufficient solubility and functionality. In the present thesis, the fractionation process was therefore designed specifically toward the recovery of a final WPC.

1.3 Technical basis of leaf protein fractionation

Leaf protein fractionation involves a series of separation steps. In the present thesis, the following principles were adapted toward the fractionation of a low-

color final WPC from kale. The process usually starts with a physical disruption of leaf tissue, such as mechanical pressing, which breaks down leaf tissue and releases intracellular fluid as green juice, while most fibrous cell wall material remains in the pulp (Nde & Foncha 2020). The green juice contains soluble WPF, including RuBisCO, as well as chloroplast fragments, pigments, phenolic compounds, salts, sugars, and other soluble components (Dijkstra et al. 2003). The main objective of the fractionation process is to isolate the soluble WPF from these non-target materials and compounds. Subsequently, the green juice must be further separated into the soluble WPF and the undesirable GPF. This step uses the different thermal sensitivity of the two fractions present in the extract. By applying a controlled heat treatment (Edwards et al. 1975; Nynäs et al. 2021), the green fraction can coagulate and be removed by centrifugation or filtration, leaving a lighter liquid fraction often referred to as white juice.

Soluble white proteins can then be recovered by acid precipitation. In this step, the pH of the white juice is adjusted to the isoelectric point of the target proteins, minimizing the electrostatic repulsion of protein molecules (Coldebella et al. 2013). The precipitated fraction can be adjusted back to and resolubilized in neutral pH (Miller et al. 1975). Their capability to remain soluble in neutral environment is particularly important for food applications because it indicates whether the recovered proteins can be incorporate into foods as soluble ingredients.

1.4 Bottlenecks and research gap in food-grade leaf protein concentrates production

Although LPCs have strong potential as sustainable food ingredients, their use in human food applications remains limited by sensory and technical barriers in fractionation. These limitations are especially important when the target product is a white protein concentrate, since food-grade ingredients must meet higher standards for sensory profile, solubility, and overall acceptability than feed-oriented products.

1.4.1 Sensory limitations: color and flavor

One of the main barriers to food-grade LPC development is its color and overall appearance. During extraction, chlorophyll, carotenoids, polyphenols, and other pigments may remain in the protein fraction, giving the final product a dark green color. Thermal processing can further alter the color of LPC because chlorophyll can be converted to pheophytin, while polyphenol oxidase activity can contribute to brown or dark coloration (Tamayo Tenorio et al. 2017). These colors make crude LPC less suitable for many industrial food applications where a light-colored ingredient is preferred.

Flavor is another major limitation. LPC may contain phenolic and volatile compounds that contribute to grassy, earthy, bitter, or astringent notes. Polyphenol oxidase could also affect sensory quality by promoting phenolic

oxidation and browning. These attributes may be acceptable in animal feed but are a major obstacle to their integration in human foods (Mayer 2006; Furia et al. 2025). Therefore, the development of food-grade LPC requires not only efficient and reproducible protein recovery but also the minimization of compounds that negatively affect the sensory profile.

1.4.2 Low extraction yield and processing complexity

A second major bottleneck is the low and variable recovery of extractable protein. Much of the leaf protein is difficult to recover because it is confined in plant cell walls. In addition, a substantial fraction of leaf protein can be insoluble or associated with thylakoid membranes, chloroplast structures, or larger cellular particles in the green juice (Tamayo Tenorio et al. 2016; Balfany et al. 2023). These structural and biochemical features make leaf protein extraction dependent on biomass type and processing conditions.

For food-grade applications, the process becomes even more demanding because the non-target components need to be reduced. More intensive purification steps may improve product quality, but they can also increase process complexity and reduce protein recovery. This is especially relevant for kale and other Brassica leafy biomass, where high levels of fiber, pigments, and phytochemicals create a complex leaf protein matrix (Bergeron et al. 2012). Therefore, fractionation process for food-grade WPC requires adaptation to the specific characteristics of each biomass.

1.5 Aim and research questions

The aim of this thesis was to evaluate selected processing methods and parameters for the fractionation of white protein concentrate from kale leaf biomass. The thesis focused on the recovery of the soluble white protein fraction, while also assessing product color and protein distribution across the fractionation process.

The study addressed the following research questions:

1. How do the temperature of thermal treatment and heating method affect protein recovery and color change in the white juice fraction?
2. Does low-temperature acid precipitation and resolubilization lead to a higher final protein recovery compared with room-temperature processing?
3. At which steps of the fractionation process do the main protein losses occur?

Rather than proposing a fully optimized fractionation process, this thesis aims to identify main limiting conditions of protein recovery and provide a methodological basis for further development of white protein concentrates from kale and other green leafy biomass within a green biorefinery framework.

2. Materials and methods

2.1 Experimental design and methodological rationale

2.1.1 Overall fractionation strategy

This thesis was designed as a methodological assessment of kale LPC fractionation rather than the direct application of standardized extraction protocols. Although LPC fractionation has been investigated in several green leafy biomass, such as lucerne, sugar beet leaves, and spinach, there is currently no universally established process for producing food-grade WPC from kale. This is particularly important because green leafy biomass differ substantially in leaf structure, fiber content, dry matter composition, phenolic profile, and protein distribution. Stødkilde et al. (2021) suggested that plant species, harvest maturity, and pressing methods can affect nitrogen recovery and the distribution of protein between liquid and fibrous fractions. Therefore, extraction parameters reported for other biomasses cannot be assumed to be directly transferable to kale. Based on this reason, the experimental design of this study was based on the selection of empirical fractionation protocols combined with process adaptation for kale biomass.

The fractionation procedure was adapted from established leaf protein fractionation principles, including the Pro-Xan process developed for lucerne protein fractionation, the Miller method for acid precipitation in low-temperature, and Nynäs' green biorefinery approach (Spencer et al. 1971; Edwards et al. 1975; Miller et al. 1975; Nynäs et al. 2021). These methods are the methodological basis for separating pressed green juice into a chlorophyll-rich green fraction and a soluble white protein fraction.

The overall fractionation process is illustrated in Figure 1, which also summarizes the abbreviations used for the resulting liquid and pellet fractions. The process stream shows the fractionation steps from juice pressing to the final resolubilized S4 fraction, while fractions removed at each separation step are indicated separately.

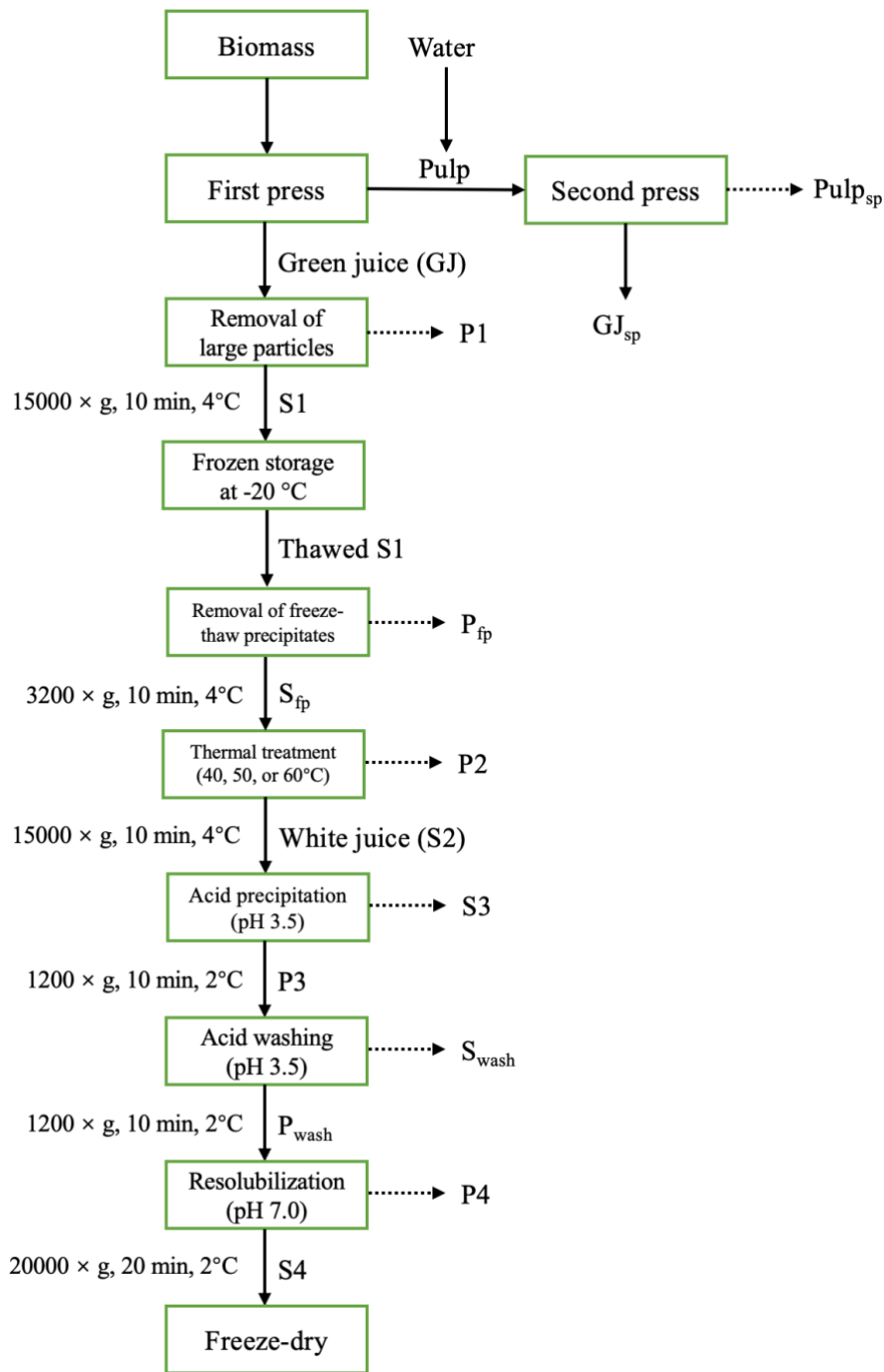


Figure 1. Overview of the kale WPC fractionation process. Fresh kale biomass was processed through a twin-screw pressing procedure to obtain green juice (GJ) and second-press green juice (GJ_{sp}). Following removal of large particles and freeze-thaw precipitates, the clarified juice fraction (S_{fp}) was proceeded to thermal treatment, acid precipitation, acid washing, and resolubilization. Fractions removed at each step are indicated by dashed arrows, while the main process stream is shown by solid arrows. Fraction abbreviations correspond to the designated names used throughout the report. Centrifugation conditions applied at each separation step are indicated adjacent to the process stream.

2.1.2 Treatment groups and comparison

Six treatment groups were included in the final experimental design, each processed in triplicate. The S_{fp} fraction was obtained by thawing S1 that had been stored at -20 °C and removing the resulting freeze-thaw precipitates by centrifugation (Fig. 1). All groups were prepared from S_{fp} because the treatments could not all be completed on same the day of juice pressing. The groups were structured around two main questions: the effect of processing temperature and the effect of thermal treatment conditions.

First, the two control groups (Control-RT and Control-ICE) were used to examine whether low-temperature acid precipitation and resolubilization improved protein recovery. The two groups underwent acid precipitation, acid washing, and resolubilization either at room temperature or under ice-water bath conditions. These two groups were included because earlier LPC fractionation processes suggested the importance of low-temperature processing for enhancing protein recovery during acid precipitation and resolubilization (Miller et al. 1975; Harrysson et al. 2019). However, the objective of the control groups in the present thesis was not to reproduce the previous fractionation process, but to isolate and evaluate the specific effect of low-temperature acid precipitation and low-temperature resolubilization on protein recovery.

Second, four thermal treatment groups (ST-40, ST-50, ST-60, WB-60) were used to evaluate the effects of thermal treatment on decolorization and protein retention prior to acid precipitation. Samples were either heated by steam injection to the designated temperature of at 40 °C, 50 °C, and 60 °C (ST-40, ST-50, ST-60), or to 60 °C in water bath (WB-60). Samples were held for 20 seconds once they have reached their designated temperatures.

2.1.3 Rationale for juice pressing method

Mechanical screw pressing was selected as the initial extraction step because it is a practical method for separating green juice from fibrous pulp that includes future industrial scalability. Compared with extraction approaches with solvent or enzyme, screw pressing is relatively simple, requires limited chemical input, and is compatible with a green biorefinery logic in which both juice and pulp fractions can be further valorized (Dijkstra et al. 2003). The second pressing step, in which the pulp was rehydrated and pressed again, was included to improve the recovery of soluble material remaining in the fibrous pulp after the first pressing (Dijkstra et al. 2003; Nynäs et al. 2021; Hansen et al. 2022; Andrade et al. 2025).

2.1.4 Rationale for thermal treatment conditions

Thermal coagulation is dependent on differences in heat sensitivity between chloroplast-associated green proteins and soluble white proteins. An appropriate level of heating can promote coagulation of chloroplast material, limit polyphenol oxidase and lipoxygenase activities, and improve visual decolorization, but

excessive heating may denature soluble proteins and reduce the following resolubilization (Xiao et al. 2017; Hadidi et al. 2019). Therefore, the temperature range of 40–60 °C was selected to balance the removal of chlorophyll-associated materials and preservation of soluble white proteins. Because separation of green and white protein fractions is highly temperature-sensitive, insufficient heating may leave green material in solution, whereas excessive heating may reduce white protein recovery (De Fremery et al. 1973; Damborg et al. 2020; Silva et al. 2023). Therefore, steam injection at 40, 50, and 60 °C represented mild, intermediate, and higher thermal conditions, respectively. Temperature of 60 °C was included as a typical thermal condition expected to enhance green fraction coagulation.

The 20-seconds holding time was selected to maintain short-time thermal exposure. The short-time heating was intended to promote rapid thermal coagulation of heat-sensitive green fraction while limiting denaturation of soluble white proteins (Damborg et al. 2020). From an industrial perspective, rapid heat treatment immediately after juice extraction may be advantageous because it could combine material transfer and heating while reducing the need for temporary storage. Reducing the processing time of thermal treatment may also prevent the endogenous enzymatic proteolysis, oxidation, or other undesirable reactions in the crude green juice (Pirie 1987). Thus, in the present study, direct steam injection was included because it provides rapid heating and has potential for scalable industrial processing (King et al. 2012). Water bath heating was included as a laboratory-scale method for reference. This design of experiment allows the effect of different heat delivery modes to be compared at the same target temperature.

2.1.5 Rationale for acid precipitation

Acid precipitation was performed by adjusting the S2 fraction to pH 3.5 using hydrochloric acid (HCl). This pH was selected because leaf protein solubility is strongly pH-dependent, and acidification is a widely used strategy for precipitating soluble proteins from green juice. Isoelectric precipitation is widely used for leaf protein recovery from the juice matrix. However, the isoelectric point of RuBisCO might not be the optimal pH because precipitation behavior in crude plant juice is influenced by the surrounding matrix, including salts, phenolic compounds, pigments, and other soluble plant components (Coldebella et al. 2013).

Previous studies have demonstrated that adjustment of precipitation conditions according to the type of green leafy biomass is often necessary. Hadidi et al. (2019), adapting the method of Hojilla-Evangelista et al. (2016), reported improved precipitation of alfalfa proteins at pH 3.5 compared with pH 4.5. In addition, Brassica leafy biomass such as kale and broccoli may require lower a pH than the theoretical isoelectric point of RuBisCO for effective precipitation (Nynäs et al. 2021). The use of pH 3.5 was therefore selected as a fixed precipitation condition to allow the comparison between treatment groups while reflecting conditions previously used in leaf protein extraction studies (Hadidi et al. 2019; Pérez-Vila et al. 2024).

2.1.6 Analytical strategy

The analytical strategy was designed to evaluate both protein recovery and visual appearance. Bicinchoninic acid (BCA) protein assay was used to determine soluble protein concentrations in liquid fractions. Since processing steps such as acid addition, washing, dilution, and resolubilization changed the volume of each fraction, concentration values alone did not correctly reflect protein recovery in each fractionation step. Therefore, BCA concentrations were converted into absolute protein masses using recorded masses of each fraction, and a mass balance approach was used to estimate protein distribution across the fractionation procedure.

Colorimetric evaluation was included because residual green color is a major limitation for the use of LPC or white protein fractions in food applications (An et al. 2023; Domokos-Szabolcsy et al. 2023). CIELAB image analysis was selected to quantify visible color changes in the liquid fractions and freeze-dried S4 powders. In the CIELAB color space, L* represents lightness, a* represents the green–red axis, and b* represents the blue–yellow axis (Commission on Illumination 2019). The a* value was used to evaluate changes along the green–red axis in S_{fp} and S2, while L* was used to evaluate the lightness of the final freeze-dried powders. Spectrophotometric chlorophyll determination provided supplementary pigment-specific evidence, and dry matter determination was used to estimate total solids carried into the final S4 fraction.

Overall, the analytical strategy allowed the treatment groups to be compared in terms of protein recovery, protein mass distribution, heat-induced color change, and final product appearance.

2.2 Materials and experimental procedures

2.2.1 Biomass material

Fresh kale leaves (*Brassica oleracea* var. *sabellica*) were purchased from a local supermarket (Maxi ICA Stormarknad, Burlöv, Sweden). The biomass was commercially grown and packaged in Spain. Each retail package contained approximately 200 g of whole kale leaves. The leaves were stored at 4 °C and processed within 24 h of purchase.

2.2.2 Juice pressing

Whole fresh kale leaves were weighed prior to juice pressing using a twin-screw Angel Juicer 5500 (Angel Co., Ltd., Busan, South Korea). The extracted green juice (GJ) and fibrous pulp were collected and weighed separately.

To assess the potential recovery of soluble proteins from the pulp fraction, the pulp was rehydrated and mixed with Milli-Q water at a 1:2 (w/w) ratio then re-fed

into Angel Juicer for a second pressing step. The resulting juice fraction was collected as green juice from second pressing step (GJ_{sp}).

GJ and GJ_{sp} samples were maintained in an ice-water bath immediately after juice pressing to minimize degradation from the activity of polyphenol oxidase and endogenous proteases.

The primary GJ fraction was clarified by centrifugation at $15,000 \times g$ for 10 min at 4 °C using a pre-cooled Sorvall Lynx 4000 superspeed centrifuge (Thermo Fisher Scientific, Waltham, MA, USA) to remove suspended solids and insoluble leaf debris. The resulting supernatant was designated as S1.

S1 and GJ_{sp} fractions were additionally vacuum-filtered through double-layered Munktell Grade 1003 filter paper (Ahlström-Munksjö, Helsinki, Finland) using a Büchner funnel assembly. Aliquots of GJ, GJ_{sp}, and S1 were stored at -20 °C for subsequent protein quantification. The remaining S1 was stored at -20 °C and later thawed for the rest of the fractionation steps (Fig. 1).

2.2.3 Thermal treatment

Frozen S1 samples were thawed under running cold water prior to thermal treatment. To remove insoluble materials formed during freeze-thaw storage, samples were centrifuged at $3,200 \times g$ for 10 min at 4 °C. The resulting freeze-thaw clarified supernatant was designated as S_{fp}. Aliquots of 40 mL S_{fp} were transferred into 50 mL conical centrifuge tubes and maintained in an ice-water bath prior to thermal processing.

Thermal treatment by conventional water bath heating was conducted using a preheated VWB 6 water bath (VWR International, Germany). Three capped sample tubes containing 40 mL S_{fp} were submerged in the water bath together with one separate capped tube containing 40 mL Milli-Q water and a calibrated thermocouple probe. When the monitoring tube reached 60 °C, the samples were held in the water bath for an additional 20 seconds.

Steam injection heating treatment was performed using direct steam injection generated from the steam rod from an AFK ESP-3 espresso maker (AFK, Germany). A calibrated thermocouple probe was immersed in each 40 mL S_{fp} sample during heating to monitor temperature changes continuously. The steam injection was initiated until the sample temperature approached approximately 2 °C below the target temperature. Steam injection was then terminated to compensate for residual heating from the steam rod.

After the holding period, all thermally treated samples were transferred to an ice-water bath and then centrifuged at $15,000 \times g$ for 15 min at 4 °C to separate heat-coagulated fractions. The resulting clarified supernatant was collected as S2. Each S2 fraction was weighed and transferred to a new pre-weighed tubes for mass balance calculations and subsequent fractionation steps.

2.2.4 Acid precipitation and acid washing

For the low-temperature treatment group (Control-ICE), all acid precipitation, washing, and resolubilization procedures were conducted under ice-bath conditions ($<2\text{ }^{\circ}\text{C}$) using pre-cooled solutions and equipment. Control-RT, ST-40, ST-50, ST-60, and WB-60 groups were processed under room temperature (approximately $18\text{ }^{\circ}\text{C}$ - $20\text{ }^{\circ}\text{C}$) in the laboratory.

The centrifuge (5804 R, Eppendorf SE, Germany) was pre-cooled to $2\text{ }^{\circ}\text{C}$ before acid precipitation. Acid precipitation was performed under continuous stirring using a RET basic magnetic stirrer (IKA-Werke GmbH & Co. KG, Germany), while pH adjustments were monitored using a Hanna Instruments FC2022 digital pH meter (Hanna Instruments, Romania).

For the thermal treatment groups (ST-40, ST-50, ST-60, and WB-60), their respective S2 fractions were used as the starting material for acid precipitation. For the control groups (Control-RT and Control-ICE), S_{fp} fractions were used without thermal treatment.

S2 of each group was acidified to pH 3.5 by drop-wise addition of 1 M HCl under continuous stirring. Once the target pH was reached, samples were equilibrated for 30 min to allow precipitation. Following equilibration, samples were centrifuged at $1,200 \times g$ for 15 min at $2\text{ }^{\circ}\text{C}$. The resulting supernatant was collected as S3, while the pellet fraction was designated as P3.

The P3 was subsequently washed using 30 mL Milli-Q water adjusted to pH 3.5 with 0.1 M HCl. For the Control-ICE group, pre-cooled Milli-Q water at pH 3.5 was used for acid washing. Washed P3 were thoroughly resuspended and centrifuged again at $1,200 \times g$ for 15 min at $2\text{ }^{\circ}\text{C}$. The supernatant, designated as S_{wash}, was collected and transferred to new empty tubes, and the remaining pellet fraction was designated as P_{wash}.

The masses of all collected fractions were recorded for subsequent mass balance calculations.

2.2.5 Resolubilization of the white protein fraction

Following acid washing, the P_{wash} was proceeded to resolubilization by pH adjustment. For the Control-ICE group, all procedures were conducted under ice-water bath conditions ($<2\text{ }^{\circ}\text{C}$) using pre-cooled 0.1 M NaOH solution. All remaining treatment groups were processed at room temperature.

The pH of each sample was adjusted to pH 7.0 by dropwise addition of 0.1 M NaOH under continuous stirring. Once neutral pH was reached, samples were stirred for an additional hour to facilitate resolubilization of the precipitated protein fraction.

Following resolubilization, samples were centrifuged at $20,000 \times g$ for 20 min at 2 °C using a pre-cooled Sorvall Lynx 4000 superspeed centrifuge (Thermo Fisher Scientific, Waltham, MA, USA) to maximize removal of residual insoluble materials and obtain a clarified supernatant. The resulting supernatant was collected and transferred to new empty tubes as S4, representing the resolubilized white protein fraction at neutral pH. The remaining pellet fraction was collected as P4.

The masses of S4 and P4 fractions were recorded for mass balance analysis.

2.2.6 Freeze-drying

After resolubilization, the S4 fractions were transferred into 50 mL conical centrifuge tubes and stored at -80 °C overnight prior to freeze-drying. Frozen samples were lyophilized using a ScanVac CoolSafe 110-4 freeze dryer (Innoguard, Höör, Sweden) until visually freeze-dried powders were obtained. The resulting freeze-dried protein powders were collected and stored for subsequent colorimetric evaluation and downstream analyses.

2.3 Protein quantification

Protein concentrations of GJ, GJ_{sp}, S_{fp}, S2, S3, S_{wash}, and S4 fraction aliquots were quantified using the Pierce BCA Protein Assay (Thermo Fisher Scientific, Waltham, MA, USA), based on the bicinchoninic acid assay described by Smith et al. (1985). Protocol was designed according to the manufacturer's instructions (Thermo Fisher Scientific 2024). The protocol used in this study included a set of acetone precipitation procedures adapted from the manufacturer's technical guidelines for elimination of interfering substances from samples in BCA protein assays (Thermo Fisher Scientific 2010).

2.3.1 Removal of interferences by acetone washing

To reduce interference from salts, phenolic compounds, phytochemicals, and other soluble compounds from plant cell, a set of acetone precipitation procedures was performed on the samples for BCA.

25 µL of each sample was transferred into 2 mL microcentrifuge tubes, followed by an addition of 200 µL -20 °C acetone. Samples were subsequently vortexed and incubated at -20 °C for 30 minutes to allow protein precipitation.

After the incubation, samples were centrifuged at $1,200 \times g$ for 10 min at 4 °C using a refrigerated microcentrifuge (5427 R, Eppendorf SE, Germany). A low centrifugation force was selected to prevent excessive compaction of the pellet, which results in a loss of protein from the acetone washing step. The supernatant was discarded, and residual acetone was allowed to evaporate under a fume hood for 10 min.

The protein pellets were resolubilized in 1 mL sodium dodecyl sulfate (SDS) buffer consisting of 3% (w/v) SDS and 0.05 M sodium phosphate monobasic monohydrate, making the samples diluted by 40X. Samples were vortexed and incubated at 65 °C for 10 min to facilitate protein resuspension. After incubation, samples were vigorously vortexed until no visible precipitate remained.

2.3.2 BCA protein assay

BCA working reagent was prepared by mixing 50 parts BCA reagent A with 1 part BCA reagent B according to the manufacturer's instructions from Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). Bovine serum albumin (BSA) standards ranging from 0–1000 µg/mL were prepared for establishing the protein standard curve. The diluent used in BSA standards was the same 3% SDS buffer used for resuspending the acetone-washed samples.

For the 96-well microplate preparation, 25 µL of resolubilized samples or BSA standards was added to each well, followed by 200 µL of BCA working reagent. Plates were incubated at 37 °C for 30 min, and the sample absorbance was measured at 562 nm using a Multiskan GO microplate spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

Protein concentrations were calculated from the BSA standard curve and corrected according to the dilution factor.

2.3.3 Mass balance calculations

A mass balance approach was applied to evaluate protein recovery throughout the fractionation process. For each processing step, the mass of the collected fraction was recorded using an analytical balance. For all collected fractions, 25 µL aliquots were separated from each sample for BCA analysis. The corresponding weight was added back to the total fraction volume during mass balance calculations.

The net mass of each fraction was calculated by subtracting the mass of the empty tube from the total mass of the tube and liquid. The mean density of the GJ, GJ_{sp}, S_{fp}, and S2 are measured as 1.006, 1.002, 1.003, and 1.003 g/mL respectively. Thus, for the purpose of mass balance calculations, the density of all liquid fractions used in BCA assay was approximated as 1.00 g/mL. The liquid mass in grams was converted as equivalent to volume in milliliters.

For aqueous, soluble fractions, including GJ, GJ_{sp}, S_{fp}, S2, S3, S_{wash}, and S4, the absolute protein mass was calculated

$$M_i = C_i \times V_i$$

where M_i is the absolute protein mass of the fraction (i) in mg, C_i is the protein concentration of the fraction (i) in mg/mL from BCA protein assay, and V_i is the calculated volume of fraction (i) in mL.

Due to the compatibility of BCA protein assay, for pellet fractions that could not be directly quantified by BCA, including P3 and P_{wash} protein mass was estimated by the mass difference using mass balance calculation.

For acid precipitation, the protein mass of P3 pellet was estimated as:

$$M_{P3} = M_{S2} - M_{S3}$$

where M_{P3} , M_{S2} , and M_{S3} are the absolute protein mass of the P3 pellet, S2 supernatant, and S3 supernatant respectively.

Similarly, for the acid washing step, the protein mass remained in the washed pellet after centrifugation was estimated as:

$$M_{P_{wash}} = M_{P3} - M_{S_{wash}}$$

where $M_{P_{wash}}$, M_{P3} , and $M_{S_{wash}}$ are the absolute protein mass of the P_{wash} pellet, P3 pellet, and S_{wash} supernatant respectively.

For protein recovery calculations, the protein mass in S_{fp} was defined as 100%.

Protein recovery at each fractionation step was calculated as:

$$Recovery_i\% = \frac{M_i}{M_{S_{fp}}} \times 100$$

All mass balance calculations were performed for each replicate before calculating mean values and standard deviations.

2.4 Colorimetric evaluation using CIELAB color space analysis

Imaged-based colorimetric analysis was performed using Trigit, a web app that allows color signal analysis of customizable region-of-interest (ROI) in multiple color spaces, including CIELAB (Tjandra et al. 2023). Images were uploaded to the web interface on <http://trigit.com.au/> for analysis in CIELAB color space.

2.4.1 Image capture

All images were captured under standardized lighting conditions to minimize variation caused by ambient light. Samples were photographed using an iPhone 15 Pro Max (Apple Inc., USA) with the 24 mm primary camera at 1× optical zoom. Images were captured in 48 MP JPEG format without digital filters. Three LED panel lights (GVM 800D-RGB, Great Video Maker, Hong Kong, China) were used as the light source and set to 5000 K. A white reference tool was included in the images to calibrate background lightness.

For thermal-treatment samples, 25 µL aliquots of S_{fp} and S2 fractions were placed on a white background and photographed under identical lighting and camera conditions. For freeze-dried S4 powders, samples were placed on white paper and gently flattened to reduce surface irregularity and shadowing before imaging. The camera was positioned perpendicular to the sample surface to minimize distortion and reflection.

For all samples, corrections were made by subtracting the values from white reference tool for photographing according to:

$$A_{corrected} = A_{measured} - A_{white_reference}$$

where $A_{measured}$ and $A_{white_reference}$ are the L^* , a^* , or b^* values of the sample and white reference tool, respectively. The result, $A_{corrected}$, was used in statistical analysis.

2.4.2 Colorimetric analysis of S_{fp} and S2 fractions

The color change from S_{fp} to S2 was used to evaluate the “decolorization” effect of thermal treatment. Images of S_{fp} and S2 liquid droplets were imported into Trigit, and the CIELAB color space was selected. ROIs were selected from the central region of each droplet using the autoselection tool at 95% sensitivity to avoid the selection of reflection spots and droplet edges.

For each treatment group, the average L^* , a^* , and b^* values were recorded. The reduction in greenness after thermal treatment was evaluated using the change in a^* value from S_{fp} to S2. Because a^* values indicate the color in the green-red axis, the percentage change in a^* was calculated in each group to assess the effectiveness of the thermal-treatment.

2.4.3 Colorimetric Analysis of Freeze-Dried S4 Powders

Freeze-dried S4 powders were analyzed to evaluate the visual color quality of the final resolubilized protein fraction. Images were imported into Trigit and analyzed in the CIELAB color space. A fixed ROI of 899 pixels was selected from each flattened powder sample to reduce variation caused by uneven powder distribution.

Average L^* , a^* , and b^* values were recorded for each sample. Higher L^* values were interpreted as higher lightness, while more negative a^* values indicated stronger residual greenness. The color profile of the freeze-dried S4 powders was therefore used as an indicator of the visual appearance of the final protein fraction.

2.5 Chlorophyll determination

Total chlorophyll content in S_{fp} and S2 fractions was determined spectrophotometrically using an acetone extraction method adapted from Arnon (1949) and Lichtenthaler (1987). All extraction procedures were conducted under dim-light conditions to minimize pigment degradation.

Blank sample was made with 80% of 80% acetone (v/v) with MilliQ water. 200 μ L aliquots of S_{fp} and S2 samples were mixed with 800 μ L of 80% acetone, which diluted the sample by 5X, and vortexed thoroughly to extract chlorophyll pigments. The mixtures were centrifuged at $3,000 \times g$ for 5 min at 4 °C to remove

proteins and other insoluble materials. The resulting acetone supernatant was collected for absorbance measurement.

Absorbance was measured at 663 nm and 645 nm using 80% acetone as the blank. To correct for background turbidity and light scattering, absorbance values at 663 and 645 nm were corrected by subtracting the corresponding absorbance at 750 nm:

$$\begin{aligned}A_{663corr} &= A_{663} - A_{750} \\A_{645corr} &= A_{645} - A_{750}\end{aligned}$$

Total chlorophyll concentration was calculated according to the equation from Arnon (1949):

$$Total\ Chlorophyll\ (\mu g/mL) = (8.02A_{663corr} + 20.2A_{645corr}) \times 5$$

where $A_{663corr}$ and $A_{645corr}$ represent corrected absorbance values at 663 nm and 645 nm, respectively.

The chlorophyll assay was used as a chlorophyll-specific assessment of the thermal treatment, complementing the image-based CIELAB colorimetric analysis.

2.6 Dry mass determination

Dry mass determination was performed to evaluate the moisture content and the recovery of the different processing fractions. Samples included in the dry mass determination were aliquots of fresh biomass (kale leaves), GJ, P, GJ_{sp}, and P_{sp}.

Aluminum foil containers were first weighed using an analytical balance with 0.1 mg readability. Samples were then added and weighed to obtain the wet mass. Samples were dried in a ventilated oven at 60 °C overnight. After drying, the oven-dried samples were reweighed and the dry mass was recorded.

2.7 Statistical analysis

All experiments were performed in independent triplicate unless otherwise specified. Protein recovery rate, colorimetric values, and mass balance calculations are presented as mean values $\pm$ standard deviation. Coefficient of variation was calculated where relevant to evaluate process reproducibility between replicate samples.

Statistical comparisons between selected treatment groups were performed using two-tailed Welch's t-tests assuming unequal variances. Statistical analyses and data processing were conducted using Microsoft Excel (Microsoft Corporation, USA). Differences were considered statistically significant at $p < 0.05$.

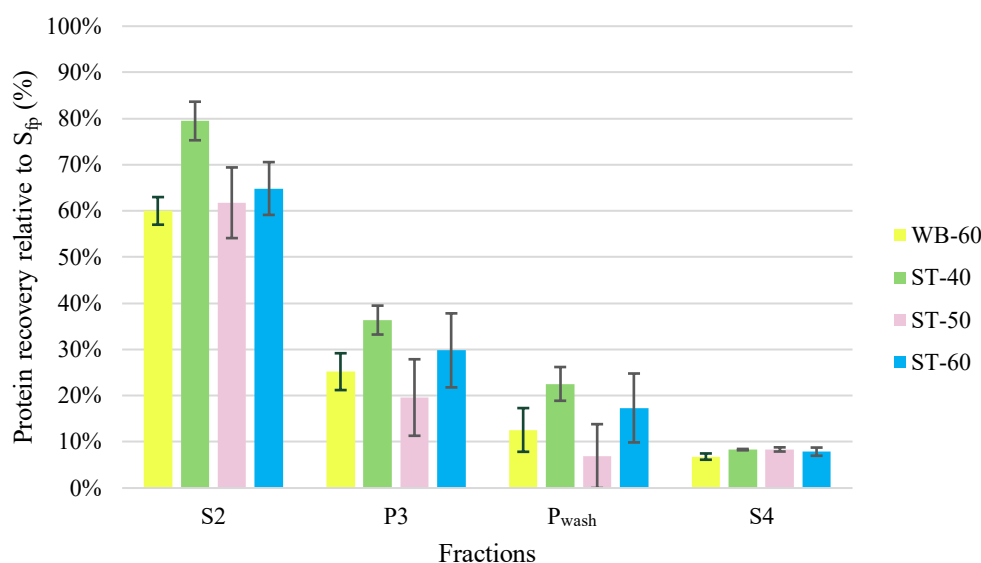


Figure 3. Protein recovery in selected process fractions of the thermally treated groups. Recovery was expressed relative to the protein mass in S_{fp} , which was defined as 100% for each group. Protein masses in S2 and S4 were measured directly using the BCA assay, whereas P3 and P_{wash} were estimated by mass balance calculations. Bars and error bars represent mean $\pm$ SD (n = 3).

The 60 °C treatments showed similar S2 protein recoveries between heating methods. WB-60 retained 60% of S_{fp} protein in S2, while ST-60 retained 65% (Fig. 3). Although ST-60 achieved 5% higher protein recovery than WB-60, this difference between the two groups was not statistically significant ($p=0.284$). This suggests that, in the S2 fraction, direct steam injection and water bath heating at 60 °C did not produce a major difference in total soluble protein recovery. However, the S2 value alone does not indicate the selectivity of decolorization or the quality of the retained protein fraction, and therefore must be interpreted together with downstream recovery and colorimetric data.

A substantial decrease in protein recovery occurred during the acid precipitation step (S2 to P3). The estimated P3 recovery values were 25% for WB-60, 36% for ST-40, 20% for ST-50, and 30% for ST-60 (Fig. 3). This indicates that the protein retained in S2 was only partially recovered in the acid precipitate, indicating a considerable proportion remained in the acid supernatant S3. Therefore, the decrease from S2 to P3 was one of the main sources of protein loss in the fractionation process.

After acid washing, further protein loss was observed. P_{wash} recovery was 13% in WB-60, 23% in ST-40, 7% in ST-50, and 17% in ST-60 (Fig. 3). The drop in protein recovery in this step from the previous step ranges from 38% to 65%, which is the second largest protein loss. The ST-50 P_{wash} value showed high variability between triplicates, with recovery values of 0.5%, 6%, and 14% in the three replicates. Since P_{wash} was calculated by mass balance rather than directly quantified, this variability may reflect accumulation of analytical and weighing

errors from S2, S3, and S_{wash} measurements. On the other hand, the acid washing step itself may have introduced practical variability through disruption or loss of P3 material into the S_{wash} . Therefore, the ST-50 P_{wash} value was interpreted cautiously and was not used as a primary indicator of process performance.

Final resolubilized S4 protein recovery was low and consistent across all thermally treated groups, with values of 6.8% for WB-60, 8.3% for ST-40, 8.4% for ST-50, and 7.9% for ST-60 (Fig. 3). On average, the loss of protein recovery from the last fractionation step was 47%. However, these values should be interpreted in the context of the mild resolubilization conditions and the complexity of the kale juice matrix. Acid precipitation may promote associations between proteins and other plant-derived components, such as phenolic compounds or pigment-associated material, which can reduce resolubility at neutral pH. Therefore, protein not recovered in S4 should not necessarily be interpreted as total process loss, as part of the protein may remain in insoluble fractions that could still be relevant for applications where solubility is less important. However, in the present study that focuses on the WPC, the main concern was the limited recovery of resolubilized protein and the substantial protein loss observed during acid washing.

Overall, the recovery profile indicates that thermal treatment was not the only determinant of final protein recovery. Instead, the largest recovery losses occurred during acid precipitation, washing, and resolubilization. This suggests that downstream pH-induced precipitation and resolubilization behavior represented a major bottleneck in the production of the final white protein fraction.

3.1.2 Final S4 protein recovery across all treatment groups

Final S4 protein recovery was compared across all treatment groups to evaluate the overall efficiency of the fractionation process. The two non-heated control groups showed higher final S4 protein recoveries than the thermally treated groups. Both Control-RT and Control-ICE recovered approximately 13% of S_{fp} protein in S4, respectively. In comparison, the thermally treated groups showed lower final recoveries, ranging from 6.8% in WB-60 to 8.4% in ST-50.

Among the thermally treated groups, final S4 recovery was relatively similar. WB-60, ST-40, ST-50, and ST-60 showed S4 recoveries of 6.8%, 8.3%, 8.4%, and 7.9%, respectively. Their differences were not statistically significant ($p>0.05$). Although the different conditions of thermal treatment altered the color profile of S2, the S4 recovery rate indicates that differences in thermal treatment temperature or heating method did not convert into large differences in final resolubilized protein recovery. Consequently, this means that the final recovery of the LPC remained constrained by the later acid precipitation, acid washing, and resolubilization steps.

The higher S4 recovery in the non-heated control groups should not be simply interpreted as evidence of better overall WPC yield. Since the two control groups did not process through the thermal treatment step, more soluble protein and non-

target soluble material may have remained available for acid precipitation and resolubilization. Therefore, final S4 recovery must be interpreted together with dry matter content and colorimetric data of the final freeze-dried S4 product.

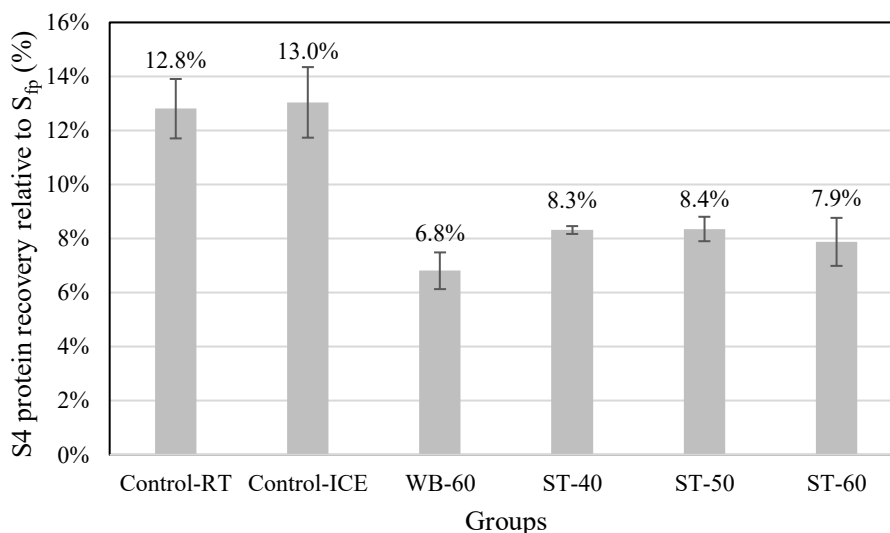


Figure 4. Final S4 protein recovery across all treatment groups. S4 protein recovery was calculated as the percentage of protein recovered in the final resolubilized S4 fraction relative to the protein mass in S_{fp} , which was defined as 100%. Values are presented as mean $\pm$ SD from triplicate samples. Control-RT and Control-ICE were processed without thermal treatment, while WB-60, ST-40, ST-50, and ST-60 were thermally treated before acid precipitation and resolubilization.

3.2 Effect of low-temperature acid precipitation and resolubilization on protein recovery

3.2.1 Room temperature group (Control-RT) versus low-temperature group (Control-ICE)

The two non-heated control groups were used to evaluate whether low-temperature acid precipitation and resolubilization improved protein recovery. Control-RT and Control-ICE were both processed directly from S_{fp} without thermal treatment.

From acid precipitation, S3 protein recovery was 53% for Control-RT and 54% for Control-ICE. The coefficients of variation were 4.9% and 1.9%, respectively. Statistical comparison showed no significant difference between the two groups for either S3 protein recovery or S3 protein concentration.

Final S4 protein recovery was also nearly identical between the two control groups. Control-RT recovered 12.8% of S_{fp} protein in S4, while Control-ICE recovered 13.0% (Fig. 4). No significant difference was observed for either S4 protein recovery or S4 protein concentration. These results indicate that, under the

conditions used in this study, conducting acid precipitation and resolubilization under a low-temperature condition under 2 °C did not significantly increase the recovery of resolubilized white protein compared with room-temperature processing.

3.2.2 Implications for room-temperature processing

The lack of a significant difference between Control-RT and Control-ICE suggests that low-temperature acid precipitation and resolubilization did not improve final S4 protein recovery under the conditions used in this study. This finding did not reproduce the higher protein recovery reported in earlier leaf protein fractionation studies using low-temperature processing conditions (Miller et al. 1975).

Several explanations are possible. First, the room-temperature exposure time in the present protocol was approximately 4 to 5 hours, which might be relatively short compared to historical protocols. The short exposure time in room temperature may have limited temperature-dependent proteolysis, aggregation, or loss of protein solubility. Second, both control groups were subjected to refrigerated centrifugation and prior storage in -20 °C, which may have reduced the effective contrast between room-temperature and low-temperature processing. Third, kale juice may behave differently from lucerne juice because of their differences in juice composition, including proteins, phytochemicals, phenolic compounds, pigments, salts, and other soluble plant constituents. Since the setting Miller et al. (1975) was based on lucerne, the difference in biomass does not guarantee methodological replicability.

From a process perspective, this result suggests that a conducting room-temperature acid precipitation and resolubilization in several hours may be worth further investigation as a lower-energy alternative to fully chilled processing. If similar recovery can be achieved without maintaining the entire acid precipitation and resolubilization step under low-temperature conditions, then energy consumption, refrigeration requirement and process complexity could potentially be reduced in industrial-scale production (Edwards et al. 1975). However, this implication should be treated cautiously because the present study was conducted at laboratory scale and did not evaluate longer processing times, microbial stability, sensory quality, or industrial-scale handling conditions.

3.3 Effects of thermal treatment on decolorization

3.3.1 CIELAB color shift from S_{fp} to S2

Colorimetric analysis in CIELAB color space was used to evaluate the color shift from S_{fp} to S2 following thermal treatment. The “a*” value was used as the main indicator of change along the green–red axis. In this dataset, thermal treatment increased a* values in all treatment groups, indicating a shift away from the initial green color of the S_{fp} fraction.

All thermal treatments produced a significant increase in a^* from S_{fp} to S_2 . The mean a^* value increased from 1.84 to 2.73 in WB-60, from 2.42 to 2.79 in ST-40, from 2.61 to 3.61 in ST-50, and from 2.32 to 3.44 in ST-60. The corresponding Δa^* values were 0.89, 0.37, 1.00, and 1.12, respectively (Fig. 5). Statistical comparison between S_{fp} and S_2 showed significant differences for all four thermally treated groups. This indicates that thermal treatment consistently changed the color of the soluble fraction of S_{fp} .

Among the groups heated by direct steam injection, ST-40 produced the smallest a^* shift, while ST-50 and ST-60 produced larger shifts. A significant difference of $S_2 \Delta a^*$ was observed between ST-40 and ST-60, while no significant difference was found between ST-40 and ST-50 ($p=0.068$), ST-50 and ST-60 ($p=0.671$). This suggests that most of the color shift in the green-red axis occurred between 40 °C and 50 °C, while there was limited change in color when the temperature of thermal treatment increased from 50 °C and 60 °C.

For the comparison of heating methods, at 60 °C, WB-60 and ST-60 resulted in similar Δa^* values, with no significant difference ($p=0.440$). This indicates that both heating methods, water bath and direct steam injection, caused a comparable shift along the green-red axis at the same target temperature. However, final $S_2 a^*$ values differed significantly between WB-60 and ST-60, which suggests that differences in their initial S_{fp} color or heat-transfer behavior may have influenced the final color of the treated S_2 fraction.

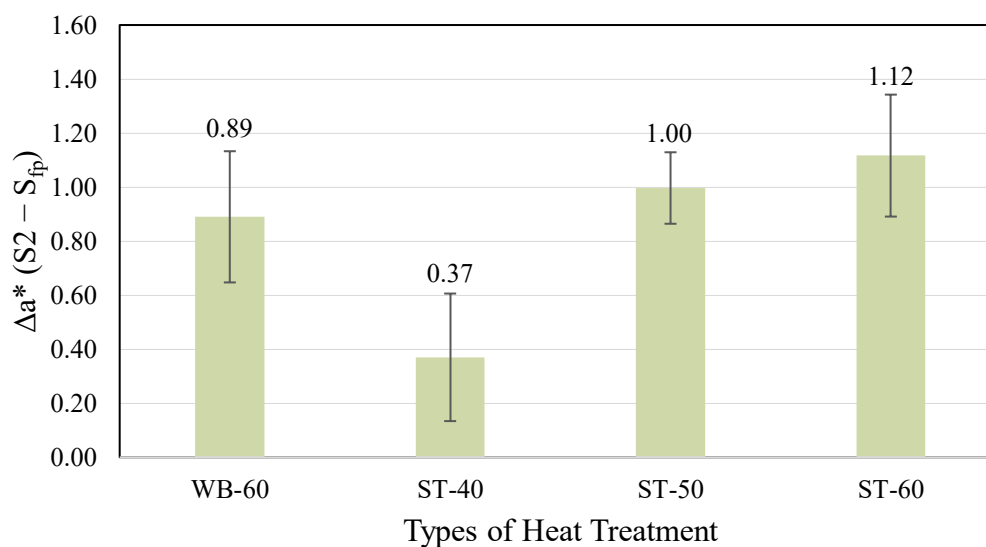


Figure 5. Change in a^* value between S_{fp} and S_2 after thermal treatment. Values represent mean $\pm$ SD from triplicate samples. Δa^* was calculated as “ $S_2 a^* - S_{fp} a^*$ ”. Higher Δa^* values indicate a stronger shift along the green-red axis after thermal treatment.

The L^* values changed only slightly after thermal treatment in all groups, suggesting that the main change in color was not primarily lightness. In contrast,

b^* values decreased in all treatments, indicating a reduction in yellow-associated color intensity.

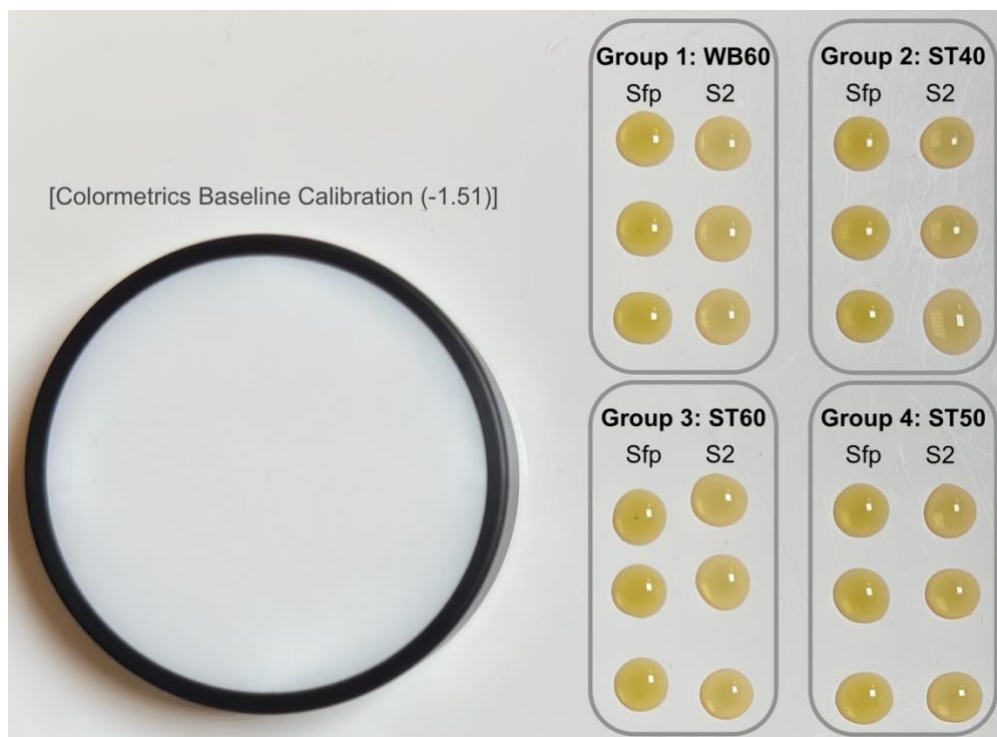


Figure 6. Photograph of S_{fp} and S_2 droplets used for CIELAB colorimetric analysis. Each treatment group contained triplicate S_{fp} and S_2 samples. S_{fp} represents the fraction before thermal treatment and S_2 represents the soluble fraction after thermal treatment and centrifugation. The image demonstrates the visual color shift from S_{fp} to S_2 across WB-60, ST-40, ST-50, and ST-60 groups under standardized photographing conditions.

In summary, the increase in a^* and decrease in b^* indicate that thermal treatment shifted S_2 away from the original green-yellow appearance of S_{fp} (Fig. 6). This supports the interpretation that thermal treatment contributed to S_2 decolorization before acid precipitation.

3.3.2 Chlorophyll determination as supplementary pigment evidence

Total chlorophyll concentration was measured in S_{fp} and S_2 fractions as a supplementary assessment of pigment removal during thermal treatment.

After A750 correction, all treatment groups showed different magnitudes of reduction in mean chlorophyll concentration in S_2 than their corresponding S_{fp} fraction. In WB-60, chlorophyll concentration decreased from $1.2 \pm 0.6 \mu\text{g/mL}$ in S_{fp} to $1.0 \pm 0.2 \mu\text{g/mL}$ in S_2 , corresponding to a 10% reduction (Fig. 7). Steam-treated samples showed larger average decreases. Chlorophyll concentration decreased from 3.8 ± 0.9 to $2.7 \pm 0.6 \mu\text{g/mL}$ in ST-40, from 3.0 ± 0.3 to 1.8 ± 1.2

$\mu\text{g/mL}$ in ST-50, and from 1.4 ± 0.5 to $0.6 \pm 0.2 \mu\text{g/mL}$ in ST-60. These corresponded to average reductions of 29%, 41%, and 55%, respectively.

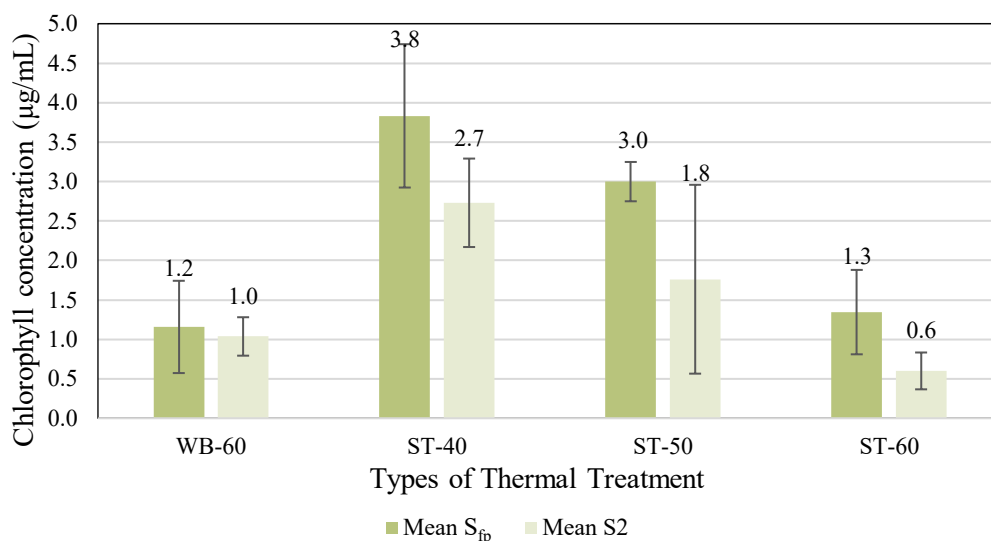


Figure 7. Total chlorophyll concentration in S_{fp} and S_2 fractions after A750-corrected spectrophotometric analysis. Values represent mean $\pm$ SD from triplicate samples. Chlorophyll concentration was calculated from A663 and A645 after subtraction of A750 to correct for background turbidity and scattering.

Among the three groups of direct steam injection, the mean reduction in chlorophyll concentration increased with treatment temperature. This indicates a trend associated to temperature, with ST-60 showing the greatest average reduction, followed by ST-50 and ST-40. WB-60 showed the smallest reduction, suggesting that direct steam injection may have been more effective than water bath heating in reducing chlorophyll concentration under the conditions tested.

However, none of the differences in chlorophyll concentration from S_{fp} to S_2 were statistically significant, with p-values ranging from 0.123 to 0.765. Therefore, the chlorophyll data should be interpreted as supportive trend evidence rather than statistically confirmed proof of chlorophyll removal during thermal treatment. Although the image-based CIELAB analysis showed more evident and reproducible color shifts from S_{fp} to S_2 , it is not compound-specific. Therefore, the results cannot confirm that the change in chlorophyll content was the cause of the observed color shifts. In contrast, the spectrophotometric assay was more specific to chlorophyll but showed higher variability in the complex kale juice matrix, possibly because chloroplast-associated particles were unevenly distributed among aliquots and extracted inconsistently. Taken together, the two methods suggest that thermal treatment altered the pigment-related color profile of S_2 , but the contribution of chlorophyll specifically remains tentative.

3.4 Final freeze-dried S4 product characteristics

3.4.1 Dry matter content of S4

The dry matter content of S4 differed substantially between non-heated controls and thermally treated groups. Control-RT and Control-ICE showed the highest S4 dry matter contents, at 1.02% and 1.04%, respectively, with no significant difference between them. The higher dry matter content indicates that a greater amount of total solids was retained in the final S4 fraction in the absence of thermal treatment. However, because dry matter includes both protein and non-protein components, these values do not reflect the protein purity of final products.

In contrast, all thermally treated groups showed much lower S4 dry matter contents. ST-40 had the highest dry matter content among the thermally treated groups, at 0.44%, followed by ST-50 at 0.34%, ST-60 at 0.16%, and WB-60 at 0.10% (Fig. 8). The comparison by pairs indicated significant differences between all thermally treated groups.

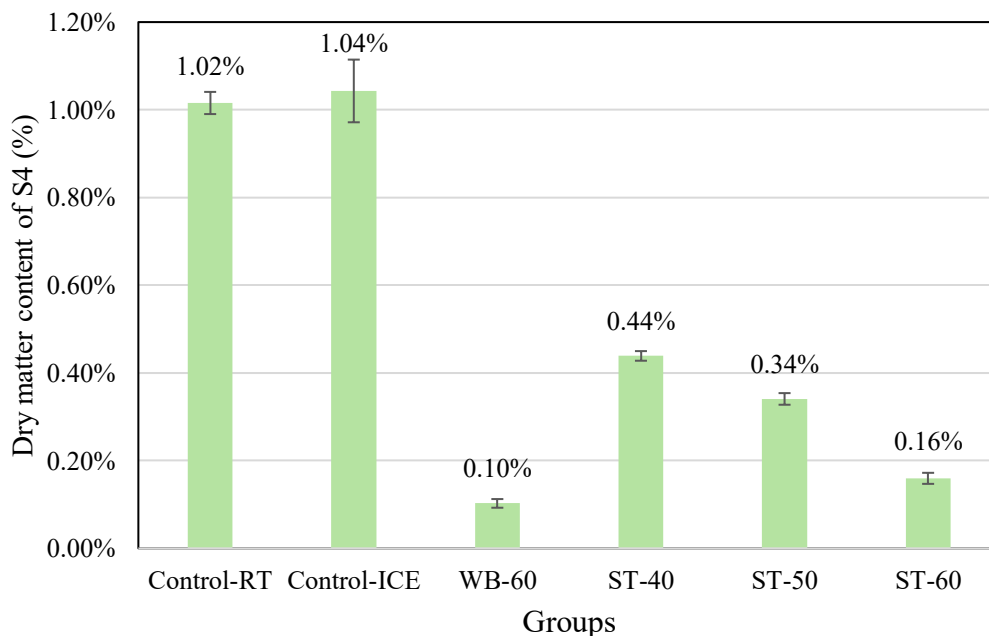


Figure 8. Dry matter content of final S4 fractions. Dry matter content was determined after freeze-drying the final S4 fractions and is expressed as percentage of the initial S4 sample mass. Values represent mean $\pm$ SD from triplicate samples. Control-RT and Control-ICE showed higher dry matter contents than the thermally treated groups, indicating greater total soluble solids in the final S4 fraction.

The higher dry matter content in the non-heated controls should not be interpreted as higher protein purity or yield. Dry matter includes all freeze-dried soluble solids, including protein, salts, carbohydrates, phenolic compounds, residual pigments, and other soluble components from plant cells. Therefore, the higher S4 dry matter content of Control-RT and Control-ICE likely reflects more soluble non-protein materials were carried to the final S4 fraction from previous fractionation steps. By contrast, the lower dry matter content in thermally treated

groups suggests that thermal treatment removed a considerable proportion of soluble solids before acid precipitation and resolubilization.

In summary, the protein recovery and dry matter results show that thermal treatment reduced the amount of total dry material reaching S4, but did not proportionally improve final protein recovery. This suggests a trade-off between the removal of impurities and the recovery of resolubilized protein.

3.4.2 CIELAB color profile of freeze-dried S4 powders

The color profile of freeze-dried S4 fractions was evaluated using CIELAB analysis. In contrast to the liquid S_{fp} and S2 fractions, the final freeze-dried products showed a yellow-brown color profile rather than a clearly green appearance. Therefore, S4 fraction color was interpreted using all three CIELAB values. Higher L* values indicate greater lightness, while higher a* and b* values indicate stronger red-brown and yellow color components, respectively.

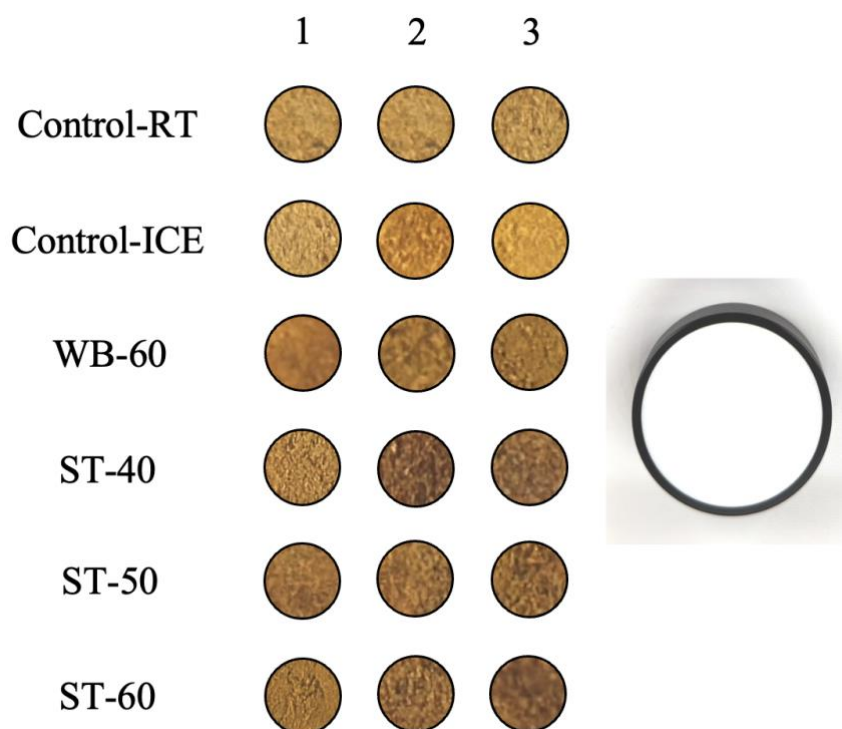


Figure 9. Photographs of freeze-dried S4 fractions used for CIELAB color analysis. Rows represent the six treatment groups, and columns 1–3 represent triplicate samples within each group. The white reference target shown on the right was included for image correction.

The non-heated control samples showed the highest L* values, with Control-RT and Control-ICE showing mean L* values of 62.96 and 65.92, respectively. However, these samples also showed relatively high dry matter contents, suggesting that their lighter appearance should not be considered directly to higher protein purity. The higher dry matter content may reflect more soluble non-protein material in the final S4 fraction.

Among the thermally treated groups, L* values were lower, ranging from 47.52 to 52.71. WB-60 showed a mean L* value of 52.71, while ST-40, ST-50, and ST-60 showed mean L* values of approximately 49.40, 50.67, and 47.52, respectively. This indicates that thermal treatment did not produce a visually whiter final product, despite reducing the dry matter content of S4.

The a* values of the S4 fractions were positive across all groups, indicating that the final freeze-dried products were not green in CIELAB terms but instead contained red-brown color components. Control-RT showed the lowest a* value, while WB-60 and ST-50 showed higher a* values. The b* values were also positive in all groups, confirming an existing yellow component in the product color. ST-40 and ST-60 showed lower mean b* values than the control groups, suggesting a reduction in yellow-associated color intensity, although variability was high in these treatments.

The colorimetric analysis results suggested that the fractionation method affected the color profile of the freeze-dried S4 fraction. Since all groups were subjected to acid precipitation and resolubilization, the lower L* values observed in the thermally treated groups suggest that combining thermal coagulation with acid precipitation may promote darker final WPF powders under the conditions used in this study. This is consistent with previous observations in leaf protein concentrate production from lucerne, where thermal coagulation has been associated with darker or browner LPC products, potentially due to protein denaturation, chlorophyll degradation, or formation of phenolic-associated products (Tamayo Tenorio et al. 2016; Furia et al. 2025).

Pairwise comparisons showed that the two non-heated control groups had significantly higher L* values than several thermally treated groups. This indicates a brighter freeze-dried S4 appearance in the samples without thermal treatments. In contrast, a* values of the thermally treated groups were not significantly different, suggesting that the main color difference between control groups and thermally treated groups was in the lightness rather than the green-red axis. In addition, since the colorimetric differences were not significant in all thermally treated groups, the S4 color results should be interpreted as descriptive trends rather than statistically confirmed treatment effects. Overall, the data of freeze-dried S4 fraction color suggest that thermal treatment reduced the proportion of dry matter in S4, but did not lead to a brighter or visually whiter final product. This indicates a trade-off between the removal of impurities, final protein recovery, and visual appearance of the freeze-dried protein fraction.

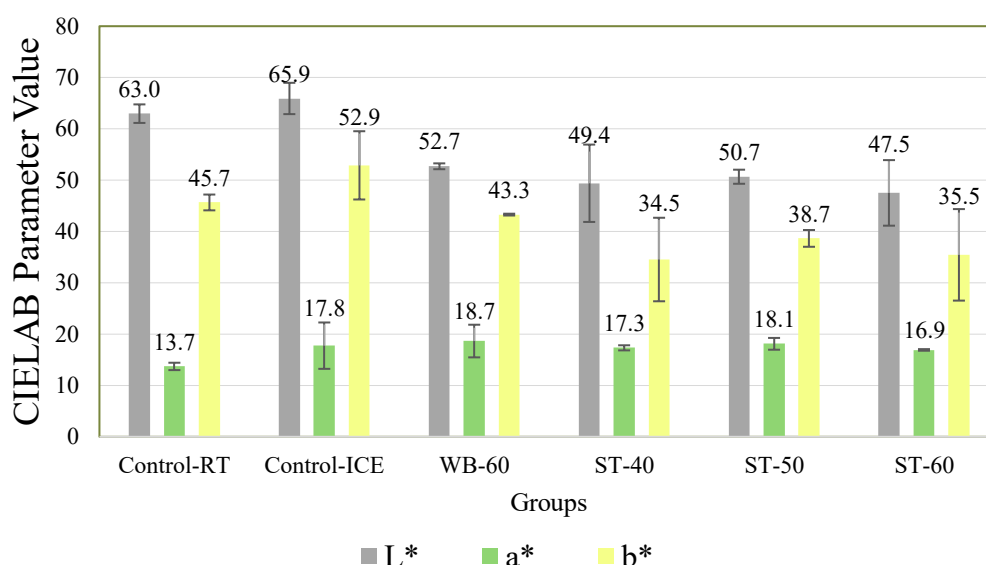


Figure 10. CIELAB color profile of freeze-dried S4 fractions. Values are presented as mean $\pm$ SD of triplicate samples. Higher L* values indicate greater lightness, while higher a* and b* values indicate stronger red-brown and yellow color components, respectively.

3.4.3 Trade-off between lightness, dry matter carry-over, and recovery

The final S4 recovery, dry matter, and colorimetric results indicate a trade-off between protein recovery, impurity removal, and visual appearance of the freeze-dried WPC product. The non-heated control groups showed higher final S4 protein recovery and higher L* values, indicating a brighter final product. However, the control group samples also showed significantly higher S4 dry matter contents. This suggests that the results of the non-heated control groups should not be concluded as higher protein recovery, since dry matter includes protein as well as salts, carbohydrates, phenolic compounds, pigments, and other soluble non-target solids.

By contrast, thermally treated groups showed much lower dry matter content in S4, suggesting that thermal treatment removed a considerable amount of soluble non-WPF material before acid precipitation and resolubilization. However, this reduction in dry matter did not result in higher final S4 protein recovery or a lighter final powder. In fact, not only the thermally treated freeze-dried S4 products showed lower L* values than the non-heated controls, they also have a noticeably darker visual appearance than those of non-heated control groups (Fig. 10). This indicates that thermal treatment did not directly enhance the final visual lightness of the freeze-dried product.

This finding is important for WPC development because the target product is not only expected to be rich in protein, but also suitable for food-related applications

where low color, low odor, and mild sensory characteristics are critical. Dark green or brown color, together with earthy or grassy flavors, has been reported as one of the major barriers of leaf protein ingredients to incorporate into human food formulations (Di Stefano et al. 2018; An et al. 2023). The current results show that decolorization in the S2 fraction does not necessarily predict the color of the final freeze-dried S4 product. This may be partly because downstream acid precipitation, washing, resolubilization, and freeze-drying can introduce additional changes in color, solids composition, and protein recovery. Residual carbohydrates and free amino acids may contribute to non-enzymatic browning reactions during processing or drying, while residual chlorophyll can be converted into pheophytin under heat or strong pH changes, resulting in darker or brownish protein products (Tamayo Tenorio et al. 2016). In addition, enzymatic browning may still occur since oxidative enzymes such as PPO are not fully suppressed. Previous studies have shown that sulfite addition can reduce PPO-induced browning and produce LPC with lighter color (Amer et al. 2021). Therefore, future optimization should not focus only on improving the appearance of the white juice fraction (S2), but should also improve the downstream acid precipitation, limit browning reactions, and enhance the visual appearance of the final WPC powder.

3.5 Process implications and methodological limitations

Overall, the results indicate that kale leaf WPC fractionation involves several trade-offs. Thermal treatment decreased the greenness of the S2 fraction and reduced the amount of dry matter carried into the final S4 fraction, but it did not increase final S4 protein recovery or produce a visually brighter freeze-dried product. The process therefore cannot be optimized by heating temperature alone. Instead, WPC fractionation requires balancing white juice decolorization, protein recovery, dry matter removal, and overall appearance of the final product.

3.5.1 Main bottlenecks in kale leaf WPC fractionation

The main bottleneck identified in this study was downstream recovery of the resolubilized white protein fraction after thermal treatment. Across the thermally treated groups, final S4 recovery remained below 9% of S_{fp} protein, despite clear treatment-related differences in S2 color and S4 dry matter content. The largest losses occurred during acid precipitation, acid washing, and resolubilization, suggesting that pH-induced precipitation and subsequent neutral redissolution were the key limiting steps in the present process.

The results reflect a broader challenge in LPC fractionation. To obtain an LPC product that balances both yield and quality, it often requires additional purification steps. However, these steps may also increase process complexity and protein loss. For instance, rigorous purification can help remove non-target compounds such as pigments, salts, phenolic compounds, carbohydrates, and

other soluble substances, but it may also reduce protein recovery or damage functional properties (Balfany et al. 2023). In the present study, this trade-off was reflected by the low final S4 recovery after acid precipitation, acid washing, and resolubilization.

The acid washing step was a particular source of uncertainty. The ST-50 P_{wash} value showed high variability, and because P_{wash} was estimated by mass balance rather than directly measured, the calculation accuracy of this intermediate fraction was susceptible to the accumulation of errors from S2, S3, and S_{wash} measurements. In practical terms, the washing step may also disturb the acid precipitate and transfer protein into the wash supernatant. This indicates that acid washing conditions require further optimization if the process is to be improved.

More importantly, the necessity and intensity of acid washing should also be re-evaluated. Historical and recent leaf protein extraction protocols differ in whether washing is included, how many washing steps are applied, and whether the wash water is pH-adjusted. Some protocols include one or several washing steps to remove salts, soluble impurities, and off-color compounds, whereas others proceed after centrifugation without a dedicated washing step (Moreno-Nájera et al. 2020; Soo et al. 2021; Patra and Arun Prasath 2024; Pérez-Vila et al. 2024). These variations infer that acid washing is not a fixed requirement, but a process variable that should be optimized according to biomass type, product target, and acceptable trade-off between purity and recovery.

For kale juice matrix, in specific, acid washing might be useful for reducing non-target soluble material and improving product quality, but excessive washing or strong pellet disruption may reduce recovery of the target protein fraction. Therefore, future work should therefore compare washing and non-washing approaches, as well as different wash volumes, wash pH values, mixing intensities, and centrifugation conditions. The objective should not be simply to maximize washing intensity, but to identify the minimum purification required to improve WPC color and composition without unnecessary loss of recoverable protein.

3.5.2 Limitations of BCA-based mass balance and chlorophyll assay

The BCA-based mass balance approach allowed protein flow to be tracked across multiple liquid fractions, but it also had several limitations. Soluble fractions such as S_{fp} , S2, S3, S_{wash} , and S4 were directly quantified by BCA protein assay, whereas pellet-associated fractions such as P3 and P_{wash} were estimated by subtraction. As a result, small errors in concentration measurement, weighing, dilution correction, or sample handling could be magnified in the calculated pellet fractions. This was particularly important for low-volume samples as small differences could produce large variation.

The BCA assay itself may also be affected by the complex of kale juice matrix. Within samples of the same fraction, the interference may have been similar and therefore be partly cancelled in treatment comparisons. However, the composition of the matrix changed across fractionation steps due to heating, acidification, and resolubilization. Therefore, the interference was not identical among all fractions. The calculated protein masses should be regarded as operational estimates rather than absolute biochemical measurements with high precision.

Interference in the BCA protein assay may have been broadly similar within the same fraction and could therefore have partly cancelled in treatment comparisons. However, matrix composition changed across fractionation steps because of heating, acidification, washing, and resolubilization, so interference was unlikely to be identical among all fractions. The calculated protein masses were therefore treated as operational estimates rather than highly precise absolute measurements.

At the same time, the choice of protein determination method is not standardized in leaf protein fractionation studies. Some of the alternative methods based on total nitrogen determination with a conversion factor, such as the Kjeldahl or Dumas method, may also have limitations because leaf extracts can contain various amounts of non-protein nitrogen (Mæhre et al. 2018). In such cases, total nitrogen does not necessarily represent true protein nitrogen. Non-protein nitrogen compounds may also influence the purification behavior and functional properties of extracted protein fractions, which makes it important to distinguish between nitrogen from protein and non-protein nitrogen when evaluating leaf protein recovery and purity (Prade et al. 2021). For this reason, BCA protein assay was used in the present study as a practical method for tracking soluble protein across multiple fractions, while the recognition of its limitations is necessary.

The chlorophyll assay was also limited by variability. Although A750 correction was applied and mean chlorophyll concentration decreased after thermal treatment, none of the S_{fp} -to-S2 differences were statistically significant. This indicates that the applicability of spectrophotometric chlorophyll determination is questionable on the kale juice matrix. Chlorophyll isolation from S_{fp} is challenging because pigments may be associated with chloroplast fragments and suspended material that are not evenly distributed between samples. The chlorophyll data were therefore treated as chlorophyll-specific evidence rather than primary proof of color-pigment removal.

Overall, the methodological limitations in this study reflect the difficulty of applying standard protein and pigment assays to a complex green juice matrix from kale and other green leafy biomass. Many historical LPC fractionation protocols were developed using lucerne or other forage biomasses, and their analytical systems might not be applicable to kale. Therefore, the mass balance and chlorophyll results should be interpreted as indications that identify major trends and bottlenecks, rather than as exact measurements of the fractionation process.

3.5.3 Interpretation of the Control-RT and Control-ICE comparison

The Control-RT and Control-ICE groups were designed to test the influence of temperature on acid precipitation and resolubilization. The control treatments are not the reproduction of the Pro-Xan process, in which the green protein fraction was precipitated and removed by thermal treatment.

This distinction is important because the composition of the material entering acid precipitation may strongly influence precipitation and resolubilization behavior. In the control groups, the acid precipitation step was applied to S_{fp} , instead of S2, which may still have contained soluble green proteins and chloroplast particles. In contrast, after thermal treatment, S2 represents a different matrix in which part of the green fraction and coagulated materials have already been removed. Therefore, it remains possible that low-temperature acid precipitation could have a stronger effect after thermal fractionation than it did when applied directly to S_{fp} .

The absence of a significant difference between Control-RT and Control-ICE should be interpreted conservatively. It suggests that, under the present conditions, maintaining the acid precipitation and resolubilization steps under ice-water bath conditions did not improve recovery when S_{fp} was used as the starting fraction. However, it does not exclude the possibility that low-temperature processing could improve recovery in thermally treated S2 fractions.

4. Conclusion

This thesis evaluated selected fractionation methods and parameters for producing a white protein concentrate from kale leaf biomass. The results showed that thermal treatment changed the color profile of the white juice (S2) fraction, with stronger decolorization observed at higher steam treatment temperatures. However, improved liquid-phase decolorization did not directly translate into higher final protein recovery or a brighter freeze-dried final product.

Final resolubilized protein recovery remained consistent but limited across all thermally treated groups, indicating that the main bottlenecks were not only related to thermal coagulation, but also to downstream acid precipitation, acid washing, and resolubilization. The comparison between Control-RT and Control-ICE showed no significant improvement from low-temperature acid precipitation and resolubilization under the conditions used in this study. However, these controls were not direct reproductions of Pro-Xan process and should be regarded as a test of processing temperature in unheated clarified green juice.

Overall, kale side-streams show potential as a raw material for white protein fraction production, but the process requires further optimization. Future work should focus on improving recovery from acid precipitation and resolubilization, reducing non-target solids and pigments, and characterizing the composition and sensory properties of the final WPC product.

5. Future aspects

Future work should focus on improving the balance between protein recovery, decolorization, and final WPF quality. The present study showed that thermal treatment can improve the color profile of the liquid S2 fraction, but this did not result in higher final S4 recovery or a brighter freeze-dried powder. Therefore, future optimization should not focus only on increasing thermal coagulation efficiency, but should take the full fractionation chain into consideration.

An additional opportunity is the use of second-press green juice (GJ_{sp}). In this study, GJ_{sp} was collected and quantified but was not processed through the full fractionation procedure. Based on its protein content, using GJ_{sp} in the full fractionation process could theoretically increase overall protein recovery by approximately 2.6–3.3%. Future techno-economic analysis should evaluate whether this additional recovery is sufficient to cover the extra water use, processing time, energy input, and handling cost.

Thermal precipitation should be further optimized because it presents a clear dilemma. Insufficient heating could leave green protein and chloroplast-associated materials in the white juice, while excessive heating may reduce protein resolubility and darken the freeze-dried final product color (Toews and Wang 2013; Tamayo Tenorio et al. 2016; Brotosudarmo et al. 2018; Ersus and Akyüz 2023). Therefore, future studies should experiment on a narrower temperature interval between 40 °C and 60 °C. Additional steps such as microfiltration or enzymatic treatments may also be tested on kale LPC fractionation to improve product color (Dos Passos and Ambye-Jensen 2024; Furia et al. 2025). Direct steam injection remains promising because of its rapid heating potential, but its strong agitation to the juice may also disrupt newly formed coagulates and affect green fraction recovery.

The following steps require particular attention. The pH of Acid precipitation, acid washing, and resolubilization should be further improved because these steps strongly limited final S4 recovery in the present study. Future work should test a narrower precipitation pH range around pH 3.5–5.0, compare acid washing and non-washing approaches, and improve resolubilization conditions.

Finally, future studies should include compositional analysis of the freeze-dried S4 powders. Measurements of protein purity, phenolic and lipid content, residual pigments, chloroplast-materials derivatives, carbohydrates, and sensory-related compounds would allow dry matter and colorimetric data to be understood more thoroughly. This is important because WPF intended for food or pharmaceutical applications requires not only protein recovery and purity, but also acceptable sensory attributes.

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Appendix 1. BSA standard preparation for BCA protein assay

Table 1. Preparation of BSA standards used for the BCA protein assay.

Vial	Volume of diluent	Volume and source of BSA	Final BSA concentration
A	0	300 μ L of stock	2000 μ g/mL
B	125 μ L	375 μ L of stock	1500 μ g/mL
C	325 μ L	325 μ L of stock	1000 μ g/mL
D	175 μ L	175 μ L of vial B dilution	750 μ g/mL
E	325 μ L	325 μ L of vial C dilution	500 μ g/mL
F	325 μ L	325 μ L of vial E dilution	250 μ g/mL
G	325 μ L	325 μ L of vial F dilution	125 μ g/mL
H	400 μ L	100 μ L of vial G dilution	25 μ g/mL
I	400 μ L	0	0 = Blank

Appendix 2. Supplementary protein recovery data and statistical comparisons

Table 2. Mean protein recovery across fractionation stages in thermally treated groups.

Group	S _{fp}	S2	P3	P _{wash}	S4
WB-60	100.0%	60.0%	25.2%	12.6%	6.8%
ST-40	100.0%	79.5%	36.4%	22.6%	8.3%
ST-50	100.0%	61.8%	19.6%	6.9%	8.4%
ST-60	100.0%	64.9%	29.8%	17.3%	7.9%

Table 3. Pairwise statistical comparisons for protein recovery

Comparison	Variable	p-value
WB-60 & ST-60	S2 recovery	0.284
Control-RT & Control-ICE	S4 protein concentration	0.816
Control-RT & Control-ICE	S4 recovery	0.825
ST-40 & ST-50	S4 recovery	0.900
ST-50 & ST-60	S4 recovery	0.468

Table 4. Pairwise statistical comparisons for CIELAB and chlorophyll values

Comparison	Variable	p-value
WB-60 S _{fp} & S2	a*	0.006
ST-40 S _{fp} & S2	a*	0.026
ST-50 S _{fp} & S2	a*	0.002
ST-60 S _{fp} & S2	a*	0.001
WB-60 & ST-60	Δa^*	0.440
ST-50 & ST-60	Δa^*	0.671
ST-40 & ST-60	Δa^*	0.049
ST-40 & ST-50	Δa^*	0.068
WB-60 S _{fp} & S2	Chlorophyll concentration	0.765
ST-40 S _{fp} & S2	Chlorophyll concentration	0.163
ST-50 S _{fp} & S2	Chlorophyll concentration	0.212
ST-60 S _{fp} & S2	Chlorophyll concentration	0.123

Appendix 3. Thermocouple calibration

To ensure temperature accuracy throughout the thermal treatment experiments, a two-point calibration of the Testo 735-2 digital thermometer (Testo SE & Co. KGaA, Germany) and its Type-T thermocouple probe was performed prior to the experiment. Calibration was conducted using an ice-water bath (0.0 °C) and boiling Milli-Q water (100.0 °C) as reference points. The thermocouple displayed 0.0 °C in ice-water bath and a measured boiling-point temperature of 100.8 °C, which indicated a slight positive offset at elevated temperatures. A linear correction model was therefore applied to all recorded temperature values according to the following equation, actual temperature (T_{actual}) is:

$$T_{actual} = m \times T_{indicated} + c$$

Therefore, the slope is calculated as:

$$m = \frac{\Delta T_{actual}}{\Delta T_{indicated}} = \frac{100.00 - 0.0}{100.8 - 0.0} = \frac{100.0}{100.8} \approx 0.992$$

And the intercept is calculated as:

$$0.0 = 0.992 \times 0.0 + c$$

Thus,

$$c = 0$$

The final linear correction:

$$T_{actual} = 0.992 \times T_{indicated} + 0$$

All temperatures reported in this study refer to corrected temperature values.