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30/4/2025

FINAL REPORT

The Leaf Protein Co: Leaf Protein Food Matrices & Model System

ENDFOODWASTE COOPERATIVE RESEARCH CENTRE

Australian Government Department of Industry, Science and Resources

AusIndustry Cooperative Research Centres Program

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EFW CRC Publication 2025

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Final Report Version 1 – 16/1/2025

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Panel Review Statement

End Food Waste CRC recognises the value of knowledge exchange and the importance of objective peer review. It is committed to encouraging and supporting its research teams in this regard. The author(s) confirm(s) that this document has been reviewed and approved by the Project Leader and Industry Partner.

This project has also been evaluated by the End Food Waste CRC publication review panel. These reviewers evaluated its:

- Methodology articulated clearly
- Positioning of findings within the current literature
- Acknowledged compliance with food safety standards
- Conclusions against results
- Relevant human and/or animal ethic approvals obtained

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Industry Partner Foreword

This project is a follow on from the "Green leaves - Wet extraction of leaf protein from fresh broccolini and other green leafy vegetable waste", which confirmed the ability to extract edible leaf protein ingredients from leafy vegetable feedstock. This follow-on project was used to assess the commercial viability of this ingredient through its application in finished products.

To be commercially viable we needed to understand the physiochemical characteristics of our leaf protein ingredient, Nüleaf Protein®, compared to other similar plant protein ingredients in market. As well as its application in different finished product formats commonly found in health foods. As such we chose to develop a beverage product and a snack product. In addition to developing basic recipes for these 2 formats we were also interested in testing the use of Nüleaf Protein® in common industrial processes: high-pressure homogenisation and dry extrusion.

The results of this project have confirmed the impact of different leaf protein extraction methods on the ingredient's functionality, and therefore application in different products. It also provided insights into potential post-processing requirements to consider in our extraction methods. Of the 2 finished product formats, we were particularly excited about the results of dry extrusion, and its potential use in newer puffed extruded snack type formats. The development of a basic recipe allows us to now work with co-manufacturers of these types of products to create a marketable finished product.

This first project demonstrating the technical application of Nüleaf Protein® in common product formats confirms for us the required extraction methodology for a high-quality ingredient as well as our initial target product applications to go to market.

Fern Ho, CEO, 8 January 2025

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Executive Summary

Food waste is a global issue, driving the food industry to explore sustainable ingredients for food applications. As such, plant-based proteins have attracted increasing interest within the food industry as consumers shift toward plant-based diets. However, not all the plant-based material is fully utilised, especially the leaves and the stem of the plant ingredient. Therefore, leaf proteins are a promising plant-based alternative ingredient, as they are sustainable and can help reduce food waste when valorised into functional food ingredients

This project consisted of four stages. The first stage focused on investigating the functional properties of proteins extracted from alfalfa leaves. The extraction process provided initial insights into how proteins can be extracted from the leaves. Key functional properties examined included particle size, zeta potential, protein solubility, rheology, and thermal properties. The results indicated that the extracted leaf proteins were not functional, particularly in terms of gelling and protein solubility, suggesting that the extraction process requires further refinement.

The second stage examined the functional properties of alfalfa leaf proteins for potential food applications. In this stage, proteins were extracted on a pilot scale, and the functional properties were analysed. In addition to the properties studied in stage one, other properties like emulsification and foaming were also assessed. The results demonstrated that the green leaf proteins exhibited higher functionality, such as better protein solubility, compared to white proteins, which is crucial for food applications. This stage laid the groundwork for the use of leaf proteins in food products.

Following the first two stages, high-pressure homogenization (HPH) was used to create leaf protein-based beverage models. This process showed the ability to reduce particle size and improve the product's stability, whether mixed with water or commercial juice. Increasing the number of passes had no significant effect when the protein was mixed with water, but when mixed with apple juice, further particle size reduction was achieved. Further studies could explore how the acidity of solutions like apple juice affects the leaf protein and its interaction with the HPH treatment.

Additionally, leaf protein was incorporated into puffed products using extrusion. A corn starch-based formulation was used to form the physical matrix, into which leaf protein was incorporated. The trial showed that up to 5% leaf protein could be added to the extruded product to create a puffed product. However, the prototype could be improved by adjusting the barrel temperature, powder feed rate, and moisture content. The formulation composition could also be optimized.

Once a prototype was developed, upscaling and reproducibility were tested using a larger extruder at CSIRO. The results showed that scaling up posed challenges, as the prototype produced at RMIT could not be reproduced with the exact process condition and additional trials are required if a new extruder is used. Additionally, the use of a larger extruder led to variations in how the formulation was fed into the machine. This raised further questions and provided additional information regarding how extrusion processes can be scaled to produce large quantities of a high-quality product.

Overall, this project provides valuable insights for industry, particularly in refining the extraction process and exploring how alfalfa leaf proteins can be used in food applications. The findings will also help companies expand their operations by developing new products that utilize leaf proteins.

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1. Introduction

1.1 Background

There is a growing demand for plant-based protein sources, with the food industry increasingly turning to alternative plant-based proteins to improve food security and environmental sustainability. Currently, large quantities of plant by-products, including leaves and stems, are discarded, contributing to food waste. In this context, green leaf waste, often regarded as agricultural byproducts, has emerged as a promising protein source, as their use addresses both global protein demand and environmental concerns about waste disposal. Green leaf waste from the leaves of vegetables is conventionally used in non-food applications such as feedstock, composting and energy production, which presents a major challenge in modern agriculture. Therefore, the food industry is looking for alternatives to transform the leaf food waste into functional ingredients for food applications (Furia et al., 2025[a]; Furia et al., 2025[b])

Leaf proteins are a current trend in food research and while most of the research on plant-based proteins has focused on crops such as soy, peas, and quinoa, there are many other varieties of leaf proteins that can be used, one of which is alfalfa leaf. Alfalfa leaf contains 20-30% protein, similar to that of pea and soy, and 30-35% fibre, comparable to that of lupin (Furia et al., 2025[a]; Lo, Kasapis, & Farahnaky, 2021; Nissen et al., 2022). Alfalfa leaf proteins represent an underexplored resource that could play a key role in this shift. While alfalfa has long been recognized as a high-quality forage crop, its protein-rich leaves offer untapped potential as a plant-based protein source for human consumption (Furia et al., 2025[a]).

Leaf protein extraction offers a sustainable alternative to traditional animal-based protein sources, of which the most commonly employed method is acid precipitation. This method is particularly valuable for utilizing plants like alfalfa, spinach, plant food wastes and other leafy greens, which are rich in protein. The extraction process involves breaking down the plant's cellular structure to release soluble proteins, followed by purification and concentration to obtain a functional protein extract. There are many technical challenges to extract protein from leaves, some of which include low yield, poor functional properties and low protein content. Therefore, examining and optimising the extraction process is critical to overcoming the technical challenges associated with leaf protein recovery (Furia et al., 2025[b]).

1.2 Research aims and questions

The food industry has gained interest in incorporating leaf proteins in many food applications. However, before it can be used in the market, the extraction process and its impact on the functional properties of leaf protein concentrates needs to be investigated (Furia et al., 2025[a]). Therefore, this project aims to:

- Determine the functional properties of extracted leaf proteins
  - How does the extraction process impact the functional properties of leaf protein?
  - How do the functional properties of leaf proteins compare to a commercial plant protein product (soy protein concentrate)?
- Incorporate leaf proteins into a food application

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- How can high pressure homogenisation be used in leaf protein applications?
- How can low moisture extrusion be used to incorporate leaf protein in food applications?

2. Methodology

2.1 Investigating the Functional Properties of Extracted Alfalfa Leaf Proteins (Stage 1)

In the first stage of the project, the functional properties of alfalfa leaf proteins extracted using different extraction methods were analyzed and compared with commercial soy protein concentrate. Chemical analyses were used to assess protein content, particle size, zeta potential, and protein solubility, while rheological, foaming, and emulsification characteristics were also evaluated.

2.1.1 Materials (Stage 1 Leaf Protein Samples)

Samples of white leaf, acid green leaf, high heat green leaf were extracted from alfalfa leaves and were compared to soy protein concentrate (control) (Figure 1) to measure the functional properties of extracted leaf proteins for the first stage of the project. The leaf samples were extracted following methods by the Leaf Protein Co. at their pilot plant (see 2.1.2).

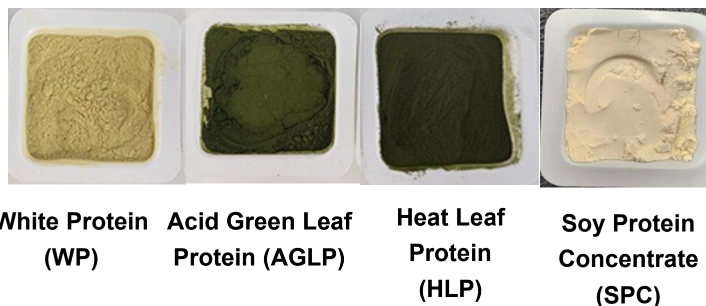

Figure 1: Extracted leaf protein and soy concentrate samples used for functional property measurements

2.1.2 Leaf Protein Extraction

White leaf protein

Green leaf juice was heated to 57ºC±1ºC, then cooled down to 25ºC±1ºC in a heat exchanger. The solution was then centrifuged to separate the liquid, termed brown liquor. The brown liquor was heated to 95ºC±1ºC for approximately 1 minute and then cooled down to 10ºC±1ºC in a heat exchanger. The solution with a solids content of 20% was then spray dried with the conditions of inlet temperature140ºC±1ºC and outlet temperature of 75ºC±1ºC. The powder was collected, vacuum packed and stored at 25ºC±1ºC for further analysis.

Acid green leaf protein

Protein extraction was extracted using the acid precipitation method. The pH of the leaf juice was adjusted to pH to 9.0±0.1 using 1.0 N NaOH and 1.0N HCl. The juice was then separated using a centrifuge and the pH of the supernatant was adjusted to pH 4.3-4.4. The suspension was centrifuged and protein in the pellet was collected. The pellet was dispersed in water to a 20% solids

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content for spray drying with the conditions of inlet temperature140ºC±1ºC and outlet temperature of 75ºC±1ºC. The powder was collected, vacuum packed and stored at 25ºC±1ºC for further analysis.

High heat green leaf protein

The green leaf juice was heated to 95ºC±1ºC for approximately 1 minute and then cooled down to 10ºC±1ºC in a heat exchanger. The solution, with a solids content of 20%, was then spray dried with the conditions of inlet temperature140ºC±1ºC and outlet temperature of 75ºC±1ºC. The powder was collected, vacuum packed and stored at 25ºC±1ºC for further analysis.

Soy protein concentrate

Commercial soy protein concentrate (SPC) was obtained from Hela Spice (Australia) with 72% protein content (dry basis), 20% total dietary fibre (20%) and 3% fat content.

2.2 Investigating the Functional Properties of Extracted Alfalfa Leaf Proteins for Food Applications (Stage 2)

In the second stage of the project, the functional properties of leaf proteins extracted using different methods were analyzed and compared with alfalfa leaf powder. Chemical analyses were used to assess protein content, particle size, zeta potential, and protein solubility, while rheological, foaming, and emulsification characteristics were also evaluated.

2.2.1 Materials (Stage 2 Leaf Protein Samples)

Samples of white leaf protein, alfalfa powder, acid leaf protein, high heat leaf protein and low heat leaf protein were extracted from alfalfa leaves (Figure 2).

Figure 2. Extracted leaf protein for food applications (Stage 2 leaf protein production)

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Alfalfa Powder

The alfalfa leaf was cut and dried under the sun for 1 day to evaporate the residual moisture. The alfalfa was then dried in a convection tunnel oven at 50ºC±1ºC for 2 days to achieve a final moisture content of 8%

White leaf protein

The alfalfa juice was heated just below 62ºC±1ºC, and the solids were separated from the liquid. The brown liquor was collected and stored at 6°C until further processing. Brown liquor was transferred to 30L pot and slowly heated on stove top while stirring and heating to 90ºC±1ºC. The sample was removed from the heat and transferred to an ice bath. The liquid in the ice bath was retained at rest to allow coagulation between the precipitated proteins, allowing for easier separation. The liquid was allowed to cool to approximately 40°C±1°C, prior to protein coagulum separation using slotted spoons and cheese cloth. Supernatant was discarded.

The sample was washed with water (5:1 water ratio to protein) and cooled down to 6ºC±1ºC for 1 hour. This process was repeated 4 times to remove the residual colour of the protein. After the 5th wash, the supernatant was nearly colourless. Protein was left to set for 24 hours before final decanting and spray drying. The solution with a solids content of 20% was then spray dried with the conditions of inlet temperature140ºC±1ºC and outlet temperature of 75ºC±1ºC. The powder is collected, vacuum packed and stored at 25ºC±1ºC for further analysis.

Acid leaf protein

Protein extraction was extracted using the acid precipitation method. The pH of the leaf juice was adjusted with distilled apple cider vinegar until the pH is 4.3-4.4. Protein was left to set for 24 hours before final decanting and spray drying. The solution with solids content of 20% was then spray dried with the conditions of inlet temperature140ºC±1ºC and outlet temperature of 75ºC±1ºC. The powder is collected, vacuum packed and stored at 25ºC±1ºC for further analysis.

High heat leaf protein

Alfalfa leaf juice was heated to 95ºC ±5ºC while stirring. The sample was then cooled down in an ice bath. The protein coagulate was scooped off from the top of the liquid. The coagulate was stored at 6ºC ±1ºC for 32 hours before spray drying. The solution, with a solids content of 20%, was then spray dried with the conditions of inlet temperature140ºC±1ºC and outlet temperature of 75ºC±1ºC. The powder is collected, vacuum packed and stored at 25ºC±1ºC for further analysis.

Low heat leaf protein

Alfalfa juice was heated to 62ºC±5ºC while stirring. The sample was cooled down in an ice bath. The protein coagulate was scooped off from the top of the liquid. The coagulate was stored at 6ºC ±1ºC for 32 hours before spray drying. The solution, with a solids content of 20%, was then spray dried with the conditions of inlet temperature140ºC±1ºC and outlet temperature of 75ºC±1ºC. The powder was collected, vacuum packed and stored at 25ºC±1ºC for further analysis.

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2.3 Extracted Leaf Protein Analysis (Stage 1 and 2)

2.3.1 Chemical Analysis of Leaf Proteins

The protein content was measured using the Kjeldahl (Kjeltec 8200-Foss, Denmark) method outlined by Lo, Kasapis, & Farahnaky (2022) where the protein factor of (N × 6.25) was used. The moisture content was measured using an infrared moisture analyser (Ohaus / Kern, MB 45 / DBS). 1g of leaf protein isolate powder was added to an aluminum plate, and dried at 105ºC. The colour analysis of the leaf proteins was analysed using the Minolta Chroma Meter CR-400 (Japan). The colour values were expressed in the L*, a*, and b* colour space. L* represents lightness (0 = black, 100 = white), a* reflects the red-green balance (positive values indicate red, negative values indicate green), and b* corresponds to the yellow-blue axis (positive values indicate yellow, negative values indicate blue).

2.3.2 Particle Size and Zeta Potential Analysis

The particle size of the leaf protein dispersions was determined using a Malvern Mastersizer 3000 Laser Diffraction Hydro MV system (Malvern Instruments Ltd, Malvern, UK). The refractive index for leaf protein was 1.46, and 1.33 for the dispersant (deionised water), with the absorbance coefficient set at 0.01.

The zeta potential was determined using the Zetasizer (Malvern Instruments Ltd, Malvern, UK) with the same refractive index for leaf protein and the dispersant as above. (Lo, Kasapis, & Farahnaky, 2024).

2.3.3 Protein Solubility of Leaf Proteins

Protein solubility was measured following the modified Lowry method as outlined by Lo, Kasapis, & Farahnaky . (2022) The sample preparation for this section is as follows: 0.1g of the isolated leaf protein was diluted into 10g of water. The pH of the solution was adjusted to 2, 4, 4.6, 6, 8 and 10 using 0.1N NaOH and 0.1 HCl. The sample was then centrifuged at 6000g for 5 minutes and 0.1mL of the solution was extracted. 1mL of the modified Lowry reagent (Pierce Modified Thermo Fisher Scientific, Australia) was then added to the sample and left to incubate for 10 minutes at ambient temperature (23ºC±1ºC). Subsequently, 0.2mL of Folin-Ciocalteu reagent (Pierce Modified Thermo Fisher Scientific, Australia) was added to the samples which was then left to incubated for 30 minutes at ambient temperature (23ºC±1ºC). After 30 minutes elapsed, the samples were taken to the Plate reader (Perkin Elmer-Lambda 35, USA) set to 750nm for analysis. The standard curve was determined using BSA standards from the modified Lowry kit provided.

2.3.4 Rheological Properties of Leaf Protein

The storage modulus of the leaf protein suspensions was measured using a Discovery Hybrid Rheometer (TA Instruments, USA). Leaf protein samples were loaded onto a 40 mm parallel plate geometry at 25ºC, and the temperature of the sample was measured from 25ºC at a rate of 1°C/minute and 1% strain to 95ºC. The sample was then cooled back down to 25ºC. The hydration and pasting properties of the leaf protein powders were analysed using a Rapid Visco Analyzer (RVA) (Model 4500, Perten Instrument, Sydney, NSW, Australia) with the method outlined by Lo, Kasapis, & Farahnaky (2024). Before the analysis, the moisture content of the powders was measured when calculating the amount of powder and water required to give a water/dry powder ratio of 25g:4 g (16 % [w/w]). The samples were mixed at 960 rpm for 100 seconds at 25ºC to disperse the powder in water and then reduced to a

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constant speed of 160 rpm. The sample was heated from 25ºC to 95ºC at a heating rate of ~10ºC and the temperature was held at 95°C for 60 seconds. The sample was then cooled down back to 25ºC.

2.3.5 Thermal Analysis Differential Scanning Calorimetry (DSC)

The thermal behaviour of Alfalfa leaf protein was evaluated using a DSC-Q2000 (TA Instruments, New Castle, DE, USA) according to the procedure described by Wang et al. (2023), with slight modifications. Approximately 3–4 mg of the sample was accurately weighed into an aluminium TA pan (Model: 901683.901), followed by adding water at a 1:2 ratio (6–8 mg). The pans were hermetically sealed and allowed to hydrate overnight.

During the differential scanning calorimetry (DSC) analysis, the temperature was increased from 20 °C to 160 °C at a rate of 10 °C/min under a nitrogen atmosphere (50 mL/min). An empty aluminium TA pan served as the reference. The onset temperature (T0), end set temperature, denaturation temperature (Td), and enthalpy change (ΔH) were determined from the thermograms using the Universal Analysis software provided by TA Instruments.

2.3.6 Foaming and Emulsion Stability

For foaming stability, 1g of leaf powder into 99g of water. The sample is high shear homogenised at 10000 rpm for 1 minute. The photo of the system was taken at timed intervals of 0, 5, 10, 20, 30, 60 and 120 minutes.

For emulsion stability, 1g of leaf protein powder into 99g of water. The sample is high shear homogenised at 10000 rpm for 1 minute. 125mL of oil was slowly added while mixing at high shear at 10000 rpm for 1 minute. The photo of the system was taken at timed intervals of 0, 5, 10, 20, 30, 60 and 120 minutes.

2.4 Incorporation of Investigating Leaf proteins Subjected to High Pressure Homogenisation (Stage 3)

In the third stage of this project, the extracted leaf protein (HHLP) was used as a functional ingredient in beverage applications. The leaf protein suspension in water was subjected to high pressure homogenisation at 350bar (35 MPa) for up to 3 cycles. The particle size and stability tests of the leaf solutions before ana after homogenization where analyzed. After that, the leaf protein was incorporated in apple juice to mimic vegetable juices out in the market. The leaf juice was subjected to high pressure homogenisation and the particle size of the juice was determined.

2.4.1 High pressure homogenisation

A 4% leaf protein (HHLP) suspension was homogenised using a two-stage homogenizer GEA Lab Homogenizer Panda PLUS 2000 (PandaPlus 2000 model, GEA,), at 350bar (35 MPa) for up to 3 cycles. The samples were collected after each cycle for analysis.

2.4.2 Particle size

The particle size of the leaf protein dispersions was determined using a Malvern Mastersizer 3000 Laser Diffraction Hydro MV system (Malvern Instruments Ltd, Malvern, UK). The refractive index for leaf protein was 1.46, and 1.33 for the dispersant (deionised water), with the absorbance coefficient set at 0.01.

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2.4.3 Stability analysis

After homogenisation, the sample was left in room temperature and photos were taken at 2 ,4 and 24 hour intervals.

2.5 Incorporation of Leaf Proteins in Extrusion Applications (Stage 4)

In the fourth stage of this project, the leaf protein was used as a functional ingredient in producing puffed snacks by using extrusion technology. The leaf protein was mixed with other ingredients such as corn flour and maltodextrin to help with the matrix of the product. After extrusion, the expansion ratio, texture and moisture content of the extrudates were analyzed. After that, the leaf mixture was taken to a larger extruder at CSIRO to determine whether this product can be upscaled on a larger production manufacturing.

2.5.1 Extrusion conditions

The powders were mixed using an Armfield powder blender for 10 minutes (Supplier, country). The extrusion process follows the method outlined by Palanisamy et al. (2019) and Naumann et al. (2021) with modifications. The HME process was carried out using a co-rotating twin screw extruder (Eurolab Thermofisher, Germany). The length of the barrel is 650 mm, 16 mm in diameter and the diameter of the screw is 16 mm. The barrel contained six heating zones and the temperature profile for the extrusion process was set to 30, 30, 70, 100, 150, 150ºC. The diameter of the die is 3mm. Corn flour (94%), maltodextrin (5%) calcium carbonate (0.5%) and salt (0.5%) was used as to produce the physical matrix. After the matrix of the system was produced. High Heat Green (HHLP) Leaf protein was incorporated into the system.

2.5.2. Texture profile analysis

The texture profile analysis of the extrudates were measured using a Texture Analyzer (TA.XT. Plus, Stable Microsystems Ltd., Godalming, UK) attached with a 35 mm diameter cylindrical aluminium probe. The sample size was kept as much as possible the same, slight variations in height and size were inevitable. Measurements were performed in triplicate for each sample as a single compression test with 50% deformation with 2mm/sec test speed.

2.5.3 Moisture analysis

The moisture content of the powder and extrudate was measured using an infrared moisture analyser (Ohaus / Kern, MB 45 / DBS).

2.5.4 Bulk density and radical expansion

The bulk density of the extrudates was calculated using the following equation

$$Bulk\ Density\ (kg/m^3) = \frac{\text{Mass of extrudate}}{Volume\ of\ extrudate}$$

$$Volume\ of\ extrudate = \pi \left(\frac{d^2}{4}\right) l$$

Where d =diameter of sample and l=sample length

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The radical expansion is calculated using the following equation

$$Radical\ Expansion = \frac{\text{Extrudate diameter}}{Die\ diameter}$$

Where die diameter =3mm

3. Results and Discussion

3.1 Investigating the Functional Properties of Extracted Alfalfa Leaf Protein Concentrates (Stage 1)

3.1.1 Chemical analysis of extracted leaf proteins

Table 1 indicates the protein content, moisture and colour analysis of the extracted leaf proteins compared to the soy concentrate. From the results, it shows that the WP has a higher protein content (65%) compared to the other green leaf AGLP (48%) and HLP (51%), with a 17% protein increase. The two-heating extraction was able to remove the impurities such as the fibre and increase the protein content. The protein content of the extracted leaf protein was compared with commercial soy protein concentrate. The findings from Table 1 indicate that SPC has a higher protein content (72%) than all leaf proteins. This could be due to the protein is easier to extract as it doesn't have a high quantity of fibre in the composition, therefore the protein content will be higher.

From Table 1 and Figure 1, the colour of the leaf and SPC was also investigated. For the green leaf samples, the HLP had a darker green colour compared to AGLP. This can also be seen in Figure 1 where the HLP is darker than AGLP. A possible suggestion would be that the denaturation of the proteins from heating caused the colour of the sample to be darker. The WP is white in colour, however it is darker than SPC, this could be due to the extraction process which impacts the colour of the sample.

Table 1: Chemical analysis of extracted leaf proteins and soy protein concentrate (control)

Samples	Protein content (%)	Moisture (%)	L*	a *	b *
Soy Protein concentrate (SPC)	72.07±0.58	7.08±0.18	90.01±0.42	0.93±0.03	14.18±0.11
White Protein (WP)	65.14±0.83	7.90±0.06	73.66±0.28	-2.86±0.60	36.26±0.60
Acid Green Leaf Protein (AGLP)	48.41±0.43	5.07±0.09	40.95±0.79	-6.31±0.23	21.81±0.67
Heat Leaf Protein (HLP)	51.67±0.61	3.67±0.40	36.54±0.41	-5.67±0.06	29.93±0.14

3.1.2 Particle size analysis

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Table 2 and 3 represent the data of particle size of the leaf and soy protein in powder and suspended in water. The D [4,3] represents the average particle size in powder or suspended in water. Table 2 shows that SPC has the largest particle size (58.70 µm) compared to all leaf proteins. This finding also correlates to figure 3A where the particle size of SPC shifted horizontally to the right, which resulted in an increase. All extracted leaf samples had a particle size ranged from 24-31µm. which suggests that the extraction process did not impact the particle size of the leaf protein powders.

The powders were then suspended into water making a 4% suspension and the particle size was analyzed and the results are shown in table 3 and figure 3B. The results show that SPC had the largest particle size, even suspended in water (110 µm), the results show that the particle size of the SPC doubled after suspending in water. This is also confirmed in figure 3B where the particle size distribution of SPC shifted horizontally to the right, hence increase in particle size. This finding indicates that the SPC protein particles can absorb and hold water, which suggests high swelling and water holding capacity. Comparing the soy to the extracted leaf protein, all leaf protein samples haves a particle size ranged between 33-43 µm which is 9-12 µm increase after suspending in water. After mixing the leaf powders in water for 2 hours, the large insoluble particles precipitate which makes the system unstable. This finding is opposite to the soy protein where the leaf protein particles can't hold water as much as SPC, hence after hydrating in water the leaf protein particle size did not increase in size largely compared to soy. This may have an impact on functional properties when leaf protein is added to food applications.

Table 2: Particle size analysis of leaf and soy protein (control) powders

Sample Name	**Dx (10) µm**	**Dx (50) µm**	**Dx (90) µm**	**D [4,3] µm**
Soy Protein concentrate (SPC)	15.40±0.11	46.90±0.14	119.00±0.74	58.70±0.25
White Protein (WP)	5.91 ±0.12	18.60±0.25	45.70±0.72	26.35±1.02
Acid Green Leaf Protein (AGLP)	10.10±0.02	23.70±0.06	51.20±0.30	24.60±0.49
Heat Leaf Protein (HLP)	14.30± 0.02	28.10±0.07	53.00±0.30	31.25±0.05

Table 3: Particle size of 4% suspension leaf and soy protein concentrates in water (4% suspension in water)

Sample Name	Dx (10) µm	Dx (50) µm	Dx (90) µm	D [4,3] µm
Soy Protein concentrate (SPC)	22.8±0.47	95.3±2.47	236.0±4.59	110.3±3.64
White Protein (WP)	10.6±0.06	30.6±0.19	66.7±0.82	36.5±0.63
Acid Green Leaf Protein (AGLP)	9.89±0.12	28.7±0.28	65.3±0.67	33.8±0.53
Heat Leaf Protein (HLP)	17.3±0.10	37.8±0.27	74.4±0.45	43.16±0.64

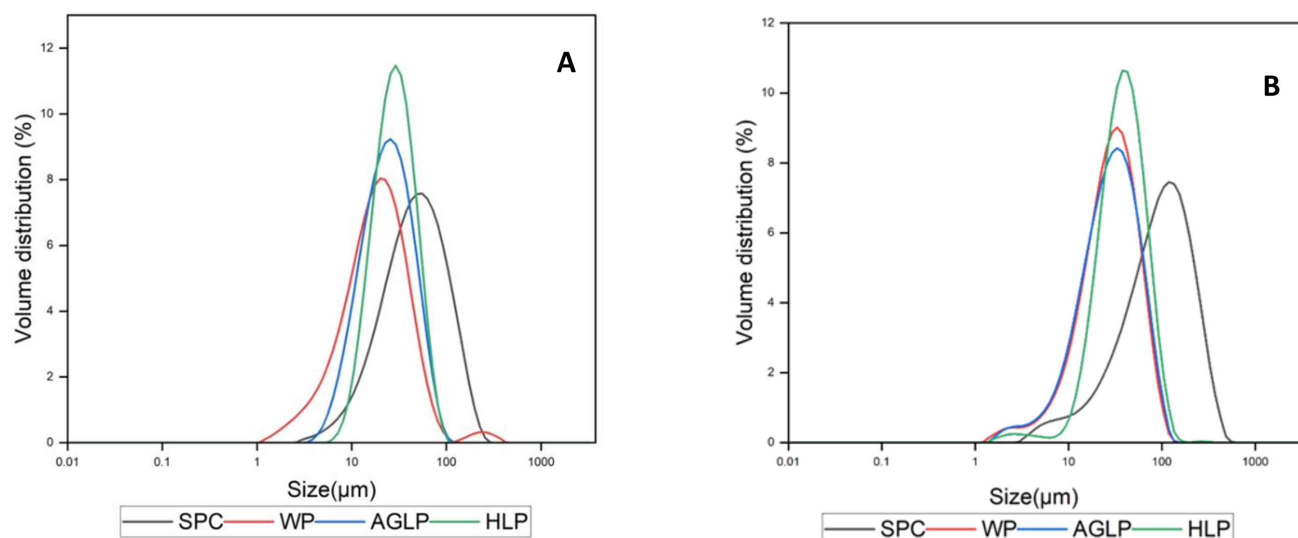

Figure 3: Particle size distribution of extracted leaf and soy protein and soy in powder (A) and in 4% water suspension (B)

The sub particle size results shown in Table 4 indicate that the SPC had the greater particle size (250nm) compared to all leaf proteins (120-160nm). This result also correlates to the particle size results in table 3 where the Dx (10), the 10th percentile of the distribution of the soy protein is higher than all leaf proteins.

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Table 4: Submicron particle size of isolated leaf and soy protein powder in a water suspension

Sample Name	Z. Ave (d.nm)
Soy Protein concentrate (SPC)	250.4±7.82
White Protein (WP)	120.9±5.75
Acid Green Leaf Protein (AGLP)	152.9±7.92
Heat Leaf Protein (HLP)	160.0±4.76

3.1.3 Zeta Potential

Figure 4 shows the zetapotential (surface charge) of the protein system particles of soy and leaf protein across a range of pH. The results show that from pH 6-10, soy protein was more negatively charged (-40 to -50 mV) than all leaf proteins. For the leaf proteins at pH 6-10, WP and AGLP were more negatively charged than HLP. The results indicate that SPC will be more stable in liquid food systems such as suspended in water compared to the leaf proteins. This finding also showed that when leaf protein concentrate is suspended in water, it was not stable, hence large insoluble particles precipitated. The findings also showed that from pH 2-4, SPC had the highest surface charge (35-40 mV), WP was the second highest and the green leaf proteins were the lowest (10 mV). The lowest surface charge for all proteins were at pH 4-4.6. At this point, the proteins are near the isoelectric point, where the proteins have close to zero net charge. Hence being the most unstable pH point.

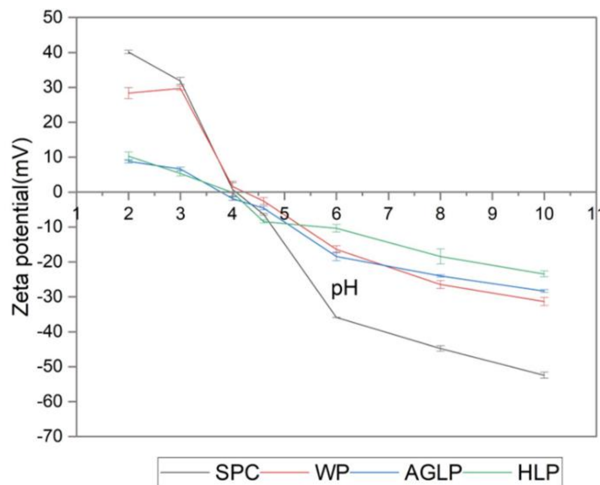

Figure 4: Zetapotential analysis of extracted leaf protein and soy protein over a range of pH (2-10)

3.1.4 Protein solubility

Protein solubility of soy and leaf proteins were investigated at a range of pH (2-10) suspended in water. The results are shown in figure 5. The results showed that SPC had the highest solubility at pH 6-10 where the solubility ranged from ~50-75%. The WP was the second highest in protein solubility (~47-55%) and the green leaf proteins were the lowest in protein solubility. This trend can also be seen from pH 2-3 where SPC had the highest solubility (55-75%) and all leaf proteins were the lowest (10-20%). At pH 4-4.6 the solubility was the lowest for all proteins as this is the isoelectric point of proteins. This result correlates to the zeta potential where at pH 6-10, the SPC high the highest charge which indicated the highest solubility. The green leaf samples had the lowest charge across of pH, hence lowest in solubility. Protein solubility is very important in food applications and from these results, green leaf proteins had the lowest in solubility, hence have an impact in food applications (Lo, Kasapis & Farahnaky, 2021).

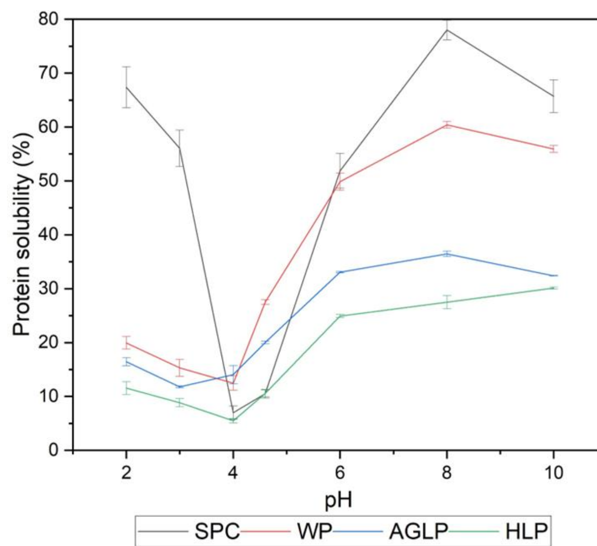

Figure 5: Protein solubility profile of soy and extracted leaf proteins at a range of pH (2-10)

3.1.5 Rheological Properties

Figure 6 represents the RVA profile of the soy and extracted leaf protein powders. The results show the pasting properties of the protein by heating the samples from 25ºC to 95ºC with continuous mixing at 160 rpm, finally cooled back down to 25ºC in an enclosed canister. The results show that before heating and only after mixing, SPC had the highest viscosity (300MPa.s) compared to all leaf samples. This shows that SPC has higher hydrating and gelling properties compared to the leaf proteins. This result can be related to the particle size findings where SPC was able to increase double in size as it is about to absorb water, whereas leaf has poor hydrating properties. At ~45ºC the WP shows an increase in viscosity (~50mMPa.s), and then after cooling back down to 40ºC, the viscosity was the highest (~150MPa.s), however SPC was still higher (~270MPa.s). This result shows heating is required

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to improve the hydrating and gelling properties of the WP. However, for all extracted green leaf proteins viscosity did not increase with or without heating, which suggests poor hydrating and gelling properties.

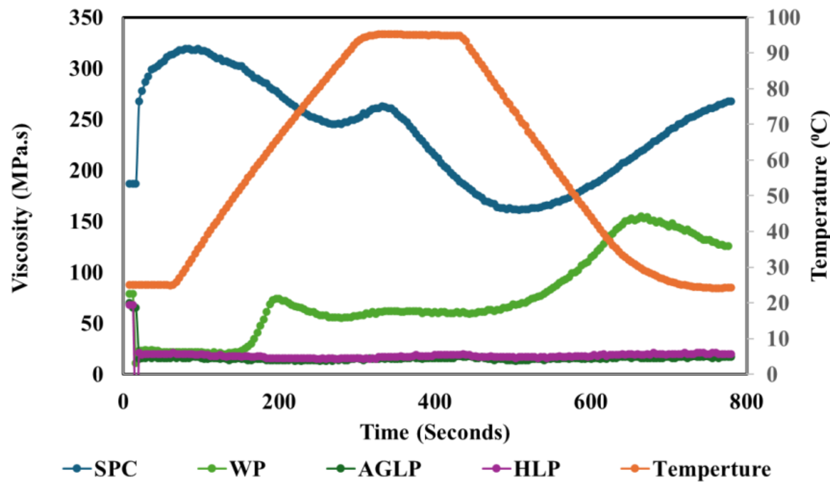

Figure 6: Pasting profile of soy and extract leaf protein heating from 25ºC to 95ºC

Figure 7 shows the results of the storage loss modulus of soy and leaf proteins. This result relates to the gelling and viscosity of proteins which involves slow heating (1ºC per minute) to 95ºC and then cooling back down to 25ºC. The results from figure 7 show that AGLP has the highest storage modulus when the protein is heated to 95ºC, even higher than SPC. When the protein is cooled back down to 25ºC, AGLP and the SPC have the highest storage moduli compared to the other leaf proteins. The results also show that when the protein is cooled down, all leaf proteins have an increase in storage modulus, which shows an increase in gelling properties. This trend can also be seen with the loss modulus where the AGLP had the highest loss modulus compared to SPC. All leaf proteins had an increase in loss modulus after cooling down from 95ºC to 25ºC, which suggests an increase in viscosity. This result shows that the heating and cooling time has an impact on improving the gelling and viscosity of leaf proteins, which can be added to food applications.

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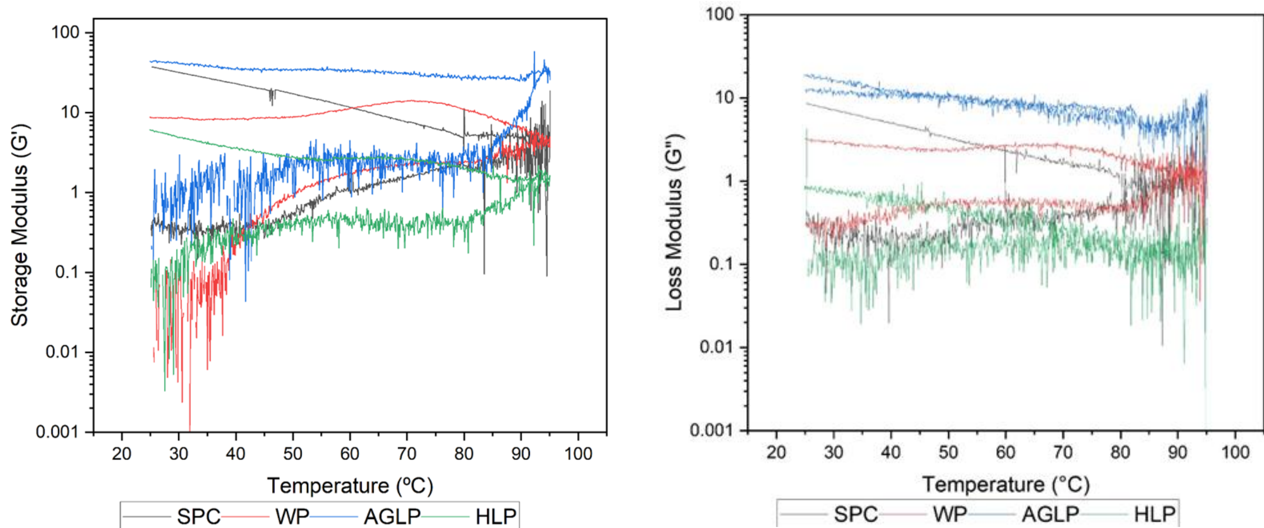

Figure 7: Storage and loss modulus of leaf protein dispersions (4% w/w) at 25-90ºC)

3.1.6 Thermal analysis of proteins

Table 5 shows the results for the thermal analysis of soy and leaf proteins. This analysis investigates the temperature when the proteins denature. The results show that SPC requires a lower temperature (41 ºC) to denature the proteins compared to AGLP (62.8 ºC). WP and HLP did not have a denaturation temperature. This suggests that during the extraction process, WP and HLP were extracted using heat denatured treatment, which denatured affected all the proteins. The temperature and time of heat treatment has an impact on the leaf protein. Based on this finding it is suggested that acid precipitation is the recommended method to maintain the natural protein structure in the leaf protein concentrate.

Table 5: Thermal denaturation temperature of soy and leaf proteins

Sample Name	Denaturation temperature (ºC)
Soy Protein Concentrate (SPC)	41.0
White Protein (WP)	ND
Acid Green Leaf Protein (AGLP)	62.8
Heat Leaf Protein (HLP)	ND

*ND=Not Detected

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3.2 Protein functionality analysis of leaf proteins for food applications (Stage 2)

3.2.1 Chemical analysis of leaf proteins

In this study, alfalfa powder (AP) is used as the control as the protein is extracted from AP. The results show that WLP has the highest protein content (71.58%). The lowest protein content were the green leaf proteins where the protein content ranged from (48-53%). Compared this to the control, the green leaf proteins had a 10.59-15.96% increase, whereas WLP had a 34.12% increase which is almost double the green leaf protein content of alfalfa powders. The results indicate that the white protein (WLP) has a higher protein content than the green leaf proteins (AP, ALP, HHLP and LHLP).

Table 6: Protein content and moisture analysis of leaf proteins of extracted leaf proteins and leaf powder

Sample Name	Denaturation temperature (ºC)
Soy Protein Concentrate (SPC)	41.0
White Protein (WP)	

Samples	Protein content (%)	Moisture (%)
Alfalfa Powder (AP)	37.46	5.50
Acid Leaf Protein (ALP)	48.05	5.38
High Heat Leaf Protein (HHLP)	53.42	4.86
Low Heat Leaf Protein (LHLP)	50.19	5.88
White Leaf Protein (WLP)	71.58	6.81

Sample Name	Denaturation temperature (ºC)
Acid Green Leaf Protein (AGLP)	62.8
Heat Leaf Protein (HLP)	ND

*ND=Not Detected

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3.2.2. Particle Size of Leaf Proteins

Table 7 shows the particle size analysis of the leaf powder and extracted leaf protein in a 4% suspension. The results show that AP has the highest average particle size (183 µm) compared to all extracted leaf proteins. This is due to the large insoluble fibres present in the system. This is shown in the Dx (90) (90 percentile of the distribution) where the particle size is 397 µm. This is also shown in figure 8 where the AP sample in the particle size distribution is shifted horizontally to the right compared to the extracted leaf samples, which suggest an increase in particle size. The extracted green leaf samples all have similar particle size (23-25 µm), regardless of if it is extracted using high and low heat or acid extraction. The WLP has a smaller particle size (19.5 µm) than all extracted leaf protein, especially in the Dx (90) (25 µm) which is 20 µm smaller. This suggests that the dual heat treatment was able to further filtrate the insoluble fibres in the white leaf system, hence reducing the particle size. After suspending all leaf proteins in water, the leaf particles precipitate rapidly, hence the system is not stable.

Table 7: Particle size of alfalfa leaf powder and extracted leaf protein in water suspension (4%)

Sample Name	Dx (10) µm	Dx (50) µm	Dx (90) µm	D [4,3] µm
Alfalfa Powder (AP)	9.97±0.24	105.02±4.05	397.00±39.00	183.00± 14.08
Acid Leaf Protein (ALP)	8.27± 0.93	19.80± 0.40	44.51±1.90	25.80 ±0.11
High Heat Leaf Protein (HHLP)	3.91± 1.89	22.10± 1.41	45.10±1.37	23.30± 0.33
Low Heat Leaf Protein (LHLP)	7.12± 0.56	21.90± 0.32	45.40±1.88	25.10± 1.02
White Leaf Protein (WLP)	4.98± 0.82	17.80± 0.88	25.00±0.86	19.50±0.42

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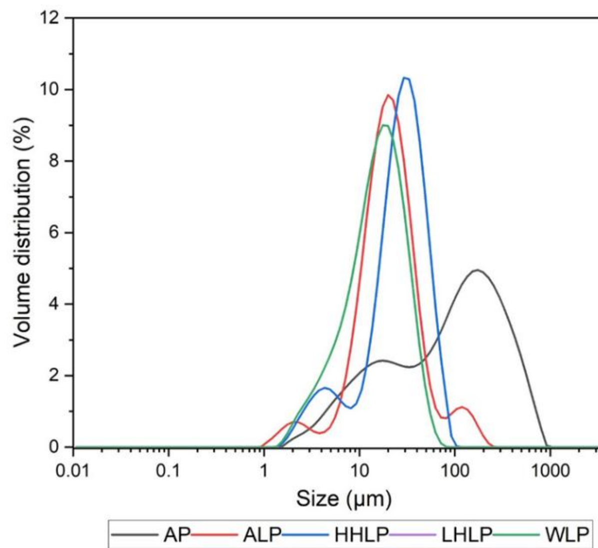

Figure 8: Particle size distribution of leaf powder and extracted leaf protein in suspension (4%)

3.2.3 Zetapotential of Leaf Proteins

Table 8 shows the results of the zetapotential which was measured on all samples at the native pH. The results show that AP, the control, the leaf sample was highly negatively charged (-19.61 mV). However, after extraction (heat or acid), the charge increases significantly (-6 to-8 mV). This range is very close to the isoelectric point where at this point, the protein has poor functional properties (Lo, Kasapis, & Farahnaky, 2022; Lo, Kasapis, & Farahnaky, 2024). This indicates that when the leaf proteins are suspended in water, it precipitates rapidly as the protein particles has poor water solubility properties.

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Table 8: Zetapotential analysis on the native pH of alfalfa leaf powder and extracted leaf proteins.

Samples	Native pH	Zetapotential (mV)
Alfalfa Powder (AP)	6.21± 0.20	-19.61± 1.01
Acid Leaf Protein (ALP)	5.10±0.10	-7.38± 1.61
High Heat Leaf Protein (HHLP)	6.45±0.30	-8.70± 0.48
Low Heat Leaf Protein (LHLP)	5.70±0.30	-7.57±0.84
White Leaf Protein (WLP)	6.13±0.20	-6.48±0.28

3.2.4 Solubility of Leaf Proteins

Figure 9 shows the protein solubility profile of the of leaf powder and extracted leaf protein at a pH range of 3-9. The results show that WLP has the lowest protein solubility across all pH 9 (<18% in protein solubility). This suggests that the dual heating of the extract process significantly reduced the protein solubility properties of WLP dispersed in water. WLP has poorer functional properties than the control (AP), which further suggests that the dual heat extraction may not be recommended as an extraction method of leaf proteins. At pH 3-4, LHLP and has the highest in protein solubility (43-45%) compared to ALP (37-44%) HHLP (24-33% and AP (19-23%). This trend can also be seen at pH 7-9. These results also correlate with the zetapotential where the surface charge of the proteins can influence the protein solubility in water.

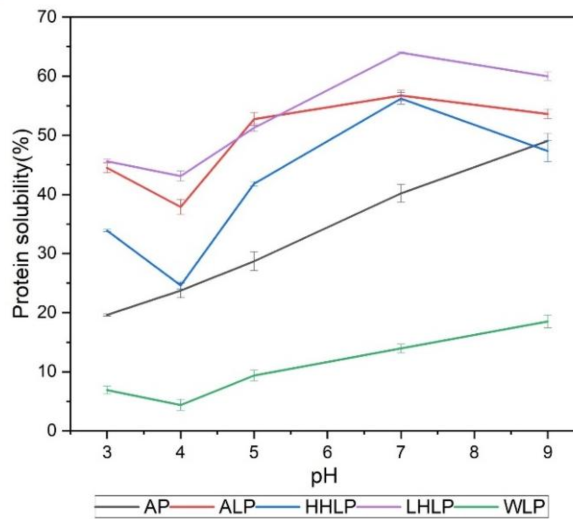

Figure 9: Protein solubility profile of alfalfa leaf powder and extracted leaf proteins at a range of pH (3-9)

3.2.5 Foaming and Emulsification Stability

Figures 10 and 11 shows the foaming stability of the leaf samples after high shear mixing and images were taken after 10 and 24 hours. The results indicated that leaf protein has high foaming properties. After high shear mixing of 10,000rpm for 1 minute, it was found that a high level of foam was formed in the system. Figure 10 shows that after 10 minutes, the foam was still present in the system and after 24 hours (Figure 11), the WLP had the highest level of foam stability in the system compared to the other green leaf proteins. This suggests that WLP has a high functionality of entrapping to air compared to the other green leaf proteins, which resulted in a higher foaming capability. This study also found that after 24 hours, the green samples had a strong fermented odour compared to the white protein. This suggested that after 24 hours, the foaming of the green leaf protein reduced due to the fermentation. This finding was not found in the white protein. This foaming functionally can also be seen in the emulsification results (Figure 13)

Figure 12 and 13 shows the emulsification stability of leaf protein. The results show that after high shear homogenisation at 10,000rpm the emulsion is not a homogeneous system, rather it is a foamed system. This result shows that the oil is trapped inside the foam which influenced the stability of the emulsion. After 24 hours storage at 25ºC±1ºC, the emulsion was separated, however, majority of the oil was still encapsulated inside the foam of the leaf system. This study also found that after the addition of oil into the leaf system, the colour of the leaf protein became a lighter colour, especially the LHLP sample.

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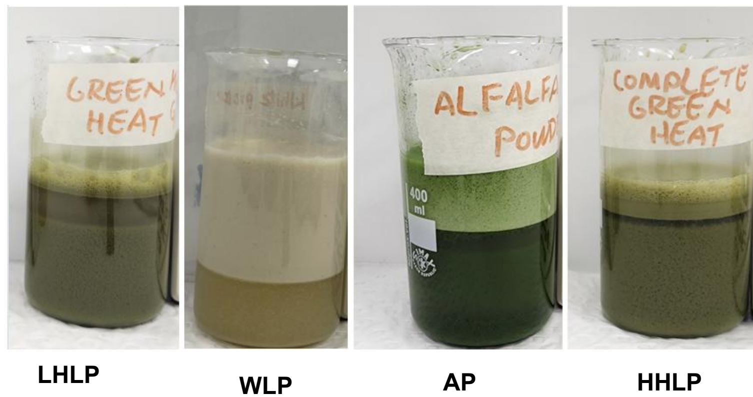

Figure 10: Foaming properties of leaf proteins after 10 mins of high shear mixing (10,000 rpm) at 25ºC±1ºC

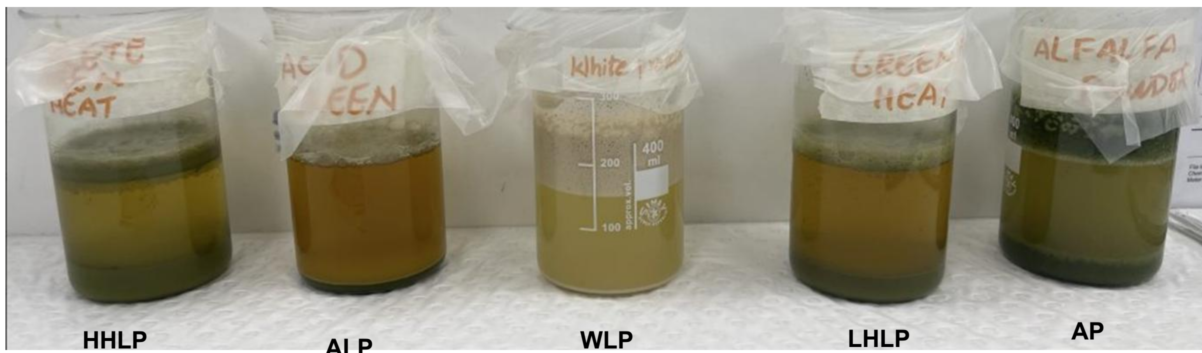

Figure 11: Foaming stability of leaf proteins after 24-hour storage at 25ºC±1ºC of high shear mixing

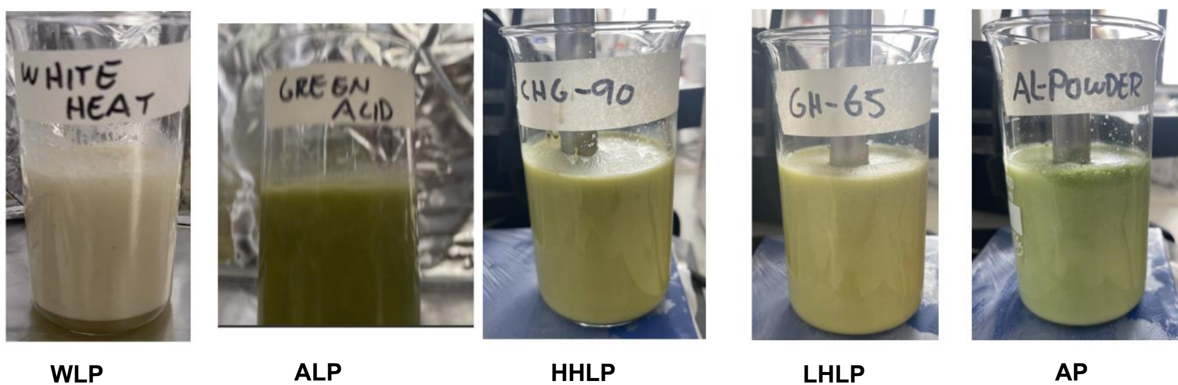

Figure 12: Emulsification stability of leaf proteins in water after 0 mins of high shear mixing

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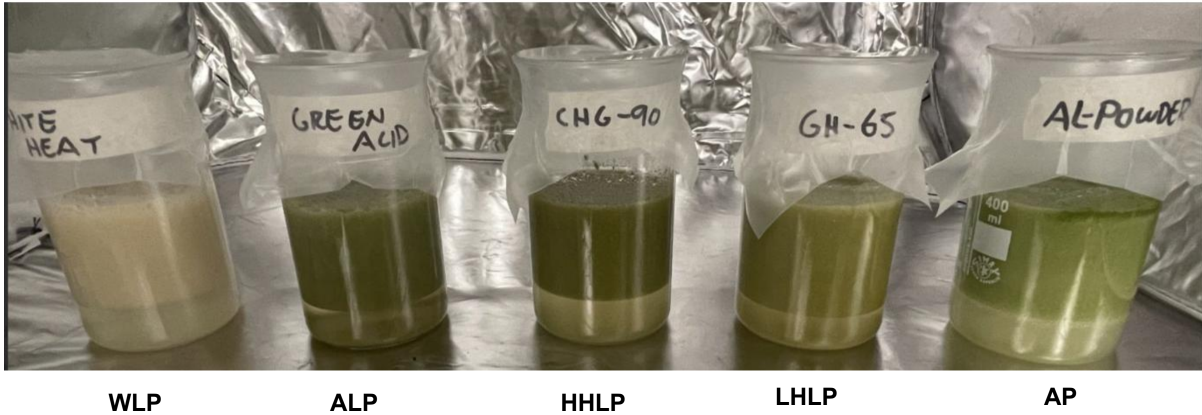

Figure 13: Emulsification stability of leaf proteins after 24 hours of high shear mixing

3.2.6 Rheological Properties of Leaf Proteins

Figure 14 shows the results of the storage loss modulus of leaf proteins dispersed in water (4% suspension). The results show that WLP and AP have the highest storage modulus when the protein is heated to 95ºC. When the system is cooled down to 25ºC, all extracted green leaf proteins have a lower storage modulus than the alfalfa powder and the white protein. This suggests that the heat and acid extracted green leaf proteins didn't improve the gelling properties. The results also show that WLP has the highest gelling properties after cooling down to 25ºC. A possible suggestion for the highest storage modulus for the white protein could be the dual heat process. This means the proteins have been denatured and the heating of the protein system increased the protein aggregation, hence, increased gelling properties. However, for the loss modulus, viscosity properties, the AP has the highest viscosity after the sample was cooled down to 25ºC. It suggests that all leaf extracted proteins do not give high viscosity, which suggests poor water hydration properties.

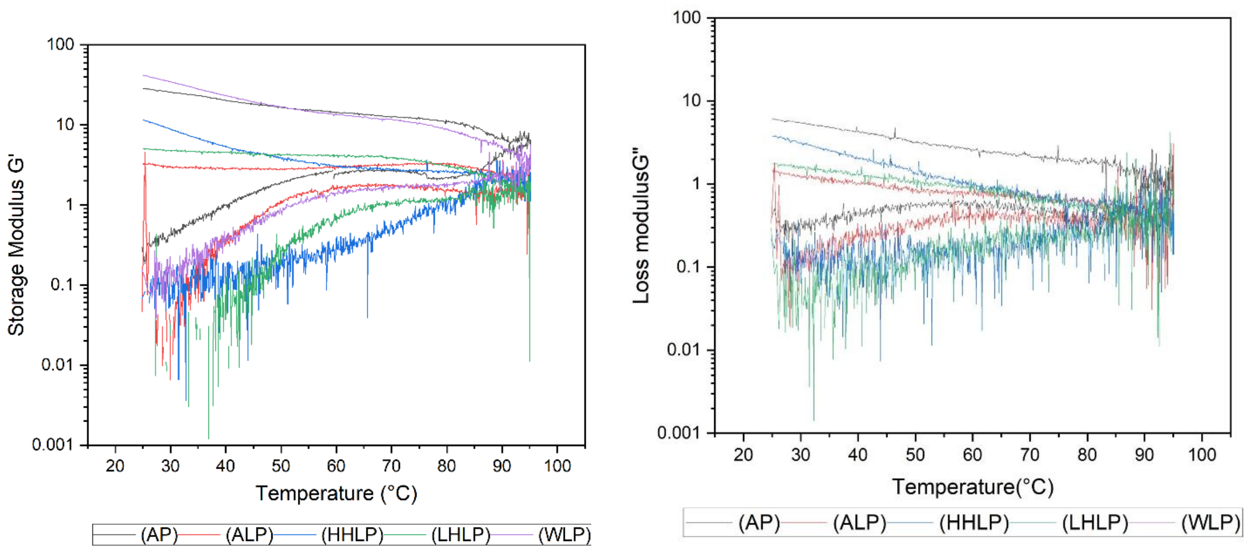

Figure 14: Storage and loss modulus of leaf protein dispersions (4% w/w) at 25-90ºC

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3.2.7 Thermal Properties of Leaf Proteins

Table 9 shows the thermal properties of leaf proteins. The results show that AP and ALP have similar results in terms of denaturation temperature (68ºC). However, ALP requires more energy (0.4 J/g) to denature the proteins compared to AP (0.21J/g). This suggests that due to the higher protein content in ALP, means more energy is required to denature the proteins. This result is different to LHLP. The results show that LHLP requires less energy to denature the proteins (0.11 J/g). A possible reason is the LHLP uses low heat extraction (~65ºC) to extract protein, which suggests that the heat has partially denatured the proteins from the extraction process. Therefore, it doesn't need much energy to denature it further. The results also found that WLP and HHLP did not show any denaturation energy or temperature for these samples. Given that the WLP protein extraction used dual heating at high temperature (95ºC) which is also the same temperature used for HHLP, this extraction procedure denatured all proteins. This finding indicates that high heat extraction damages all the proteins in the system which results in poor functional properties, therefore impacting the use in food applications.

Table 9: Thermal analysis of leaf proteins

Sample Name	Onset (ºC)	Off set (ºC)	Peak temperature (ºC)	J/g
Alfalfa Powder (AP)	59.63	76.08	68.22	0.21
Acid Leaf Protein (ALP)	61.49	77.11	68.83	0.40
High Heat Leaf Protein (HHLP)	ND	ND	ND	ND
Low Heat Leaf Protein (LHLP)	69.31	81.08	74.76	0.11
White Leaf Protein (WLP)	ND	ND	ND	ND

*ND=Not Detected

3.3 Incorporation of Leaf Proteins in High Pressure Homogenisation (Stage 3)

3.3.1 Impact of High-pressure Homogenisation on Leaf Protein in Water System

After the analysis of the leaf protein in stage 2, the HHLP sample was used in food applications. One example of the application used is high pressure homogenisation (HPH) in making leaf beverages. The first step is determining whether HPH can be used in processing leaf proteins after suspending in a 4% suspension (4% is used to represent solids content in beverage systems). The

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protein system is homogenised at 350BAR, and it went under 3 homogenisation cycles. The sample is collected after each pass and the particle size and stability analysis was determined.

Table 10 shows the particle size of the HHLP before and after HPH. The results show that the initial particle size, control was 23.30 µm and after the first homogenisation pass, the particle size reduced to 1.66 µm. This indicates that there was a ~93% reduction in particle size. After the second and third pass, the particle size didn't significantly change. This suggests that only one homogenisation pass would be sufficed in particle size reduction.

Table 10: Particle size before and after HPH with leaf protein concentrates suspended in water.

Sample Name	Dx (10) µm	Dx (50) µm	Dx (90) µm	D [4,3] µm
Control (4% leaf protein concentrate in water-no homogenisation)	3.91 ± 1.89	22.10 ± 1.41	45.10 ±1.37	23.30 ± 0.33
Homogenisation Pass 1	0.10 ± 0.02	1.04 ± 0.65	4.14 ± 0.19	1.66 ± 0.18
Homogenisation Pass 2	0.11 ± 0.01	1.38 ± 0.17	4.20 ± 0.08	1.72 ± 0.08
Homogenisation Pass 3	0.11 ± 0.02	1.76 ± 0.36	4.50 ± 0.25	1.94 ± 0.24

Figure 15 shows the stability of the leaf protein beverage model after HPH. The results show that in the control (no HPH treatment) the particles precipitate very quickly which relates back to the results obtained in table 7. However, after HPH treatment, the stability of the system has improved substantially. The system is very stable even after 4 hours of storage. The system begins to separate after 24 hours of storage, which shows that HPH not only decreases the particle size, but only improves the stability.

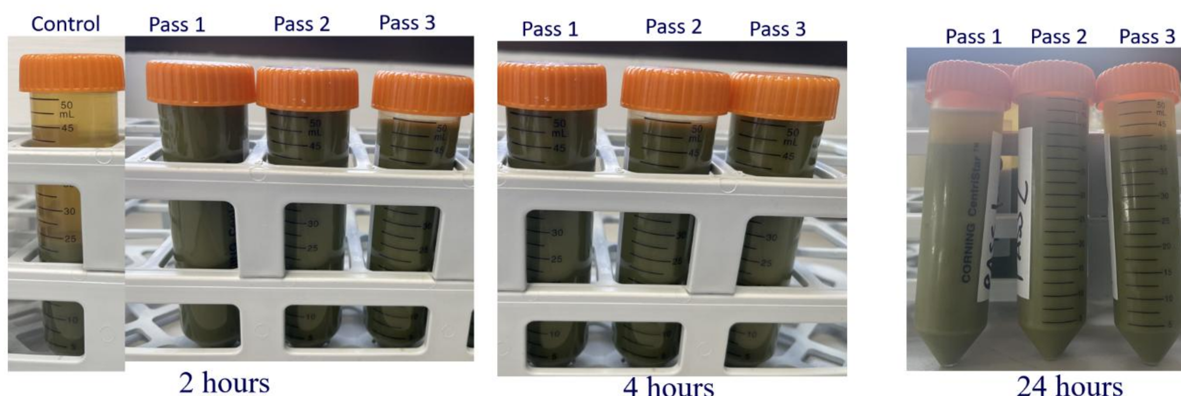

Figure 15: Stability analysis of leaf protein dispersions subjected to high pressure homogenisation on leaf protein

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3.3.2 Impact of High-pressure Homogenisation on Leaf Protein in Beverage Application

Based on the study using leaf protein in water, the next step is to apply leaf protein in a beverage system. In this study, a 4% leaf protein (HHLP) suspension was made in apple juice. Most vegetable juice systems use fruit juice as the main ingredient. Therefore, in this study, leaf protein was suspended in apple juice. The homogenisation condition is the same as the previous study. The particle size of the leaf juice beverage is shown in table 11. The results show that the particle size of the leaf protein mixed with commercial apple juice (control) was 341.50 µm. After the first homogenization pass, the particle size reduced to 28 µm. The second and third homogenization pass reduced it to 8 and 5 µm respectively. This study suggests that mixing the apple juice with leaf protein increased in particle size compared to adding water. HPH was able to reduce the particle size, but not as small as when leaf protein is mixed with water. A possible solution would be to homogenise the apple juice before mixing it with the leaf protein. Also, another study would be to investigate the impact of pH on the mixing with leaf proteins. Given that apple juice is acidic, it may impact on how it solublises the leaf protein.

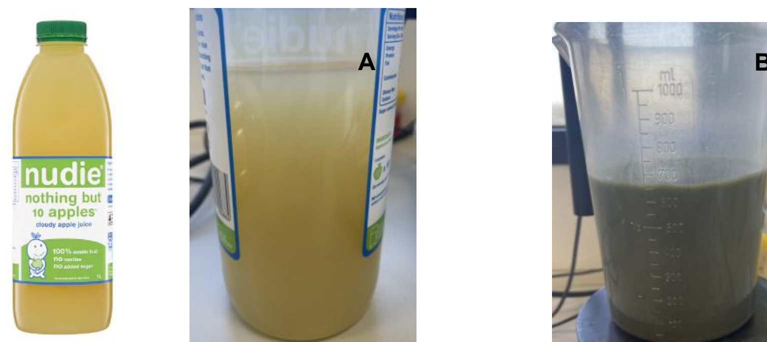

Figure 16: Commercial apple juice (Nudie apple juice) (A) and leaf protein suspended in apple juice (i.e., mixture of apple juice and leaf protein concentrate) (B)

Table 11: Particle size before and after HPH with leaf suspended in apple juice

Sample Name	Dx (10) µm	Dx (50) µm	Dx (90) µm	D [4,3] µm
Control (4% leaf protein in apple juice)	42.30 ± 1.70	225.50 ± 26.16	809.00 ± 54.15	341.50 ± 58.69
Apple+leaf protein (Pass 1)	10.80	25.80	49.10	28.00
Apple+leaf protein (Pass 2)	2.39	4.40	8.38	8.29
Apple+leaf protein (Pass 3)	2.33	4.25	7.77	5.75

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3.4 Incorporation of Leaf Proteins in Extrusion Applications (Stage 4)

3.4.1 Creating a corn flour-based matrix by extrusion processing

In this study, leaf protein was used in extrusion to make puffed snacks. However, before leaf can be added into the system, a physical matrix needs to be determined. Table 12 shows the ingredients to make a corn flour-based matrix. Corn flour is chosen as most of the commercial ingredients are corn based. Maltodextrin is used as a filler in the puffed product and calcium carbonate helps with the puffing of the product. The impact of changing the moisture added into the extruder is shown in figure 17. By changing the moisture of the product, has an impact on the product. The results show that, low moisture added increases the puffing of the product. Based on this preliminary study, the matrix using corn has been achieved. The next step is to incorporate leaf protein into the system.

Table 12: Ingredients used to make a corn-based matrix

Ingredients	%
Corn Flour	94.00%
Maltodextrin (DE18)	5.00%
Calcium carbonate	0.50%
Salt (NaCl)	0.50%

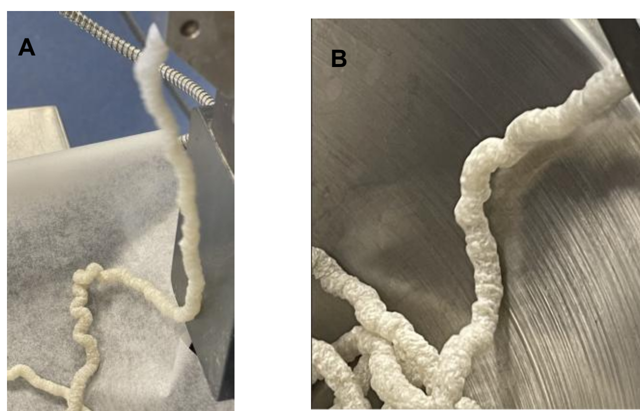

Figure 17: Corn-based extrudates coming out of the extruder. High moisture added (A) and low moisture added into the extruder (B)

3.4.2 Incorporation of Leaf Protein with the Corn-based Matrix Extrusion

Table 13 shows the formation of a 5% leaf puffed product extrudate. This study shows that leaf protein can be extruded and that 5% added can be used to make a puffed product. Figure 18 shows the leaf protein puffed product and the impact of moisture added into the extruder. The results show that by decreasing the water added into the extruder, the extrudate becomes lighter and the product becomes more puffed. Analysis such as texture (hardness), bulk density and radical expansion was analysed on the 3 products outlined in figure 18. Table 14 and 15 shows the results for bulk density, radical expansion, texture analysis and moisture content of the leaf extrudate compared to the corn-based matrix. The results show that by reducing the moisture added to the extruder, the bulk density decreases. The results also show that the addition of the leaf protein could improve the expansion of the product. This also decreased the hardness of the product compared to the control. Decreasing the moisture added, the hardness of the product also decreased. Which suggests that the product becomes more brittle, which is an important property in puffed products.

Table 13: Addition of 5% leaf protein powder (HHLP) incorporated with the corn flour matrix

Ingredients	%
Corn Flour	89.00%
Maltodextrin (DE18)	5.00%
Leaf protein powder (HHLP)	5.00%
Calcium carbonate	0.50%
Salt	0.50%

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High moisture

Medium moisture

Low moisture

Figure 18: The impact of moisture added to 5% leaf protein corn-based matrix

Table 14: Bulk density and radical expansion of leaf protein extrudates

Sample Name	Extrudate Diameter (Sample) (m)	Die Diameter (m)	Radical Expansion (Extrudate diameter/Die Diameter)	Length (l) (m)	Volume of Extrudate $\pi x(d^2/4)xl$	Mass of Extrudate (kg)	Bulk Density (Mass of extrudate) /(Volume of extrudate) (kg/m^3)
Control	0.00601	0.003	**2.00**	0.05020	0.000001	0.00136	**959.29**
Leaf 1 (High Moisture)	0.00713	0.003	**2.37**	0.03164	0.000001	0.00099	**780.57**
Leaf 2 (Medium Moisture)	0.00715	0.003	**2.38**	0.05875	0.000002	0.00149	**631.12**
Leaf 3 (Low Moisture)	0.00736	0.003	**2.45**	0.05009	0.000002	0.00136	**638.83**

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Table 15: Texture and moisture analysis of leaf protein extrudates

Sample Name	Hardness (N)	Moisture Content (%)
Control	290.03	3.38
Leaf 1 (High Moisture)	185.73	3.97
Leaf 2 (Medium Moisture)	111.89	4.57
Leaf 3 (Low Moisture)	97.41	4.50

3.4.3 Upscaling and Reproducibility Using a Large Pilot Scale Extruder at CSIRO

After the extrusion trials conducted at RMIT, the next step was to reproduce and upscale the extrusion processing. Two formulations were made for the extrusion at CSIRO. The extruder at CSIRO was also a twin screw extruder which can produce up to 4kg/hour of product. The screw was 30mm in size, double the one at RMIT. The barrel temperature of the extruder ranged from 30ºC to 150ºC (die temperature). The 5% leaf protein formulation was first applied in the extruder. The formulation used in the extruder did not produce a puffed product, instead it produced a viscous paste (figure 19). This also caused the extruder to continuously blocked. A possible reason for the blockage of the extruder is due to the small particle size of the leaf and corn flour in the formulation.

The next trial was mixing the 10% leaf formulation with 1kg corn grits to form a 5% leaf protein formulation (figure 20). In this trial, corn grits were used to stablise the extruder. The puffed product is shown in before feeding the 5% leaf powder with corn grits into the extruder (figure 21A). The leaf extruded leaf protein mix with corn grits is shown in figure 21B. The product is not puffed, and it is flexible and rubbery. After 5 minutes of extrusion, the extruder became unstable, and blockage started to occur. The product that came out had a higher level of puffing but there was no texture in the product and there was burning on the surface of the product. This study showed that the low moisture formulation that was used from the RMIT extruder could not be upscaled using a high moisture formulation system. This needs to be further investigated on how the formation that was used on a smaller extruder can be upscaled. The other factor is the extrusion parameters needs to be investigated such as screw size and the heating temperatures also needs to be investigated.

Overall, this upscaling trial did not produce a puffed product but provided valuable information and further investigation needs to be carried out before it can be taken into industry production.

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Table 16 A shows 5% leaf protein used at the upscaling extrusion at CSIRO

Ingredients	%
Corn Flour	89.00%
Maltodextrin (DE18)	5.00%
Leaf protein powder (HHLP)	5.00%
Calcium carbonate	0.50%
Salt	0.50%

Table 17 B shows 5% leaf protein used at the upscaling extrusion at CSIRO

Ingredients	%
Corn Flour	84.00%
Maltodextrin (DE18)	5.00%
Leaf protein powder (HHLP)	10.00%
Calcium carbonate	0.50%
Salt	0.50%

Figure 19: 5% Leaf protein product coming out of the extruder

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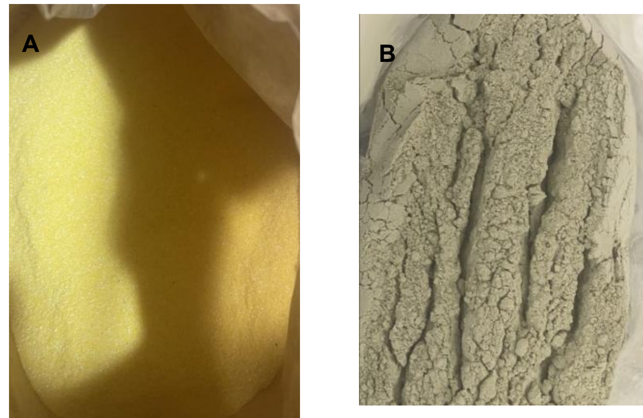

Figure 20: Corn grits (A) used to improve the formulation and leaf protein mixed with corn grits (B)

Figure 21: Extruded corn grits (A) and extruded leaf protein mixed with corn grits with high moisture (B) and after extruder was blocked (C)

4. Conclusion

In this project, there were 4 stages. The first stage investigated the functional properties of extracted alfalfa leaf proteins. This extraction process provided the preliminary findings on how leaf protein production conditions impact extracted proteins from leaves. The functional properties that were investigated were, particle size, zeta potential, protein solubility, rheology and thermal properties. The findings from this study shows that the leaf proteins were not functional, especially the gelling and protein solubility

properties. This indicates that the extraction process needs to be further investigated if leaf protein with high physical functionality is required.

The second stage of the project investigated the functional properties of extracted alfalfa leaf proteins for food applications. In this study, leaf protein was extracted in a pilot scale and the functional properties were determined. The same functional properties from stage 1 were analysed with the investigation of additional functional properties such as emulsification and foaming. The results showed that the green leaf proteins had a higher functionality (e.g. protein solubility) compared to the white proteins, which is important in food applications. This study also provided the foundation for using leaf proteins in food applications. Stage 3 and 4 of the project investigated how leaf proteins can be applied into food applications. High pressure homogenisation was chosen to provide relevant information for making leaf protein beverages. HPH showed that it has the capability to reduce the particle size and improve the stability of the product, regardless if it was mixed with water or a commercial fruit juice. Increased passes didn't have an impact when the leaf was mixed with food, however when mixed with apple juice the particle size can be further decreased. However, further studies can be conducted on how the acidity of the solution (e.g. apple juice) can impact leaf protein stability, also how it can impact the HPH treatment. Leaf protein was also incorporated with extrusion to make puffed products. A corn based formulation was made to set the physical matrix of the product. Once the matrix has been produced, leaf protein was incorporated. The trial showed that a 5% leaf protein can be added into the extruded and a puffed product can be produced. However, this prototype could be further improved by altering the barrel temperature, powder feed rate and moisture added into the system. Also, the composition of the formulation can also be altered in the prototype. Once a prototype was a made, upscaling and reproducibility of the product was investigated using a larger extruder at CSIRO. This study showed that upscaling using a high moisture system was a challenge as the prototype made at RMIT (low moisture based) could not be reproduced. The other challenge was the extruder was different, larger extruder, which resulted in changes with the formulation was fed into the extruder. This provides another research question on how to upscale the extrusion processing to produce large scale products. This project can help industry to improve on their extraction process and how the extracted leaf protein be used in food applications. Furthermore, the outcomes will also help the company to expand on their operations with producing new products using leaf proteins.

5. Impact and Ongoing Monitoring

This project helps to confirm the use of leaf protein as a functional food ingredient. As such, waste leafy vegetable feedstock can become a valuable input source for leaf protein ingredients to contribute to food waste reduction. One of the promising uses of food waste feedstock for leaf protein are contract grown "standing - waste" herbs, normally grown to excess for retail distribution. As a next step, The Leaf Protein Co. will work with local growers to convert the waste herbs into leaf protein ingredients for the prototype finished products.

We are working with local contract growers to setup a supply schedule which will help determine how much of the waste herbs is used to produce leaf protein ingredients

6. Acknowledgements

We would like to thank and acknowledge Dr Danyang Ying, Dr Jordan Pennells and Dr Regine Stockmann from CSIRO for their time and organizing us to use their extruder The work has been supported by End Food Waste

RMIT Classification: Trusted

Cooperative Research Centre whose activities are funded by the Australian Government's Cooperative Research Centre Program. This is EFW CRC Document 20**/***"

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