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# Harnessing the potential of green leaves

## Agricultural biomass as a source of sustainable food protein

Anna-Lovisa Nynäs

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Cover: A broccoli leaf fractionated into valuable puzzle pieces
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# Harnessing the potential of green leaves


## Abstract

Demand for sustainable protein-rich food sources is currently increasing to meet the nutritional requirements of a growing population, while also considering climate challenges. Green leafy biomass (GLBM), in the form of side-streams and main crops, is a widely available protein source with potential food value. Extended use of GLBM, *e.g*. through a biorefinery process targeting leaf protein concentrates (LPCs), could add direct values, *e.g*. economic revenues from side-stream valorisation, or indirect values, *e.g*. reduced greenhouse gas emissions from higher resource utilisation.

In this thesis, several types of GLBM were successfully subjected to an extraction protocol targeting water-soluble proteins, although the outcomes, *e.g.* yield, differed significantly between GLBM types. The major protein component in LPC was the enzyme RuBisCO. A pre-feasibility assessment revealed insufficient recovery rates on upscaling the process. To achieve economic viability, further process development is needed and additional compounds and products should be targeted.

The use of LPCs in food applications is of interest due to their nutritional aspects, *i.e*. high protein content and good amino acid profile. Another area of interest is their potential as a functional ingredient, *e.g*. their foam stabilising ability, which was demonstrated for LPCs from several GLBM types in this thesis. Air-water interfacial properties, which can serve as an indicator of foam stabilising capacity, did not differ significantly between LPCs from the GLBM types evaluated. Further, no major differences in interfacial properties were observed between the LPCs and egg white.

Green leafy biomass can be viewed as a valuable resource with great potential and extending the use of GLBM through LPC production could contribute to a more sustainable food production system.



Keywords: Green leafy biomass, protein fractionation, leaf protein concentrate, side-stream valorisation, protein foam stabilisation.



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# Grön bladbiomassa – en hållbar proteinkälla

## Sammanfattning

Efterfrågan på hållbara proteinkällor ökar till följd av en växande befolkning i kombination med allt tydligare klimatutmaningar. Grön bladbiomassa (*green leafy biomass*, GLBM) har potential som en lättillgänglig proteinkälla, både i form av sidoströmmar och som huvudgrödor. Ett ökat nyttjande av GLBM, t.ex. genom utvinning av bladproteinkoncentrat (*leaf protein concentrates,* LPC), kan tillföra direkta värden, såsom ekonomiska intäkter från onyttjade sidoströmmar, eller indirekta värden i form av minskade växthusgasutsläpp i.o.m. en högre nyttjandegrad av investerade resurser.

Inom ramen för denna avhandling har proteiner framgångsrikt utvunnits från flera olika sorters GLBM, genom en utvinningsprocess inriktad på vattenlösliga proteiner. Det huvudsakliga proteinet i LPC:erna var RuBisCO. En genomförbarhets-bedömning visade dock tydligt att utvinningsgraderna av LPC i en sådan uppskalad process var otillräckliga. För att ekonomisk lönsamhet ska kunna uppnås krävs därför ytterligare processutveckling och fler säljbara slutprodukter.

Tack vare det höga näringsvärdet, d.v.s. högt proteininnehåll och god aminosyraprofil, är det lämpligt att använda LPC i olika livsmedelstillämpningar. Därutöver har LPC en stor potential som en funktionell ingrediens, eftersom de kan användas till att stabilisera t.ex. skum, vilket kunde påvisas för LPC från flera olika sorters grödor. Koncentratens ytstabiliserande egenskaper – en indikator på deras skumstabiliserande förmåga – skiljde sig inte mellan LPC från olika sorters GLBM. Inga betydande skillnader kunde heller konstateras mellan de olika LPC:erna och äggvita.

Grön bladbiomassa bör ses som en värdefull resurs med stor potential. Ökad nyttjandegrad av GLBM, t.ex. genom utvinning av LPC, skulle kunna bidra till ett mer hållbart livsmedelssystem.

Nyckelord: Grön bladbiomassa, proteinutvinning, bladproteinkoncentrat, sidoströmsvalorisering, skumstabilisering av proteiner.

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# Contents

# List of publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I. Berndtsson, E., Nynäs, A. L., Newson, W. R., Langton, M., Andersson, R., Johansson, E., & Olsson, M. E. (2019). The underutilised side streams of broccoli and kale — valorisation via proteins and phenols. In *Sustainable Governance and Management of Food Systems: Ethical Perspectives*, Wageningen Academic Publishers, pp. 74-81.

II. Nynäs, A. L., Newson, W. R., & Johansson, E. (2021). Protein Fractionation of green leaves as an underutilized food source — protein yield and the effect of process parameters. *Foods*, 10(11), 2533.

III. Nynäs, A.L., Newson, W. R., Langton, M., Wouters, A., & Johansson, E. (2022). Leaf protein concentrates at the air-water interface and concentrate properties at food-relevant pH. *(manuscript)*

IV. Prade, T., Muneer, F., Berndtsson, E., Nynäs, A. L., Svensson, S. E., Newson, W. R., & Johansson, E. (2021). Protein fractionation of broccoli (*Brassica oleracea*, var *Italica*) and kale (*Brassica oleracea*, var. *Sabellica*) leaves — a pre-feasibility assessment and evaluation of fraction phenol and fibre content. *Food and Bioproducts Processing*, 130, 229-243.

The contribution of Anna-Lovisa Nynäs to the papers included in this thesis were as follows:

I. Gathered reference articles and wrote the manuscript together with EB, with input from the co-authors on the final version.

II. Designed the study together with the co-authors. Performed all data collection and data analysis. Wrote the final version of the manuscript together with the co-authors.

III. Designed the study together with the co-authors. Performed most of the data collection and all data analysis. Wrote the final version of the manuscript together with the co-authors.

IV. Performed data collection regarding protein extraction yields and fraction analysis. Contributed to the final manuscript.

# Abbreviations

| | |
|---|---|
| BJ | Brown juice |
| CPF | Combined protein fraction |
| DM | Dry matter |
| GAE | Gallic acid equivalents |
| GHG | Greenhouse gas |
| GJ | Green juice |
| GLBM | Green leafy biomass |
| GPF | Green protein fraction |
| LPC | Leaf protein concentrate |
| LS | Large subunit |
| N | Nitrogen |
| P | Pellet |
| pI | Isoelectric point |
| RuBisCO | Ribulose-1,5-bisphosphate carboxylase/oxygenase |
| S | Supernatant |
| SDS | Sodium dodecyl sulphate |
| SDS-PAGE | SDS-polyacrylamide gel electrophoresis |
| SE-HPLC | Size exclusion-high performance liquid chromatography |
| SS | Small subunit |
| WJ | White juice |
| WPC | White protein concentrate |

# 1. Introduction

With the increasing demand for food due to a growing global population, combined with the environmental pressures caused by the current food production systems, it is essential to develop sustainable food production practices. A commonly presented way of mitigating these challenges is to shift from consumption of animal proteins to plant proteins, while another route is to reduce the amount of under-exploited biomass in the agricultural system (Springmann *et al.* 2018). Using green leafy biomass (GLBM), especially biomass types not currently utilised as food, as a protein source has the potential to form part of both these mitigation routes.

The work presented in this thesis focused mainly on upcycling green leafy harvest residues, such as broccoli and sugarbeet leaves, but the scope extended to crops today used as animal feed, *e.g*. lucerne, as there is potential in extending their utilisation. Use of GLBM as a source of protein for food was explored from four different angles: i) benefits from an environmental/sustainability perspective (Paper I), ii) extraction of proteins from different types of GLBM (Paper II), iii) utility of the proteins in food applications, *e.g*. as foam stabilisers (Paper III), and iv) the economic feasibility of extracting water-soluble leaf proteins (Paper IV).

# 2. Background

## 2.1 Reduced waste of resources by wasting less produce

Food production is one of the most resource-demanding activities globally in terms of use of energy, farmland, fertilisers and water (Westhoek *et al.* 2016). It also has significant environmental impacts in the form of deforestation, acidification, greenhouse gas emissions, eutrophication, soil erosion and biodiversity loss (Figure 1). In order to feed a global population of 9.8 billion people, as expected by 2050 (United Nations 2019), more food will have to be produced, making the demand for resources even greater (FAO 2018). The food production system is currently one of the most significant sources of anthropogenic greenhouse gas emissions, contributing an estimated one-third of total global emissions (Crippa *et al.* 2021; Xu *et al.* 2021). Intensification of food production, if not done sustainably, will aggravate these environmental impacts.

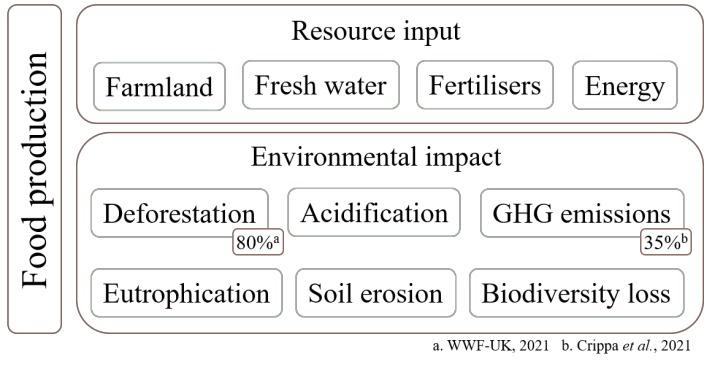


*Figure 1*. Resource inputs required and environmental impacts of food production. The percentages shown indicate share of the total anthropological impact of food production. GHG: Greenhouse gas.

Adding to the large resource demand, food production is a sector where up to one-third of the produce is lost along the value chain (Meybeck *et al.* 2011). This corresponds to 1200 million tonnes of wasted food at farm level each year, or 2.2 Gt of $CO_2$ equivalents, which is 16% of all agricultural greenhouse gas emissions (WWF-UK 2021). Another important aspect of food waste is the lost nutrition, with 25% of all calories produced ending up in waste streams (Kummu *et al.* 2012). Production of these wasted calories in turn requires approximately one-quarter of the total resources invested in food production (Figure 2). By reducing the amount of food waste at all levels in the food supply chain, a more sustainable food system would be achieved (Bajželj *et al.* 2020).

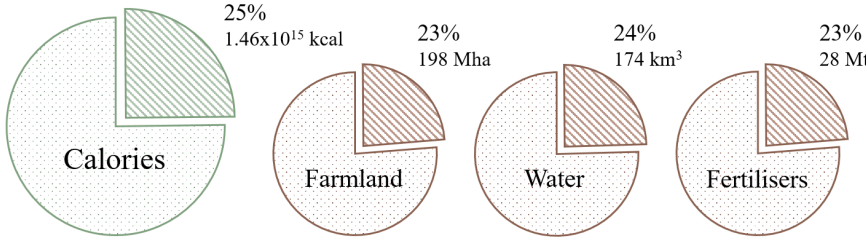


*Figure 2.* Amount of wasted calories in food produced today and amounts of resources wasted in producing these calories. Based on data from Kummu *et al*. (2012).

All biomass produced in the field requires considerable amounts of resources (see Figure 1). However, agricultural side-streams, such as leaves, are seldom included in food waste estimates. One example is broccoli (*Brassica oleracea* var. *italica*) leaves, which are not considered as food in Sweden and are ploughed back into the soil after the broccoli florets have been harvested (Figure 3). Another example of a wasted leafy side-stream is leaves of kale (*B. oleracea* var. *sabellica*) that are not harvested, mainly due to cosmetic reasons (Figure 4). Leafy biomass types used mainly as animal feed, such as ley grasses and legumes (*e.g*. lucerne (*Medicago sativa*) and clovers (*Trifolium* species)), are also not included in waste estimates, even though they could be exploited more efficiently.

Extended utilisation of all actual agricultural produce, including leafy side-streams and other leafy biomass, could mitigate the environmental impact of the food system even further if combined with a general reduction in food waste.

*Figure 3.* Residues left in the field after harvest of broccoli heads.

*Figure 4.* Different harvest stages of a kale field. Far left: All harvest residues have been ploughed down. Left centre: Harvest residues of kale before being ploughed down. Right: Kale plants awaiting harvest.

## 2.2 Green leafy biomass as a resource and raw material

Green leaves are one of the most widely available types of agricultural biomass globally in terms of both main crops and side-stream materials. Their high abundance makes GLBM interesting as a resource in the food industry, for example as a raw material in a fractionation process. Such a biorefinery provides great potential for increasing the value of many GLBM types, as they could be utilised as a source of protein and other valuable compounds (Muneer *et al.* 2021; Møller *et al.* 2021). Many leafy side-streams are highly nutritious, *e.g.* broccoli leaves (Berndtsson *et al.* 2020), and contain high levels of protein, phenolic compounds and dietary fibre, all of which are potentially valuable products.

The interest in using GLBM more extensively does not originate solely from the potential value of sellable compounds extracted from the biomass. A large factor is the sustainability aspects related to using agricultural produce more efficiently. Using GLBM as a raw material for protein fractionation, followed by sequential anaerobic digestion to produce biogas and biofertilisers, would provide environmental savings (Parajuli *et al.* 2018), and several environmental services are provided by the cultivation of *e.g.* ley grasses and legumes (Martin *et al.* 2020).

For many agricultural crops, leafy harvest residues comprise a substantial part of total crop biomass. For example, in the case of broccoli, only a small part of the plant biomass is actually harvested, as illustrated in Figure 3, while the remaining parts are left in the field (Liu et al. 2018; Berndtsson 2020). It is difficult to estimate the amount of leafy harvest residues, *i.e.* any part of the plant not harvested and left on the field, as studies on the matter are scarce. In the case of broccoli, different studies suggest that 36% (Berndtsson 2020) or 47% (Liu et al. 2018) of total plant biomass consists of leaves, with the corresponding proportion for the broccoli heads, *i.e.* the main product, being 15% or 30%, respectively. According to these estimates, approximately 4100 tonnes of broccoli leaves are generated every year in Sweden (Table 1). Sugarbeet and beetroot (two cultivars of *Beta vulgaris*) are other examples of crops with large amounts of residual leaves (see Table 1) that could be utilised in a better way. For sugarbeet, the leaves are estimated to constitute 20-34% of total plant biomass (Tamayo Tenorio 2017), and a similar range can be assumed for beetroot. Other agricultural crops resulting in large amounts of leafy harvest residues are carrot (*Daucus carota*), kale and cabbage (*B. oleracea* var. *capitata*).

*Table 1.* Examples of green leafy biomass (GLBM) types available in Sweden. Based on data from Jordbruksverket (2022). Average values for 2018-2020 unless otherwise indicated.

| | **Cultivated area** *(ha)* | **Main crop** *(t/ha)* | **Leaves as % of total biomass** [b] | **GLBM** *(t/ha)* | **Total GLBM** *(t)* |
|---|---|---|---|---|---|
| Sugarbeet | 29 750 [a] | 66 | 20 | 16 | 480 000 |
| Broccoli | 343 | 9.0 | 40 | 12 | 4 100 |
| Beetroot | 496 [a] | 37 | 20 | 9.2 | 4 600 |
| Ley | 837 700 [a] | 4.7 | 100 | 4.7 | 4 000 000 |

[a] Value for 2020 only. [b] Estimated values. For broccoli, 30% of the total biomass was assumed to be the main produce, 40% leaves and 30% stems.

Even though considerable amounts of biomass are available in the form of leafy harvest residues, a significantly larger proportion of cultivated GLBM is in the form of ley (Jordbruksverket 2022), with a total harvest of 4 million tonnes per year in Sweden (Table 1). Ley is commonly a mixture of different perennial grasses (*e.g.* ryegrass (*Lolium multiflorum* L.)), and legumes (*e.g.* lucerne and clovers) and the harvested biomass is today mainly used as animal feed. Depending on the plant mixture, ley can be grown on most kinds of soil, even on marginal land (Carlsson *et al.* 2017), and in most climate zones. Inclusion of perennial ley in a crop rotation provides ecosystem services, *e.g.* in the form of soil organic carbon sequestration (Brady *et al.* 2021) and has been shown to sustain cereal yields in a changing climate (Marini *et al.* 2020).

Other significant sources of GLBM are cover crops and catch crops, which in 2016 were grown on 8% of arable land in the European Union (EUROSTAT 2020) and in 2018 were grown on 70 000 ha in Sweden (Asplund & Svensson 2018). Cover crops are included in crop rotations to maintain a green cover on the fields between the main crops. In this role, they contribute to many environmental services, such as reduced leakage of fertilisers and pesticides to the surroundings, weed control, decreased soil erosion and carbon sequestration in soil (EUROSTAT 2020). Cover crops can be inserted into existing crop rotation systems without interfering with the main crops. Some examples of cover crops are buckwheat (e.g. *Fagopyrum esculentum*), Persian clover (*Trifolium resupinatum*), oil radish (*Raphanus sativus*) and phacelia (*Phacelia tanacetifolia*) (Hansson *et al.* 2021), and cereals can also be used as cover crops.

Different GLBM types have been tested as raw material in various protein fractionation processes. The two most common types reported in the literature are lucerne (De Fremery *et al.* 1973; Miller *et al.* 1975; Wang &

Kinsella 1976; Fiorentini & Galoppini 1981; Hood *et al.* 1981; Koschuh *et al.* 2004; Lamsal *et al.* 2007; Hojilla-Evangelista *et al.* 2016; Santamaria-Fernandez *et al.* 2017; Nissen *et al.* 2021), and sugarbeet (Merodio & Sabater 1987; Jwanny *et al.* 1993; Kiskini *et al.* 2016; Tamayo Tenorio *et al.* 2016; Martin *et al.* 2018). Other GLBM sources used include spinach (*Spinacia oleracea*) (Merodio *et al.* 1983; Barbeau & Kinsella 1986), duckweed (*Lemma gibba*) (Nieuwland *et al.* 2021), Jerusalem artichoke (*Helianthus tuberosus)* (Kaszás *et al.* 2020), white and red clover (*Trifolium repens*, *T. pratense*) (Santamaria-Fernandez *et al.* 2017; Amer *et al.* 2020; Stødkilde *et al.* 2021), oilseed radish (Santamaria-Fernandez *et al.* 2017), escarole lettuce (*Cichorium endivia)* (Ducrocq *et al.* 2022), cauliflower (*B. oleracea* var. *botrytis*), cabbage, broccoli, and beetroot (Sedlar *et al.* 2021), to mention but a few examples. The wide range of GLBM types studied illustrates the wide interest in using GLBM as a raw material and also the great versatility within this group of biomass types.

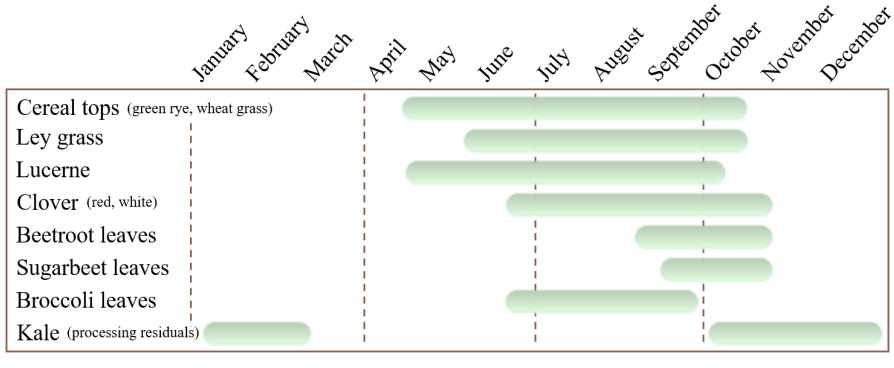


*Figure 5.* Estimated availability of different types of green leafy biomass in southern Sweden through the year.

Having access to a large variety of different GLBM types would be beneficial when using it in a biorefinery approach, as it would prolong the processing season. The availability of GLBM through the year is heavily dependent on the season, with most GLBM types available during late spring, summer and early autumn (Figure 5). The main limitation on the processing season is the perishability of fresh green leaves, which will deteriorate if not stored cooled or frozen. As such storage is costly (Tamayo Tenorio *et al.* 2017), GLBM should be processed as soon as possible after harvest.

## 2.3 Proteins in green leaves

The concept of using green leaves as a protein source first emerged in the 1940s (Pirie 1942) and has received increasing attention during the past few decades. Fresh green leaves contain ~1-3% protein, corresponding to approximately 10-30% protein on a dry matter basis (Paper II), and large variation can be expected between GLBM types. The proteins can be roughly divided into a *white* fraction consisting of water-soluble proteins and a *green* fraction consisting of insoluble proteins.

The main protein in the white protein fraction is the enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO), which plays a major role in photosynthesis and is found in all photosynthetic organisms. In many plants, up to 50% of the soluble protein is RuBisCO (Patel & Berry 2008). In duckweed the content is even higher, with ~50% of the total protein being RuBisCO (Nieuwland *et al.* 2021). The essential carbon fixating role of the enzyme, in combination with rather low efficiency, makes RuBisCO the most abundant protein in the world (Ellis 1979).

All photosynthetic organisms have some version of the enzyme RuBisCO. In higher plants, the most common type is a hexadecameric protein with eight large subunits (LS) of 50-55 kDa and eight small subunits (SS) of 12-18 kDa (Andersson & Backlund 2008). The subunits connect with each other through non-covalent interactions, which are interrupted in the presence of disruptive agents, *e.g.* sodium dodecyl sulphate (SDS), causing disassembly of the subunits. Due to this, in many protein analyses, the subunits are separated and appear as two different bands in SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and two different peaks in size exclusion-high performance liquid chromatography (SE-HPLC) in the presence of such agents.

RuBisCO is a highly abundant protein found all over the world. Leaf protein concentrates (LPCs), in which the protein is enriched, have several other properties adding to the potential value. These properties include a nutritionally good amino acid profile, high solubility and promising properties in food applications, *e.g.* as foam stabilisers, emulsifiers and gelling agents (Hood *et al.* 1981; Martin *et al.* 2014; Hojilla-Evangelista *et al.* 2016; Nieuwland *et al.* 2021; Ducrocq *et al.* 2022). The term LPC is defined in this thesis as concentrates consisting mainly of the water-soluble proteins in the leaf, *i.e.* the *white* protein fraction, where RuBisCO is the major constituent, but not the only one. In many studies, the term LPC

includes both the *white* and *green* protein fraction, but such concentrates are here referred to as the *combined protein fraction* (CPF).

## 2.4 Leaf protein extraction

The protein content in fresh leaves is relatively low (~1-3%) (Papers II and IV) in comparison with the content of fibre (~4-6%)(Paper IV). This makes it difficult to meet the nutritional protein requirement of monogastric animals, including humans, with a diet consisting predominantly of leaves (Møller *et al.* 2021). In order to utilise the nutritional potential of leaf proteins, an extraction process is necessary to increase the protein content and decrease the fibre content. Another consequence of the relatively low protein content in fresh leaves is the large amount of raw material required for producing LPCs. A generalised version of an LPC extraction process is described in the following sections and depicted in Figure 6.

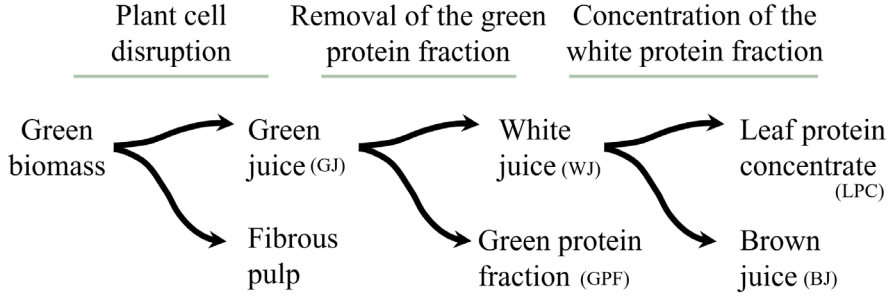


*Figure 6.* The general protein extraction process used in this thesis.

### 2.4.1 Plant cell disruption releases water-soluble protein

RuBisCO, the main protein in the white protein fraction, is a water-soluble protein located inside the chloroplasts (Ellis 1979). Hence, the first step in a leaf protein extraction process is disruption of the plant cells to release the intracellular liquid which, when combined with the intercellular liquid, is called the green juice (GJ). Cell disruption can be achieved by screw pressing, where GJ is pressed from the leaves, leaving a fibrous pulp containing most of the solid matter. The GJ contains both the green and the white protein fractions and all the soluble compounds present in the leaf, including chlorophyll, as well as cell debris and other insoluble components.

### 2.4.2 Removal of the green protein fraction

The white and green proteins precipitate at different temperatures. The green proteins coagulate at temperatures around 50-55 °C, while RuBisCO is more thermally stable and denatures at temperatures around 61°C (Nieuwland *et al.* 2021). This difference can be exploited for separation of the protein fractions. In a process aiming to produce LPC with only the soluble proteins, the green protein fraction (GPF) is removed from the GJ. Gentle heating of the GJ at temperatures around 50-55 °C causes coagulation of the GPF, while RuBisCO (and many other proteins) remain soluble (Merodio *et al.* 1983; Lamsal *et al.* 2003; Martin *et al.* 2014; Tamayo Tenorio *et al.* 2016; Nieuwland *et al.* 2021). The coagulum can be removed by centrifugation or filtration, and the white protein fraction, including RuBisCO, is found in the clarified liquid, in this thesis called the white juice (WJ).

### 2.4.3 Concentrating the white protein fraction

Once the green protein fraction has been removed, the remaining proteins in the WJ are further concentrated to obtain an LPC (see Figure 6). Methods used for this include heat, isoelectric precipitation or different filtration techniques. When isoelectric precipitation is chosen, the pH is adjusted with acid to 3.5-4.5 (Lamsal *et al.* 2007; Nissen *et al.* 2021). When concentrating the proteins by heating, a temperature of 80 °C can be used (Edwards *et al.* 1975). In both cases, the LPC can be separated from the brown juice (BJ) by centrifugation. Acid-precipitated protein can be redissolved by neutralising the pH, which may improve the functional properties of the resulting LPC (Lamsal *et al.* 2007). Ultrafiltration or diafiltration are additional process steps in which contaminants and undesired compounds can be removed to achieve higher purity of the LPC (Ducrocq *et al.* 2022), which may also improve the functionality.

When targeting a protein concentrate consisting of both the green and white protein fractions, *i.e.* the combined protein fraction (CPF), a similar approach can be applied. If heating is used to concentrate the proteins in the GJ, higher temperatures (80 °C, 95 °C) are applied (Koschuh *et al.* 2004; Kaszás *et al.* 2020), which causes precipitation of both protein fractions. Isoelectric precipitation can also be applied, and the decrease in pH can be achieved by addition of acid or by microbial fermentation (Santamaria-Fernandez *et al.* 2017).

## 2.5 Leaf proteins in food

### 2.5.1 LPC in food applications

Proteins in LPCs, in common with many other proteins, have the ability to stabilise foams and emulsions, a property that is of great importance in many food products, *e.g.* meringues. In this thesis, only the foam stabilising ability of the LPC is investigated in more detail, but other researchers have reported promising results for LPCs from various GLBM sources as emulsifiers and gelling agents (*e.g.* Nieuwland *et al.* 2021, Sheen *et al.* 1991, Knuckles & Kohler 1982).

### 2.5.2 Foam stabilisation by proteins

Foams consist of air bubbles dispersed in a continuous water phase. To enable formation of a foam the air bubbles need to be stabilised by some form of surface active agent, preventing them from immediate coalescence and/or disruption (Damodaran 2005). Proteins are amphiphilic molecules, *i.e.* they have both hydrophobic and hydrophilic regions, making them highly surface-active (Dickinson 1999).

Proteins stabilise foams by diffusing to and adsorbing at the air-water interface, which is the first stage in formation of an interface stabilising protein layer (Zayas 1997)(Figure 7). At the air-water interface, the hydrophobic regions of the protein are oriented away from the water phase. The proteins assemble into a viscoelastic film as non-covalent interactions are formed, and continued adsorption of proteins at the interface results in the formation of multilayers. The properties of the resulting interfacial protein film are determined by the protein-protein and protein-interface interactions, which in turn are dictated by the capability of the proteins for diffusion to and adsorption at the interface and by their propensity for forming interactions (Zayas 1997).

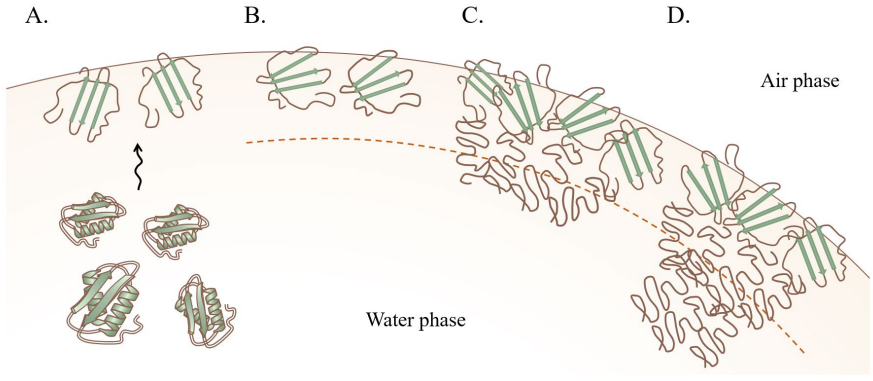


*Figure 7*. Different stages in foam stabilisation by proteins. A: Protein diffusion to and adsorption at the air-water interface. B: Reorientation and conformational change of the protein to direct hydrophobic regions towards the air phase. C: Development of protein-protein interactions and formation of a second protein layer. D: Formation of multilayers.

### 2.5.3 Understanding protein interfacial behaviour

When proteins, or other surface-active agents, adsorb to the air-water interface, the surface tension (γ) is reduced (Damodaran 2005). Measuring γ for a newly formed interface over time, *e.g.* by using an optical tensiometry technique such as the pendant drop method, can provide insights into the properties of a protein solution in terms of diffusion rate of the constituents to the interface and rate of adsorption at the interface. At the interface, the proteins form a viscoelastic film, the properties of which can be assessed by dilatational surface rheology measurements (Wierenga & Gruppen 2010), *e.g.* using the oscillating pendant drop method.

## 2.6 Evaluating the economic feasibility of GLBM fractionation to produce LPC

Before investing large amounts of time and capital in an upscaled industrial process, such as fractionation of GLBM to produce LPC, an economic pre-feasibility study is advisable. Such a study should assess the economic viability of the process by estimating the costs linked to the process and possible revenues from the products (Bals & Dale 2011; Johansson *et al.* 2015; Muneer *et al.* 2021). The estimates on revenues and costs can be based

on, *e.g.* literature values or market analyses of similar products and for comparable processes.

To estimate the total costs of a process with sufficient accuracy, the costs of all individual operations need to be included, considering the requirements for equipment, energy and labour. In the case of LPC production from GLBM, this includes harvesting GLBM (and possibly also cultivation), transport to the processing facility, all processing steps and treatment of the end-products (Muneer *et al.* 2021). Potential revenues from the products can be difficult to estimate, as there are no directly comparable products on the market (or, for the case considered in this thesis, available today in regular supermarkets in Sweden). Hence, estimates of possible revenues have to be based on comparisons with similar products.

Besides giving insights into the economic viability of a process, an economic feasibility study can identify valuable aspects regarding lack of knowledge or necessary process development. The economic model used for the feasibility study can also be applied in sensitivity analysis to test the effect of changing different parameters, such as the process size required to reach viability, or to compare different process pathways (Bals & Dale 2011; Muneer *et al.* 2021). The assessment can also provide clues as to the overall sustainability of the process, as an economically costly process may be associated with high environmental impact, *e.g.* high energy requirements.

# 3. Thesis objectives

The overall objective of the work presented in this thesis was originally to assess how to utilise green leafy biomass as a source of food protein and to investigate how these proteins could be used in food applications. During the initial research, questions emerged regarding the different values linked to viewing green leaves as a resource. With these questions included, the overall aim of the work expanded to broadening the knowledge and understanding of using green leaves as a food source.

The individual papers (I-IV) on which this thesis is based each contributed to achieving the overall aim. Specific objectives in Paper I-IV were as follows:

I. Review and discuss the ethical aspects of utilising broccoli and kale side-streams more extensively.
II. Explore the use of nine different types of green leafy biomass in a protein fractionation process targeting water-soluble proteins and establish a basis for an upscaled process with cues to enable further process development.
III. Investigate the air-water interfacial behaviour of leaf protein concentrates from six different biomass types, and assess the solubility and aggregation behaviour of the concentrates at food-relevant pH values.
IV. Assess the economic feasibility of upscaling fractionation of broccoli and kale leaf residues, and evaluate the use of the resulting protein, fibre, and phenolic compounds in food and feed products.

# 4. Methods

In this chapter, the methods used in the work are briefly described. For more detailed descriptions, the reader is referred to the *Material and methods* sections in Papers II, III and IV.

## 4.1 Green leafy biomass

Green leafy biomass (GLBM) of nine different crops was included in the study described in Paper II (Table 2). GLBM of six of these crops was further studied in Paper III, while only kale and broccoli were investigated in Paper IV. The biomass was collected soon before or after harvest of the main crop, except for mangold (*B. vulgaris* subsp. *vulgaris* var. *cicla)*, lucerne and spinach. The collected mangold leaves were over-mature and considered unfit as food, mainly due to cosmetic reasons. Lucerne was collected in late spring, at the time of the first cut, while spinach was purchased from a supermarket. In all studies, frozen and thawed leaves were used.

Table 2. *Green leafy biomass sources included in the studies described in Papers II, III, and IV.*

| **Leaf source** | **Latin name** | **Included in Paper** |
|---|---|---|
| Beetroot | *Beta vulgaris,* subsp. *vulgaris,* var. *Red hawk* | II, III |
| Broccoli | *Brassica oleracea,* var. *italica* | II, IV |
| Cabbage | *Brassica oleracea,* var. *capitata* | II |
| Kale | *Brassica oleracea,* var. *sabellica* | II, III, IV |
| Mangold | *Beta vulgaris,* subsp. *vulgaris,* var. *cicla* | II, III |
| Sugarbeet | *Beta vulgaris,* subsp. *vulgaris,* var. *Lombok* | II, III |
| Carrot | *Daucus carota* subsp. *sativus* | II |
| Lucerne | *Medicago sativa* | II, III |
| Spinach | *Spinacia oleracea* | II, III |

## 4.2 Protein extraction

The complete leaf protein extraction protocol, with all the different processing steps, is presented in Figure 8. The first step of the extraction was to break the plant cells to release the intracellular liquid. For this, a kitchen model twin-screw press was used, and the leaves were fractioned into a green juice (GJ) and a fibrous pulp.

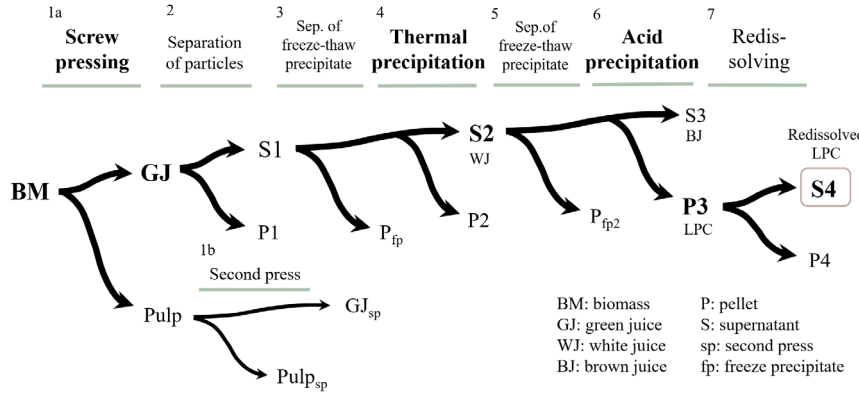


*Figure 8.* The leaf protein concentrate (LPC) extraction process used in Paper II, with the main process steps in bold font.

In order to remove the green colour and some other undesired compounds (cell debris and other insoluble compounds), the GJ was heated gently to ~55°C. This heating caused coagulation of the *green* protein fraction (GPF) and particles, and the resulting coagulated GPF was removed by centrifugation. The supernatant contains the *white* proteins and is referred to as the *white* juice (WJ), regardless of its actual colour. In Paper II, the most suitable temperature to use in this thermal treatment was examined, with the aim of finding a suitable temperature where as much of the green protein as possible, but as little of RuBisCO as possible, was removed. This was assessed by heating aliquots of GJ to different temperatures and measuring the protein concentration and composition in the supernatant.

To concentrate the proteins in the WJ, isoelectric precipitation was applied. The most suitable pH for acid precipitation was assessed by recording the size of the aggregate particles at different pH values, which was done using an autotitration unit coupled with a dynamic light scattering instrument, where the particle size was measured at pH intervals of 0.5 units. A pH of 4.5 was then used for the isoelectric precipitation, and the

precipitated white proteins were separated by centrifugation, resulting in a pellet rich in protein and a supernatant, named the brown juice (BJ), which was low in protein.

The pellet was dispersed in water and the white protein was redissolved as the pH was neutralised. The proteins which did not dissolve were removed by centrifugation and the supernatant was lyophilised in order to obtain a dry leaf protein concentrate (LPC), also referred to as white protein concentrate (WPC) in Paper IV. In Paper III the acid-precipitated pellet was washed twice to remove impurities (sugars, salt, *etc*.) before redissolving.

In Paper II, the mass balances (wet mass, dry matter (DM), nitrogen (N)) for three replicate extractions were recorded. This included some extra process steps: i) Centrifugation of the first GJ to assess the amount of particles, ii) centrifugation of the frozen and thawed particle-free GJ to assess the amount of freeze-thaw precipitate and to remove any precipitated protein prior to the thermal treatment, and iii) centrifugation of thawed WJ to remove any freeze-thaw precipitate prior to isoelectric precipitation.

## 4.3 Compositional analyses

Dry matter (DM) content was determined by recording the mass of a sample before and after drying. Nitrogen (N) content was analysed using the Dumas method. In Paper IV, a conversion factor of 5.8 was used to calculate the protein content, while a conversion factor of 6.25 was used in Paper III. A bicinchoninic acid assay was applied to the liquid supernatant samples from the thermal treatment test described above to determine the protein concentration. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was used for assessing the protein composition. The content of free and bound phenolic compounds and the content of dietary fibre were determined as described in Paper IV.

## 4.4 Effect of pH on LPC behaviour

The solubility of LPCs from six different GLBM (Table 2) at three food-relevant pH values (7.0, 5.0, 3.0), under both non-reducing and reducing conditions was determined in Paper III using size exclusion-high performance liquid chromatography (SE-HPLC). The LPC aggregation behaviour at different pH values and the total particle charge (zeta potential)

were studied using an autotitration system coupled with a dynamic light scattering instrument measuring the particle size and charge.

## 4.5 Interfacial behaviour

In Paper III, a preliminary foaming test with LPC mixed with water was performed using graduated cylinders and a kitchen model milk frother. To study the ability of the LPC constituents to diffuse to and adsorb at the air-water interface optical tensiometry was used. In those experiments, the shape of a pendant drop was recorded over time and based on the shape the surface tension ($\gamma$) was monitored. The viscoelastic nature of the protein film at the air-water interface was calculated by recording $\gamma$ while changing the volume of the pendant drop in an oscillating manner (Paper III).

## 4.6 Economic feasibility assessment

A cost-benefit analysis was performed for an industrial scale fractionation of broccoli and kale field residues (Paper IV). The model included all necessary machinery operations in the field, transport, storage and processing for a theoretical process. As a basis for the model, the amount of available field residues from broccoli and kale production was estimated, and samples of harvest residues were fractionated using the protein extraction process described in section 4.2. The content of protein, dietary fibre and free and bound phenolic compounds was also determined for each fraction, and rates of these were calculated.

Three different processing pathways with an assumed processing capacity of 100 t/h were evaluated in the cost-benefit analysis: (i) dried and milled biomass, (ii) production of a green protein fraction (GPF) and a LPC (referred to as a white protein concentrate (WPC) in Paper IV), and (iii) a combined protein fraction (CPF) consisting of co-recovered green and white protein fractions. The final products were (i) a fine powder intended as a protein-rich niche health product, (ii) a further purified LPC powder with a protein content of 85% to be sold as a high-value food ingredient, (ii and iii) dried and milled green protein powder (GPF and CPF, respectively) intended as high-protein horse feed additive, ensiled pulp to be used as feed for ruminants, and brown juice (BJ) that could potentially be used as a biogas substrate.

# 5. Research outcomes

## 5.1 The value of agricultural green leafy biomass

The primary aim of the work described in this thesis was to enable the use of green leafy biomass (GLBM) as a source of food protein. During the course of the research, ethical questions (Paper I) and economic questions (Paper IV) emerged concerning the usefulness of GLBM and the potential value in extending its use.

### 5.1.1 Under-utilised produce representing wasted resources

Production of agricultural GLBM requires large inputs of resources (farmland, energy, fertilisers, water), while also giving rise to large environmental impacts (*e.g.* greenhouse gas emissions, soil erosion, deforestation) (see Figure 1). This is the case for any GLBM, whether in the form of side-stream material from cultivation of *e.g.* broccoli, kale or sugarbeet, or in the form of perennial ley grasses or cover crops. Despite the large demand for resources for production, the potential of extending the use of many GLBM types has not been exploited at all, or at least not fully, leading to a waste of the resources used for their production, and to a less sustainable food system (Paper I).

### 5.1.2 An ignored waste…

Under-utilised GLBM is a neglected resource, but one could also argue that under-utilisation in itself is neglected when discussing the environmental impacts of the food system (Paper I). Side-stream biomass types, such as those from broccoli and kale cultivation, are not considered food waste. Hence their impact is not included in claims by *e.g.* IPCC that reduced food

waste is one of the least controversial actions for making the food system more sustainable (Mbow *et al.* 2019). It has been estimated that a 50% reduction in food waste could alleviate the environmental pressure by 6-16%, and a 75% reduction would alleviate it by 9-24% (Springmann *et al.* 2018). The total mitigation effect would be higher if these side-streams and other GLBM types was to be included.

### 5.1.3 …with riches to be revealed

Green leafy biomass contains high levels of potentially valuable and obtainable constituents, *e.g.* dietary fibre, phenolic compounds and protein (Table 3). By applying a biorefinery approach targeting such compounds, valorisation of GLBM was achieved (Papers I, II and IV). If GLBM were to be recognised as a raw material for a biorefinery process, economic value would be added to side-stream biomass from *e.g.* broccoli cultivation, a biomass type currently left in the field and used as a green fertiliser (Papers I and IV). Extending the use of GLBM originally grown as feed for cattle could also add value. Fractions of higher monetary value could be extracted from the biomass before using the residuals (*i.e.* fibrous pulp) as feed (Paper IV; Damborg *et al.* 2019). In this solution, the original application is not hampered, while the total value of the GLBM is increased substantially.

Table 3. *Protein, phenolics and fibre content (dry matter (DM) basis) of some leafy green biomass types. GAE: Gallic acid equivalents.*

| | **Protein** *Nx5.8 (%)* | **Phenolic compounds** *(mg GAE/g DM / $Fe^{2+}$µmol/gDM)* | **Dietary fibre** *(%)* | |
|---|---|---|---|---|
| Broccoli leaves | 12 | 8.2 / 108 | 35 | Paper IV |
| Kale leaves | 15 | 7.7 / 88 | 41 | Paper IV |
| Sugarbeet leaves | 17 | n.d. | n.d. | Paper II |
| Lucerne | 16 | n.d. | n.d. | Paper II |

Valorisation of under-utilised GLBM can do more than increase profitability for agriculture, *e.g.* the associated increase in productivity reduces the required resource input and lessens the environmental pressures caused by the food production system (Eriksson *et al.* 2021). Cover crops and ley grasses are GLBM types that also have the capability to provide environmental and ecosystem services in the form of soil carbon sequestration, prevented soil erosion, reduced leakage of fertilisers and pesticides from the fields, preserved and promoted biodiversity, and

enhanced weed control (Carlsson *et al.* 2017; EUROSTAT 2020; Chen *et al.* 2022). Extended utilisation through fractionation of such GLBM types would hence contribute to both higher profitability and more sustainable food production. It has also been suggested that fractionation of cover crops, where fractions of lower value are used for production of biogas and biofertilisers, would reduce the total greenhouse gas emissions by 2.5-fold compared with using the biomass directly as green fertilisers (Hansson *et al.* 2021). However, total removal of GLBM from the fields can impair soil carbon sequestration, as organic matter plays an important role in the microbial processes binding carbon to the soil (Witzgall *et al.* 2021). Finding a good balance when considering all sustainability aspects is essential in extended use of GLBM.

## 5.2 Proteins from green leafy biomass – a sustainable option

As suggested in the previous section, GLBM is a widely available raw material and extended utilisation through fractionation would be beneficial for the sustainability of the food production system. In the research described in Papers II-IV, the focus was on soluble proteins from GLBM. Thus, the discussion below centres primarily on some properties of LPC from GLBM and how they could be produced.

### 5.2.1 Leaf protein concentrates

*LPCs can be extracted from a wide range of GLBM*

In Papers II-IV in this thesis, LPCs (see examples in Figure 9) were successfully produced from seven different GLBM types using the protocol presented in Figure 8. These GLBM types included forms currently regarded as harvest residues (leaves from beetroot, broccoli, and sugarbeet, rejected kale and mangold) and some main crops (spinach, lucerne). In addition, several other ley and cover crops (*e.g.* oil radish leaves, crimson clover (*Trifolium incarnatum*), phacelia) were found to be successful substrates for the fractionation process (unpublished results). However, the proposed protein extraction protocol did not work for all biomass types tested in Paper II, with no protein recovered from carrot and cabbage leaves.

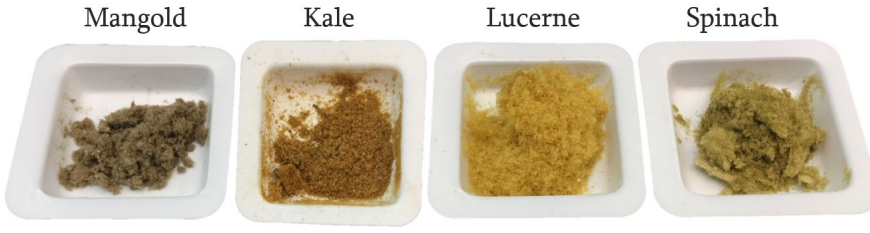


*Figure 9.* Lyophilised leaf protein concentrates from mangold, kale, lucerne and spinach.

*LPC composition and nutritional value*

The LPCs obtained in Papers II and III were light yellow to dark brown in colour (Figure 9) and had a protein composition dominated by RuBisCO (Figure 10). RuBisCO was not the only protein in the concentrates, as indicated by other protein bands detected in the SDS-PAGE analysis, but isolating pure RuBisCO was not the intention; the target for the extraction process was the full white protein fraction, *i.e.* all soluble proteins in the GLBM.

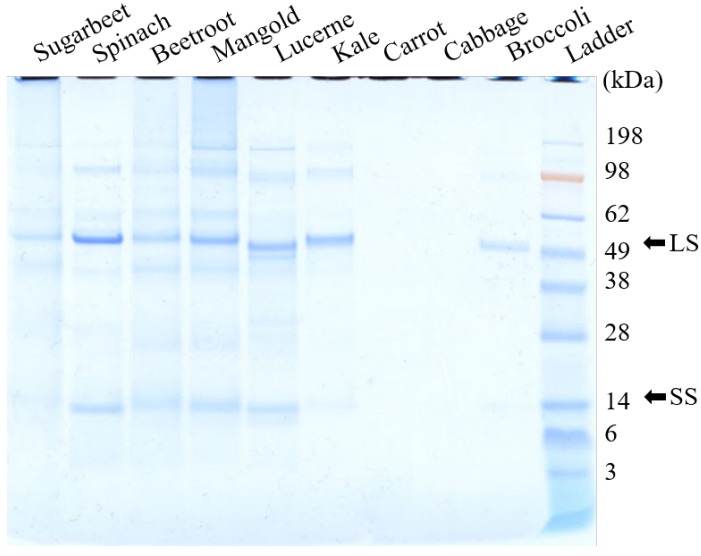


*Figure 10.* Leaf protein concentrates from different green leafy biomass types, as analysed using SDS-PAGE. The arrows indicate large and small subunits (LS and SS) of RuBisCO. Diagram based on results published in Paper II.

Leaf protein concentrates from many different GLBM sources are suggested to be highly nutritious foodstuffs, mainly due to the high protein content and good amino acid profile (Betschart & Kinsella 1974; Sheen 1991; Hojilla-Evangelista *et al.* 2016; Nieuwland *et al.* 2021). The LPCs obtained in Papers II and III had rather differing nitrogen content (Table 4). A conversion factor of *e.g.* 5.8 (Nieuwland *et al.* 2021) can be applied to calculate the protein content of the LPCs. The difference in protein content between the LPCs was probably caused by the washing step applied to the precipitated protein prior to redissolving the protein in Paper III. The nitrogen content in the LPCs studied in Paper III and that in the unredissolved fraction (P3) in Paper II were more comparable, both with each other and with previously reported nitrogen contents of ~11-15% for lucerne LPCs (Miller *et al.* 1975; Wang & Kinsella 1975; Martin *et al.* 2018). The LPCs also contained phenolic compounds and dietary fibre, which may contribute to their nutritional value (Paper IV).

Table 4. *Nitrogen (N) content in leaf protein concentrates (LPCs) obtained from different types of green leafy biomass (GLBM) types in Paper II and III, and from the unredissolved protein (P3 fraction) in Paper II. Means ± standard deviations for the process triplicates in Paper II, other values are means of technical replicates.*

| **GLBM type** | **P3 fraction** | **LPC** | |
|---|---|---|---|
| Study | Paper II | Paper II | Paper III |
| Beetroot | 9.2±0.7 | 3.3±0.7 | 14.0 |
| Kale | 7.9±0.4 | 2.6±0.4 | 11.6 |
| Mangold | 7.2±0.6 | 3.6±0.9 | 13.3 |
| Lucerne | 7.2* | 4.0* | 10.8 |
| Spinach | 8.1±1.8 | 3.3±0.5 | 12.9 |
| Sugarbeet | 2.6±1.5 | 2.3±0.9 | 12.1 |

* No process replicates

The nutritional characteristics alone would not generate sufficiently high revenue to make LPC production profitable (Paper IV). However, as discussed below and in Paper III, the LPCs from many GLBM types show promising foam stabilising properties. In combination with the good nutritional aspects and the possibility of LPCs being accredited as a local and sustainable option, these properties should increase the revenues considerably. With this in mind, an extraction pathway aiming at an LPC consisting of only the water-soluble white protein fraction could be more feasible.

### 5.2.2 LPCs in food applications

*LPCs as foam stabilisers*

Most proteins have the ability to stabilise foams, including LPCs from many different GLBM sources, *e.g.* lucerne (Figure 11). This was illustrated by a whipping test in Paper III. The test was a preliminary study and no exact foam volumes were recorded, but it was clear from the experiment that foams with up to three times the initial volume of the LPC solution could be formed. The foams were also stable over time, although visible drainage occurred after one minute for the least stable foam. These findings indicate that LPCs from many different GLBM types could be used as a foam stabilising food ingredient. This is in line with results presented by other researchers, who have reported foam stabilising properties comparable to those of whey and soy for *e.g.* lucerne and sugarbeet LPCs (Hojilla-Evangelista *et al.* 2016; Martin *et al.* 2018).

*Figure 11*. Foam stabilised by leaf protein concentrate from lucerne.

*Air-water interfacial properties of LPCs*

The foam stabilising properties of proteins are linked to their air-water interfacial properties (Murray 2020). As a way of evaluating and comparing LPCs from different GLBM sources, the ability of the constituents to stabilise an air-water interface was assessed in Paper III. The surface tension ($\gamma$) reduction rate of different LPC solutions was determined using optical tensiometry. From this experiment it was clear that LPCs from all GLBM types evaluated had the ability to reduce $\gamma$ in a similar way to egg white, as illustrated for kale and spinach LPCs in Figure 12.

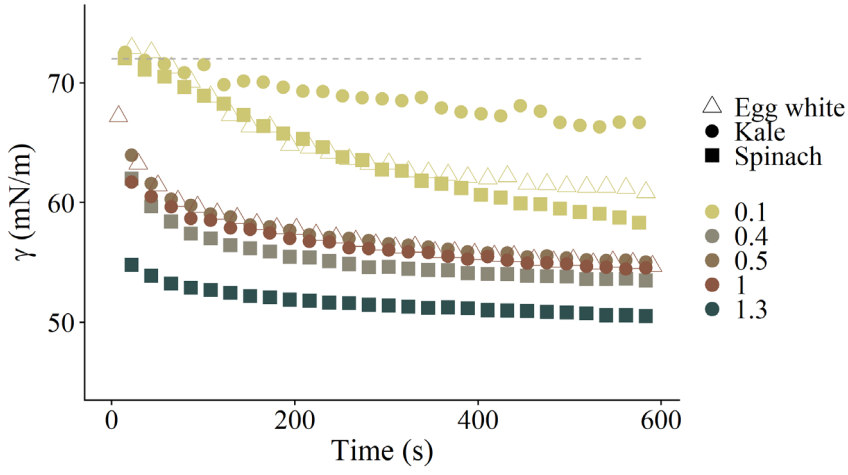


*Figure 12*. Surface tension (γ) as a function of time for spinach and kale leaf protein concentrates and for egg white in water, as measured with the pendant drop method at different protein concentrations. The horizontal line represents the γ of pure water at room temperature. Diagram based on data included in Paper III.

The LPCs not only reduce γ similarly to egg white, but several of them, *e.g.* spinach LPC, also had a stronger effect on γ at a protein concentration of 1 mg/ml (Figure 12). Another interesting finding in Paper III, as also illustrated in Figure 12, was that some LPCs, *e.g.* that from kale, seemed to reach saturation at the air-water interface, as γ was reduced similarly for LPC solutions with protein concentrations of both 0.5 and 1.0 mg/ml.

In Paper III, it was clear that the LPC source had little effect on the γ reduction rate, indicating promising foam stabilising properties for LPCs independent of GLBM type. This would be beneficial for an industrial protein fractionation set-up since the resulting LPC, regardless of the source, could be marketed as a foam stabilising ingredient.

*LPC at different food-relevant pH values*

The behaviour of proteins at different pH values is an important indicator of their behaviour in many food applications, not least since protein solubility is strongly affected by pH. When the solubility of LPCs at three different neutral to acidic pH values (7.0, 5.0, 3.0) was evaluated in Paper III, the highest solubility was seen at pH 7 for all GLBM types tested. This is well in line with finding in many other studies on LPC solubility (*e.g.* Sheen &

Sheen 1985; Lamsal *et al.* 2007; Martin *et al.* 2018; Nieuwland *et al.* 2021). The solubility at different pH values can be partly linked to the aggregation behaviour of the proteins (Figure 13). Aggregation was initiated around pH 4.5 for all LPCs evaluated in Paper III, which is reasonable given that the proteins in the LPCs were originally concentrated by isoelectric precipitation at that pH.

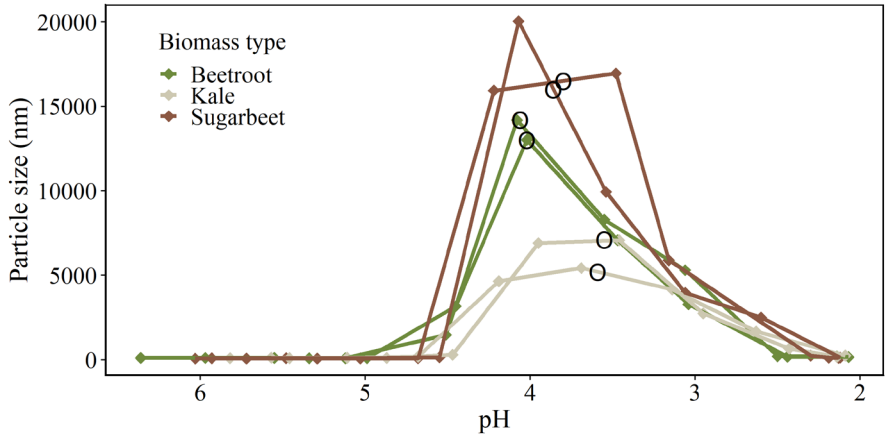


*Figure 13*. Average particle size of leaf protein concentrates from different biomass types during titration with hydrochloric acid. The lines represent technical replicates. The isoelectric point (pI) of replicates is marked with a circle. Diagram based on data included in Paper III.

The solubility of the LPCs at pH 7 ranged between 41% and 68%, which is low in comparison with values reported by others of *e.g.* 90% (Martin *et al.* 2018) and 97% (Lamsal *et al.* 2007). Protein solubility in LPC is tightly linked to the processing history, and precipitation with acid or heat reduces the solubility of the leaf proteins (Nieuwland *et al.* 2021; Tanambell *et al.* 2021). Enzymatic browning is another factor potentially decreasing the solubility (Amer *et al.* 2020). The brown colour of the LPCs shown in Figure 9 is probably due to the occurrence of enzymatic browning during production of the concentrates. As high solubility is an important aspect for the functionality of food proteins, the value and quality of LPCs could be increased by adapting the process to avoid enzymatic browning through the use of antioxidants, *e.g.* sodium sulphite (Tanambell *et al.* 2021), or by developing extraction methods in which precipitation is avoided, *e.g.* by using filtration techniques (Nieuwland *et al.* 2021).

## 5.3 Extraction of leaf proteins

In a biorefinery process targeting leaf proteins, the extraction protocol developed should preferably be versatile in terms of raw material, as different types of GLBM will be available depending on the season (see Figure 5). The process should also be efficient, inexpensive, easily scalable and suitable for food products. The LPC extraction protocol developed and evaluated in this thesis (see Figure 8) was based on literature methods (*e.g.* Martin *et al.* 2018; Hojilla-Evangelista *et al.* 2016; Tamayo Tenorio *et al.* 2016; Sheen, 1991; Fiorentini & Galoppini, 1981; Edwards *et al.* 1975).

### 5.3.1 Extraction protocol and performance of GLBM

The protocol devised in Paper II (depicted in Figure 8) was successful for seven of the nine GLBM types evaluated regarding the presence of RuBisCO in the final LPCs (see Figure 10). However, the overall nitrogen recovery rates (proportion of nitrogen in the original biomass recovered in the LPC) obtained for broccoli and kale GLBM (0.1%-0.4%) were not sufficiently high to make an industrial process economically feasible (Paper IV). Higher recovery rates, of 1.5% and 1.9% respectively, were found for lucerne and mangold in Paper II, but the losses throughout the process were still large in these cases. Higher recovery rates are needed to make protein fractionation feasible, and for that further process development is required. Process adaptation for individual GLBM types, or even for GLBM of different maturity stages, might be needed to reach sufficient protein yields.

### 5.3.2 Establishing and evaluating the LPC extraction protocol

*Obtaining soluble leaf proteins*

The very first step in a leaf protein extraction process is to disrupt the plant cells and release the intracellular liquid (green juice, GJ), as this contains the water-soluble *white* protein fraction. If cell disruption is incomplete, the proteins are not recoverable and will end up in the fibrous pulp. In Paper II, the nitrogen yield in GJ pressing was found to vary between the GLBM types, ranging from 15% (sugarbeet) to 53% (mangold). These differences were suggested to depend on the structure of the leaves. GLBM types with softer leaves, *e.g.* baby spinach, had poor separation of GJ and pulp, with a probable explanation being that the wet and soft consistency of the thawed leaves resulted in improper feeding through the screw press. For GLBM with

harder stems, *e.g.* lucerne, the fibrous texture of the stems may explain the low separation rate. For two of the GLBM types studied in Paper II (carrot and cabbage), a substantial part of the nitrogen in the GJ was removed with the particle fraction, indicating presence of intact plant cells and chloroplasts due to insufficient cell disruption.

Poor performance in separation of GJ and fibrous pulp was identified as the most important issue to be addressed to render an economically feasible industrial protein fractionation aimed at soluble leaf proteins (Paper IV). One possible way to improve performance would be to add a second screw-pressing step to the process. In the case of lucerne, such a second press increased nitrogen recovery in GJ from the original GLBM from 52% to 67%, which corresponded to an increase in recovery of 29% (Paper II).

*Removal of the GPF*

The green protein fraction (GPF) can be removed by gentle heat treatment of the GJ followed by centrifugation, resulting in a non-green white juice (WJ) containing the water-soluble white protein fraction. This process should remove as much of the green colour (*i.e.* chlorophyll) as possible, but as little of the RuBisCO and other soluble proteins as possible. The experimental work in Paper II revealed variations in the thermal sensitivity of protein from different GLBM types. These differences are exemplified in the upper panels in Figure 14, where the intensity of the protein bands in the SDS-PAGE gel is clearly fading at 60 °C for beetroot, but at 65 °C for spinach. A similar pattern can be seen in the corresponding protein concentration diagrams (lower panels in Figure 14).

Based on the experimental results, experiences from unpublished pilot studies and literature methods (*e.g.* Tamayo Tenorio *et al.* 2016; Martin *et al.* 2014), a temperature of 55 °C was chosen for further processing, as it removed the green colour from all samples while the protein content in the WJ was not too adversely affected. However, as illustrated in Figure 14, the proteins from different GLBM types showed differences in sensitivity to thermal denaturation. Hence, finding the lowest temperature (or the shortest treatment time) at which the GPF precipitates for each GLBM type would probably increase the overall protein yield, thus enhancing the economic profitability.

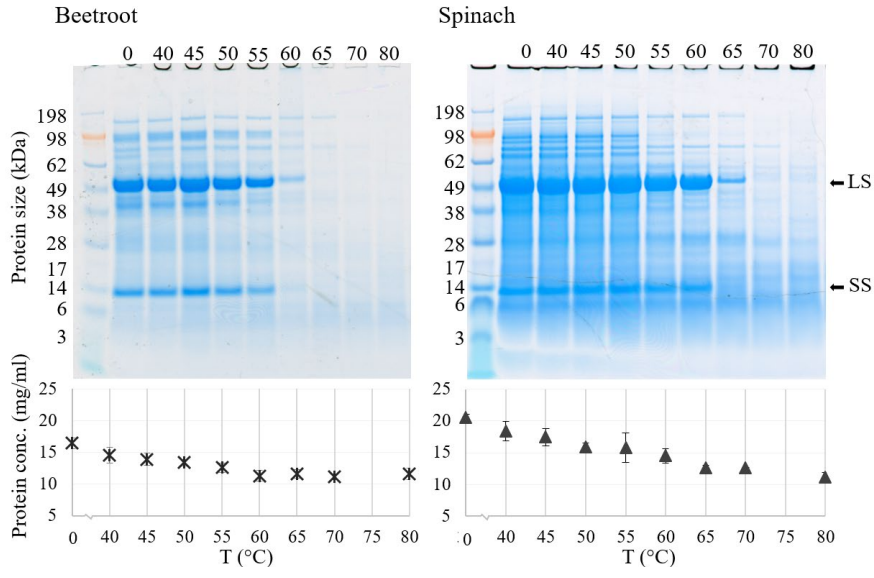


*Figure 14.* (Upper panels) SDS-PAGE gels and (lower panels) protein concentration in beetroot and spinach green juice treated at different temperatures. The images of the SDS-PAGE gels are from Supplementary Figure S1 in Paper II.

*Concentrating the white protein fraction through isoelectric precipitation*

In this thesis work, the chosen method for concentrating the WJ proteins was isoelectric precipitation. The precipitation pattern for the WJ components during titration with acid was investigated for the different GLBM types using dynamic light scattering. As can be seen in Figure 15, the size of the WJ aggregates increased at pH values approaching 3.5 for sugarbeet, while aggregation was initiated already at around pH 4.5 for beetroot, kale and most of the other GLBM types studied in Paper II.

The isoelectric point (pI) of the WJs from the different GLBM types included in Paper II ranged from 2.2 to 4.3 (with a few examples presented in Figure 15), which is significantly lower than the theoretical isoelectric point of spinach RuBisCO (pI = 6.03) (Paper II). However, the pI value determined in Paper II was that of the full WJ, a matrix consisting of RuBisCO and a range of other proteins, salts, sugars and other charged components. Selecting a pH value that is closest to that of RuBisCO from within the range of observed pI values for the WJ, *i.e.* a value of 4.5, should result in an LPC high in RuBisCO. Due to this, a pH of 4.5 was considered

suitable for protein concentration in the LPC extraction protocol developed in Paper II.

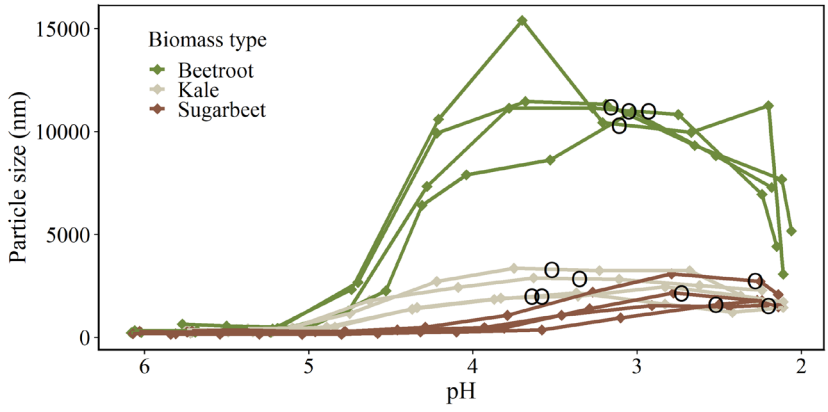


*Figure 15*. Average particle size during titration with acid of white juice from different sources, measured using dynamic light scattering. The lines represent technical replicates. The isoelectric point (pI) of replicates is marked with a circle.

Nitrogen yield in the precipitation step in the extraction protocol (see Figure 8) ranged from ~11% to ~22% for the GLBM types considered successful in LPC extraction (*i.e.* beetroot, broccoli, kale, lucerne, mangold, spinach and sugarbeet), corresponding to approximately 2-3% of the nitrogen in the initial biomass. As in the case of the thermal removal of the GPF, an industrial-scale process would most likely benefit from further GLBM specific process development, for which the different aggregation patterns presented in this thesis would provide a starting point.

*Additional factors affecting the overall nitrogen yields*

In all experimental studies (Papers II-IV), the intermediate juices (GJ and WJ) and the initial GLBM were frozen for practical reasons. It became clear that the freezing and subsequent thawing were responsible for losses in the process, due to protein precipitation. To isolate the effects of thermal removal of the GPF (step 4 in Figure 8) and isoelectric precipitation of the soluble proteins (step 7), several additional steps (2, 3 and 5) were included in the extraction protocol in Paper II. These extra steps in themselves decreased the overall yield in Pape II, but if the process were to be run continuously, without intermediate freezing, these losses would be avoided and higher protein recovery could be achieved.

## 5.4 Values beyond proteins

The economic pre-feasibility study in Paper IV clearly indicated that a fractionation process producing an LPC containing only the water-soluble proteins from the original GLBM is unlikely to be profitable. Utilising GLBM more efficiently would in many ways be beneficial from an environmental perspective, but to make the fractionation path economically viable additional revenues are needed. The revenues from the fractionation would be increased by higher protein recovery rates, but also by exploiting the values and properties of other process streams.

Additional revenues from the LPCs could derive from claims made for the product. Substantiation of health benefits of phenolic compounds in the LPCs, but also in the other fractions, could be one additional claim-increasing value. Locally produced food has gained interest in recent years (Nemes *et al.* 2021), and emphasising the local origin of GLBM, in combination with sustainability claims relating to using an under-utilised protein source, could increase consumer willingness to pay extra for such products. Greater emphasis on the functionality of the LPCs in food applications, extending from foaming to emulsification and gelation, could also increase the revenues if successful functionality can be demonstrated. Phenolic compounds in leaf extracts from various plant sources have been shown to have antioxidant capacity (Burri *et al.* 2017), and leaf extracts, such as LPCs, could potentially be used as plant-based antioxidants in food applications.

Much of the GLBM available today is used directly as an animal feed. By fractionating the GLBM in a biorefinery process as suggested in this thesis, the feed value for animals could actually be enhanced. The fibrous pulp fraction could serve as an excellent feed for lactating cows (Damborg *et al.* 2019; Larsson 2021), and the GPF has a protein profile that makes it suitable as a feedstuff for non-ruminants, *e.g.* pigs, which otherwise cannot degrade most GLBM (Olsson & Magnusson 2021). The brown juice contains soluble dietary fibre, of which the fructo-oligosaccharides have great value as a pre-biotic supplement for pigs (Feeney *et al.* 2021).

The potential uses of GLBM are not restricted to food and feed. The different process streams contain various compounds that could be useful in cosmetics, *e.g.* as anti-ageing agents (Prawitz 2020). In such applications, the revenues for the fractions would be significantly higher. One compound group represented in high amounts in all fractions from the extraction

process, but not least the brown juice fraction, was phenolic compounds (Paper IV). Brown juice is suggested to be a good substrate for anaerobic digestion, producing biogas as an energy source and digestate that can be used as fertiliser (Santamaria-Fernandez *et al.* 2020). Through further refinement of this process side-stream, prior to anaerobic digestion, phenolic extracts and other compounds of potentially high value could be obtained (Paper IV).

# 6. Future paths towards green protein

The work presented in this thesis indicated that leaf protein concentrates (LPCs) can be a sustainable food protein option. However, the work also raised further questions about what really defines sustainable food. Extended use of green leaves would provide great possibilities to harness their full potential, but other requirements might need to be fulfilled to guarantee LPCs as a sustainable option, from both an economic and environmental perspective. The environmental viability of both the process in itself, but also the full concept, needs to be addressed in further studies, in which both the negative and positive impacts should be considered.

Two requirements for economic viability were mentioned in this thesis: i) higher protein recovery rates and ii) a wider range of target products with high revenues. To meet these two requirements, the fractionation process used needs further process development, transforming it from a protein extraction process into a biorefinery. In such development, properties of the compounds other than proteins, must be considered to maintain their value and ensure proper separation. It is also necessary to focus on improved process yields and product purity, and on protein functionality.

# 7. Conclusions

The major conclusions from this thesis are as follows:

- Vast amounts of resources are spent on producing food, so not using the biomass produced to its fullest potential is a waste of those resources.
- Green leafy biomass is one of the largest biomass sources globally and could be used more optimally. One way would be to use it as a raw material in a biorefinery process, where protein is one of the target outputs.
- Proteins can be extracted from a wide range of different green leafy biomass sources.
- The resulting leaf protein concentrates have foam stabilising properties, which could increase their value beyond the simply nutritional aspects.
- Further development of the protein extraction process is needed to reach higher protein yields, as otherwise the proposed process, aiming mainly for leaf protein concentrates, would not be economically feasible.
- Extending the use of green leafy biomass has the potential to decrease environmental pressures from the food production system.

# References


Amer, B., Juul, L., Møller, A., Møller, H. & Dalsgaard, T. (2020). Improved solubility of proteins from white and red clover–Inhibition of redox enzymes. *International Journal of Food Science & Technology*. https://doi.org/10.1111/ijfs.14632

Andersson, I. & Backlund, A. (2008). Structure and function of Rubisco. *Plant Physiology and Biochemistry,* 46(3), 275-291. https://doi.org/10.1016/j.plaphy.2008.01.001

Asplund, L. & Svensson, Å. (2018). *Ersättning för minskat kväveläckage, En uppföljning av stöd inom landsbygdsprogrammet 2014–2020 baserad på uppgifter fram till och med den 18 juni 2018*. Jordbruksverket.

Bajželj, B., Quested, T.E., Röös, E. & Swannell, R.P. (2020). The role of reducing food waste for resilient food systems. *Ecosystem services,* 45, 101140. https://doi.org/10.1016/j.ecoser.2020.101140

Bals, B. & Dale, B.E. (2011). Economic comparison of multiple techniques for recovering leaf protein in biomass processing. *Biotechnology and bioengineering,* 108(3), 8. https://doi.org/10.1002/bit.22973

Barbeau, W.E. & Kinsella, J.E. (1986). Physical behavior functional properties: Relationship between surface rheology and foam stability of ribulose 1,5-bisphosphate carboxylase. *Colloids and Surfaces,* 17(2), 169-183. https://doi.org/10.1016/0166-6622(86)80244-X

Berndtsson, E. (2020). *Content of dietary fibre and phenolic compounds in broccoli side streams.* Licentiate thesis. Faculty of Landscape Architecture, Horticulture and Crop Production Science, Department of Plant Breeding. Swedish University of Agricultural Sciences.

Berndtsson, E., Andersson, R., Johansson, E. & Olsson, M.E. (2020). Side streams of broccoli leaves: A climate smart and healthy food ingredient. *International Journal of Environmental Research and Public Health,* 17(7), 2406. https://doi.org/10.3390/ijerph17072406

Betschart, A.A. & Kinsella, J.E. (1974). Influence of storage on composition, amino acid content, and solubility of soybean leaf protein concentrate. *Journal of Agricultural and Food Chemistry,* 22(1), 116-123. https://doi.org/10.1021/jf60191a013

Brady, M.V., Land, M. & Scharin, H. (2021). *Växtföljders påverkan på inlagring av organiskt kol i jordbruksmark: En systematisk översikt och samhällsekonomisk analys*. (F1:2021). FORMAS.

Burri, S.C., Ekholm, A., Håkansson, Å., Tornberg, E. & Rumpunen, K. (2017). Antioxidant capacity and major phenol compounds of horticultural plant materials not usually used. *Journal of functional foods,* 38, 119-127. https://doi.org/10.1016/j.jff.2017.09.003

Carlsson, G., Mårtensson, L.M., Prade, T., Svensson, S.E. & Jensen, E.S. (2017). Perennial species mixtures for multifunctional production of biomass on marginal land. *GCB Bioenergy,* 9(1), 191-201. https://doi.org/10.1111/gcbb.12373

Chen, J., Lærke, P.E. & Jørgensen, U. (2022). Land conversion from annual to perennial crops: A win-win strategy for biomass yield and soil organic carbon and total nitrogen sequestration. *Agriculture, Ecosystems & Environment,* 330, 107907. https://doi.org/10.1016/j.agee.2022.107907

Crippa, M., Solazzo, E., Guizzardi, D., Monforti-Ferrario, F., Tubiello, F. & Leip, A. (2021). Food systems are responsible for a third of global anthropogenic GHG emissions. *Nature Food,* 2(3), 198-209. https://doi.org/10.1038/s43016-021-00225-9

Damborg, V.K., Jensen, S.K., Johansen, M., Ambye-Jensen, M. & Weisbjerg, M.R. (2019). Ensiled pulp from biorefining increased milk production in dairy cows compared with grass–clover silage. *Journal of dairy science,* 102(12), 8883-8897. https://doi.org/10.3168/jds.2018-16096

Damodaran, S. (2005). Protein stabilization of emulsions and foams. *Journal of Food Science,* 70(3), R54-R66. https://doi.org/10.1111/j.1365-2621.2005.tb07150.x

De Fremery, D., Miller, R.E., Edwards, R.H., Knuckles, B.E., Bickoff, E.M. & Kohler, G.O. (1973). Centrifugal separation of white and green protein fractions from alfalfa juice following controlled heating. *Journal of Agricultural and Food Chemistry,* 21(5), 886-889. https://doi.org/10.1021/jf60189a020

Dickinson, E. (1999). Adsorbed protein layers at fluid interfaces: interactions, structure and surface rheology. *Colloids and Surfaces B: Biointerfaces,* 15(2), 161-176. https://doi.org/10.1016/S0927-7765(99)00042-9

Ducrocq, M., Morel, M.-H., Anton, M., Micard, V., Guyot, S., Beaumal, V., Solé-Jamault, V. & Boire, A. (2022). Biochemical and physical-chemical characterisation of leaf proteins extracted from *Cichorium endivia* leaves. *Food Chemistry,* 381. https://doi.org/10.1016/j.foodchem.2022.132254

Edwards, R.H., Miller, R.E., De Fremery, D., Knuckles, B.E., Bickoff, E.M. & Kohler, G.O. (1975). Pilot plant production of an edible white fraction leaf protein concentrate from alfalfa. *Journal of Agricultural and Food Chemistry,* 23(4), 620-626. https://doi.org/10.1021/jf60200a046

Ellis, R.J. (1979). The most abundant protein in the world. *Trends in Biochemical Sciences,* 4(11), 241-244. https://doi.org/10.1016/0968-0004(79)90212-3

Eriksson, M., Bartek, L., Löfkvist, K., Malefors, C. & Olsson, M.E. (2021). Environmental assessment of upgrading horticultural side streams—The case of unharvested broccoli leaves. *Sustainability,* 13(10), 5327. https://doi.org/10.3390/su13105327

EUROSTAT (2020). *Agri-environmental indicator - soil cover*. https://ec.europa.eu/eurostat/statistics-explained/index.php?title=Agri-environmental_indicator_-_soil_cover

FAO (2018). *The future of food and agriculture – Alternative pathways to 2050*. Rome.

Feeney, F., Buckley, E., Gaffey, J., Hayes, D. & Gottumukkala, L. (2021). *Report on the potential of recirculated grass whey as a nutrient fertilizer and opportunities for grass whey in biogas production.* https://biorefineryglas.eu/wp-content/uploads/2021/03/Biorefinery-Glas-D2.6.pdf

Fiorentini, R. & Galoppini, C. (1981). Pilot plant production of an edible alfalfa protein concentrate. *Journal of Food Science,* 46(5), 1514-1517. https://doi.org/10.1111/j.1365-2621.1981.tb04209.x

Hansson, D., Svensson, S.-E. & Prade, T. (2021). *Etableringstidpunktens inverkan på sommarmellangrödors ogräsbekämpande egenskaper, markkolsbidrag och potential som biogasråvara–fältförsök Norra Åsum 2018*. (915768992X). Sveriges lantbruksuniversitet, F.f.l., trädgårds- och jordbruksvetenskap, I.f.b.o. & teknologi. http://urn.kb.se/resolve?urn=urn:nbn:se:slu:epsilon-p-110749

Hojilla-Evangelista, M.P., Selling, G.W., Hatfield, R. & Digman, M. (2016). Extraction, composition, and functional properties of dried alfalfa (*Medicago sativa* L.) leaf protein. *Journal of the Science of Food and Agriculture,* 97(3), 882-888. https://doi.org/10.1002/jsfa.7810

Hood, L., Cheng, S., Koch, U. & Brunner, J. (1981). Alfalfa proteins: Isolation and partial characterization of the major component—Fraction I protein. *Journal of Food Science,* 46(6), 1843-1850. https://doi.org/10.1111/j.1365-2621.1981.tb04501.x

Johansson, E., Prade, T., Angelidaki, I., Svensson, S.-E., Newson, W.R., Gunnarsson, I.B. & Hovmalm, H.P. (2015). Economically viable components from Jerusalem artichoke (*Helianthus tuberosus* L.) in a biorefinery concept. *International Journal of Molecular Sciences,* 16(4), 8997-9016. https://doi.org/10.3390/ijms16048997

Jordbruksverket (2022). Jordbruksverkets statistikdatabas. https://statistik.sjv.se/PXWeb/pxweb/sv/Jordbruksverkets%20statistikdatabas [2022-02-24].

Jwanny, E.W., Montanari, L. & Fantozzi, P. (1993). Protein production for human use from sugarbeet: Byproducts. *Bioresource Technology,* 43(1), 67-70. https://doi.org/10.1016/0960-8524(93)90085-P

Kaszás, L., Alshaal, T., Kovács, Z., Koroknai, J., Elhawat, N., Nagy, É., El-Ramady, H., Fári, M. & Domokos-Szabolcsy, É. (2020). Refining high-quality leaf protein and valuable co-products from green biomass of Jerusalem artichoke (*Helianthus tuberosus* L.) for sustainable protein supply. *Biomass Conversion and Biorefinery*, 1-16. https://doi.org/10.1007/s13399-020-00696-z

Kiskini, A., Vissers, A., Vincken, J.-P., Gruppen, H. & Wierenga, P.A. (2016). Effect of plant age on the quantity and quality of proteins extracted from sugar beet (*Beta vulgaris* L.) leaves. *Journal of Agricultural and Food Chemistry,* 64(44), 8305-8314. https://doi.org/10.1021/acs.jafc.6b03095

Koschuh, W., Povoden, G., Thang, V.H., Kromus, S., Kulbe, K.D., Novalin, S. & Krotscheck, C. (2004). Production of leaf protein concentrate from ryegrass (*Lolium perenne x multiflorum*) and alfalfa (*Medicago sauva* subsp. *sativa*). Comparison between heat coagulation/centrifugation and ultrafiltration. *Desalination,* 163(1-3), 253-259. https://doi.org/10.1016/S0011-9164(04)90197-X

Kummu, M., De Moel, H., Porkka, M., Siebert, S., Varis, O. & Ward, P.J. (2012). Lost food, wasted resources: Global food supply chain losses and their impacts on freshwater, cropland, and fertiliser use. *Science of the total environment,* 438, 477-489.

Lamsal, B.P., Koegel, R.G. & Boettcher, M.E. (2003). Separation of protein fractions in alfalfa juice: Effects of some pre-treatment methods. *Transactions of the ASAE,* 46(3), 715-720. https://doi.org/10.13031/2013.13572

Lamsal, B.P., Koegel, R.G. & Gunasekaran, S. (2007). Some physicochemical and functional properties of alfalfa soluble leaf proteins. *LWT - Food Science and Technology,* 40(9), 1520-1526. https://doi.org/10.1016/j.lwt.2006.11.010

Larsson, M. (2021). *Performance of dairy cows fed grass-clover silage or biorefined silage pulp of grass-clover silage.* Master thesis. Department of Animal Environment and Health, Faculty of Veterinary Medicine and Animal Sciences. Swedish University of Agricultural Sciences. http://urn.kb.se/resolve?urn=urn:nbn:se:slu:epsilon-s-17226

Liu, M., Zhang, L., Ser, S.L., Cumming, J.R. & Ku, K.-M. (2018). Comparative phytonutrient analysis of broccoli by-products: The potentials for broccoli by-product utilization. *Molecules,* 23(4), 900. https://doi.org/10.3390/molecules23040900

Marini, L., St-Martin, A., Vico, G., Baldoni, G., Berti, A., Blecharczyk, A., Malecka-Jankowiak, I., Morari, F., Sawinska, Z. & Bommarco, R. (2020). Crop rotations sustain cereal yields under a changing climate. *Environmental Research Letters,* 15(12), 124011. https://doi.org/10.1088/1748-9326/abc651

Martin, A.H., Castellani, O., de Jong, G.A.H., Bovetto, L. & Schmitt, C. (2018). Comparison of the functional properties of RuBisCO protein isolate extracted from sugar beet leaves with commercial whey protein and soy protein isolates. *Journal of the Science of Food and Agriculture*. https://doi.org/10.1002/jsfa.9335

Martin, A.H., Nieuwland, M. & de Jong, G.A.H. (2014). Characterization of heat-set gels from RuBisCO in comparison to those from other proteins. *Journal of Agricultural and Food Chemistry,* 62(44), 10783-10791. https://doi.org/10.1021/jf502905g

Martin, G., Durand, J.-L., Duru, M., Gastal, F., Julier, B., Litrico, I., Louarn, G., Mediene, S., Moreau, D. & Valentin-Morison, M. (2020). Role of ley pastures in tomorrow's cropping systems. A review. *Agronomy for Sustainable Development,* 40(3), 1-25. https://doi.org/10.1007/s13593-020-00620-9

Mbow, C., Rosenzweig, C., Barioni, L.G., Benton, T.G., Herrero, M., Krishnapillai, M., Liwenga, E., Pradhan, P., Rivera-Ferre, M.G., Sapkota, T., Tubiello, F.N. & Xu, Y. (2019). *Food security*. (Climate Change and Land: an IPCC special report on climate change, desertification, land degradation, sustainable land management, food security, and greenhouse gas fluxes in terrestrial ecosystems ). IPCC. https://www.ipcc.ch/srccl/chapter/chapter-5/

Merodio, C., Martin, M. & Sabater, B. (1983). Improved separation of green and soluble leaf proteins by pH shift. *Journal of Agricultural and Food Chemistry,* 31(5), 957-959. https://doi.org/10.1021/jf00119a009

Merodio, C. & Sabater, B. (1987). Preparation and properties of a white protein fraction in high yield from sugar beet (*Beta vulgaris* L) leaves. *Journal of the Science of Food and Agriculture,* 44(3), 237-243. https://doi.org/10.1002/jsfa.2740440305

Meybeck, A., Cederberg, C., Gustavsson, J., van Otterdijk, R. & Sonesson, U. (2011). *Global food losses and food waste: extent, causes and prevention*. Rome, Italy: FAO.

Miller, R.E., de Fremery, D., Bickoff, E.M. & Kohler, G.O. (1975). Soluble protein concentrate from alfalfa by low-temperature acid precipitation. *Journal of Agricultural and Food Chemistry,* 23(6), 1177-9. https://doi.org/10.1021/jf60202a037

Muneer, F., Hovmalm, H.P., Svensson, S.-E., Newson, W.R., Johansson, E. & Prade, T. (2021). Economic viability of protein concentrate production from green biomass of intermediate crops: A pre-feasibility study. *Journal of cleaner production,* 294, 126304. https://doi.org/10.1016/j.jclepro.2021.126304

Murray, B.S. (2020). Recent developments in food foams. *Current Opinion in Colloid & Interface Science*, 101394. https://doi.org/10.1016/j.cocis.2020.101394

Møller, A.H., Hammershøj, M., Dos Passos, N.H.M., Tanambell, H., Stødkilde, L., Ambye-Jensen, M., Danielsen, M., Jensen, S.K. & Dalsgaard, T.K. (2021). Biorefinery of green biomass─How to extract and evaluate high quality leaf protein for food? *Journal of Agricultural and Food Chemistry*. https://doi.org/10.1021/acs.jafc.1c04289

Nemes, G., Chiffoleau, Y., Zollet, S., Collison, M., Benedek, Z., Colantuono, F., Dulsrud, A., Fiore, M., Holtkamp, C. & Kim, T.-Y. (2021). The impact of COVID-19 on alternative and local food systems and the potential for the sustainability transition: Insights from 13 countries. *Sustainable Production and Consumption*. https://doi.org/10.1016/j.spc.2021.06.022

Nieuwland, M., Geerdink, P., Engelen-Smit, N.P., Van Der Meer, I.M., America, A.H., Mes, J.J., Kootstra, A.M.J., Henket, J.T. & Mulder, W.J. (2021). Isolation and gelling properties of duckweed protein concentrate. *ACS Food Science & Technology*. https://doi.org/10.1021/acsfoodscitech.1c00009

Nissen, S.H., Schmidt, J.M., Gregersen, S., Hammershøj, M., Møller, A.H., Danielsen, M., Stødkilde, L., Nebel, C. & Dalsgaard, T.K. (2021). Increased solubility and functional properties of precipitated alfalfa protein concentrate subjected to pH shift processes. *Food Hydrocolloids,* 119, 106874. https://doi.org/10.1016/j.foodhyd.2021.106874

Olsson, A.-C. & Magnusson, M. (2021). *Proteinkvalitet i grönjuice och grönprotein extraherad från vallbiomassa i pilotanläggningen inom projektet Växtproteinfabriken i Alnarp*. (LTV-fakultetens faktablad). http://urn.kb.se/resolve?urn=urn:nbn:se:slu:epsilon-p-111628

Parajuli, R., Dalgaard, T. & Birkved, M. (2018). Can farmers mitigate environmental impacts through combined production of food, fuel and feed? A consequential life cycle assessment of integrated mixed crop-livestock system with a green biorefinery. *Science of the total environment,* 619, 127-143. https://doi.org/10.1016/j.scitotenv.2017.11.082

Patel, M. & Berry, J.O. (2008). Rubisco gene expression in C4 plants. *Journal of Experimental Botany,* 59(7), 1625-1634. https://doi.org/10.1093/jxb/erm368

Pirie, N.W. (1942). The direct use of leaf protein in human nutrition. *Chemistry and Industry,* 61, 45-48. https://repository.rothamsted.ac.uk/item/8www3/the-direct-use-of-leaf-protein-in-human-nutrition

Prawitz, L. (2020). *How substances in agricultural waste material can be used as active ingredients in skin care products.* Master thesis. Department of Food Technology, Engineering and Nutrition, Faculty of Engineering. Lund University. http://lup.lub.lu.se/student-papers/record/9020005

Santamaria-Fernandez, M., Molinuevo-Salces, B., Kiel, P., Steenfeldt, S., Uellendahl, H. & Lübeck, M. (2017). Lactic acid fermentation for refining proteins from green crops and obtaining a high quality feed product for

monogastric animals. *Journal of cleaner production,* 162, 875-881. https://doi.org/10.1016/j.jclepro.2017.06.115

Santamaria-Fernandez, M., Ytting, N.K., Lübeck, M. & Uellendahl, H. (2020). Potential nutrient recovery in a green biorefinery for production of feed, fuel and fertilizer for organic farming. *Waste and Biomass Valorization,* 11(11), 5901-5911. https://doi.org/10.1007/s12649-019-00842-3

Sedlar, T., Čakarević, J., Tomić, J. & Popović, L. (2021). Vegetable by-products as new sources of functional proteins. *Plant Foods for Human Nutrition,* 76(1), 31-36. https://doi.org/10.1007/s11130-020-00870-8

Sheen, S.J. (1991). Comparison of chemical and functional properties of soluble leaf proteins from four plant species. *Journal of Agricultural and Food Chemistry,* 39(4), 681-685. https://doi.org/10.1021/jf00004a011

Sheen, S.J. & Sheen, V.L. (1985). Functional properties of fraction 1 protein from tobacco leaf. *Journal of Agricultural and Food Chemistry,* 33(1), 79-83. https://doi.org/10.1021/jf00061a023

Springmann, M., Clark, M., Mason-D'Croz, D., Wiebe, K., Bodirsky, B.L., Lassaletta, L., De Vries, W., Vermeulen, S.J., Herrero, M. & Carlson, K.M. (2018). Options for keeping the food system within environmental limits. *Nature,* 562(7728), 519-525. https://doi.org/10.1038/s41586-018-0594-0

Stødkilde, L., Lashkari, S., Eriksen, J. & Jensen, S.K. (2021). Enhancing protein recovery in green biorefineries through selection of plant species and time of harvest. *Animal Feed Science and Technology,* 278, 115016. https://doi.org/10.1016/j.anifeedsci.2021.115016

Tamayo Tenorio, A., Gieteling, J., de Jong, G.A., Boom, R.M. & van der Goot, A.J. (2016). Recovery of protein from green leaves: Overview of crucial steps for utilisation. *Food Chemistry,* 203, 402-408. https://doi.org/10.1016/j.foodchem.2016.02.092

Tamayo Tenorio, A. (2017). *Sugar beet leaves for functional ingredients.* Doctoral thesis. Wageningen University.

Tamayo Tenorio, A., Schreuders, F., Zisopoulos, F., Boom, R. & Van der Goot, A. (2017). Processing concepts for the use of green leaves as raw materials for the food industry. *Journal of cleaner production,* 164, 736-748. https://doi.org/10.1016/j.jclepro.2017.06.248

Tanambell, H., Møller , A.H., Corredig, M. & Dalsgaard, T.K. (2021). RuBisCO from alfalfa–Native subunits preservation through sodium sulfite addition and reduced solubility after acid precipitation followed by freeze-drying. *LWT*, 112682. https://doi.org/10.1016/j.lwt.2021.112682

United Nations, D.o.E.a.S.A., Population Division (2019). *World Population Prospects 2019: Highlights*. (ST/ESA/SER.A/423).

Wang, J. & Kinsella, J.E. (1975). Composition of alfalfa leaf protein isolates. *Journal of Food Science,* 40(6), 1156-1161. https://doi.org/10.1111/j.1365-2621.1975.tb01041.x

Wang, J.C. & Kinsella, J.E. (1976). Functional properties of novel proteins: Alfalfa leaf protein. *Journal of Food Science,* 41(2), 286-292. https://doi.org/10.1111/j.1365-2621.1976.tb00602.x

Westhoek, H., Ingram, J.S.I., Van Berkum, S., Özay, L. & Hajer, M. (2016). *Food systems and natural resources. A Report of the Working Group on Food Systems of the International Resource Panel/UNEP*. UNEP.

Wierenga, P. & Gruppen, H. (2010). New views on foams from protein solutions. *Current Opinion in Colloid & Interface Science,* 15(5), 365-373. https://doi.org/10.1016/j.cocis.2010.05.017

Witzgall, K., Vidal, A., Schubert, D.I., Höschen, C., Schweizer, S.A., Buegger, F., Pouteau, V., Chenu, C. & Mueller, C.W. (2021). Particulate organic matter as a functional soil component for persistent soil organic carbon. *Nature Communications,* 12(1), 1-10. https://doi.org/10.1038/s41467-021-24192-8

WWF-UK (2021). *Driven to waste: The Global Impact of Food Loss and Waste on Farms*. WWF-UK.

Xu, X., Sharma, P., Shu, S., Lin, T.-S., Ciais, P., Tubiello, F.N., Smith, P., Campbell, N. & Jain, A.K. (2021). Global greenhouse gas emissions from animal-based foods are twice those of plant-based foods. *Nature Food,* 2(9), 724-732. https://doi.org/10.1038/s43016-021-00358-x

Zayas, J.F. (1997). *Functionality of food proteins*. New York: Springer-Verlag Berlin Heidelberg.

# Popular science summary

*Eating green - for a sustainable future!*

Green leaves might not be the first foodstuff that comes to mind when thinking about the protein sources of tomorrow. One may even think that they do not contain any protein at all, but they do, and as there are plenty of green leaves around they might even be one of the larger protein sources available worldwide. However, a problem with green leaves as a protein source is the low protein content in relation to other leaf components. A fresh leaf, for example a sugarbeet leaf, contains ~95% water, ~4% fibre and only ~1-2% protein. The average human stomach can probably not hold enough sugarbeet leaves to fulfil the dietary need for protein.

A solution to this problem is to make a leaf protein concentrate (LPC) where the majority of the other leaf components are removed and the relative protein content is increased. Such an LPC would not only be nutritious, but could also have great functional properties as a food ingredient, as the leaf proteins have good foam stabilising ability, which was demonstrated in this work. This could, in the long run, make LPC a good option to *e.g.* egg white in many food applications.

In this thesis, a method for extracting water-soluble proteins from crop leaves (not only sugarbeet leaves) was developed. Nine different green leafy crops were evaluated as raw material for this process, and protein was successfully extracted from the majority of them. The emphasis in method development was not on finding the best possible way to produce LPCs, but to find a way suitable for a range of different leafy crops. For protein extraction industrially in a biorefinery set-up, a versatile extraction protocol would be necessary to maintain supply, as the availability of different kinds of leaves varies widely over the seasons, especially in Sweden and other Nordic countries. However, the protein extraction method used did not give

high enough protein recovery rates to make an upscaled process economically viable – yet. Hence, further process development, as well as targeting additional products, *e.g.* antioxidants, of potential high value, is needed.

Using green leaves from agriculture as a raw material in a biorefinery set-up would add monetary value to the produce and could also be part of the transition to a more sustainable food production system. Resources in the form of water, farmland, energy, and fertilisers are spent on producing the whole plant, and are wasted if all the plant biomass is not utilised in the best possible way. Hence, eating protein from green leaves may be part of our route to a sustainable future!

# Populärvetenskaplig sammanfattning

*Ät grönt – för vår framtids skull!*

Gröna blad är kanske inte det första man kommer att tänka på när man tänker på framtidens proteinkällor. Man kanske till och med tänker att de inte innehåller några proteiner över huvud taget; men det gör de. Eftersom gröna blad dessutom finns nästan överallt, kan de till och med vara världens största proteinkälla. Ett problem med gröna blad som livsmedel, och särskilt som proteinkälla, är dock den relativt låga halten av just protein i förhållande till de övriga beståndsdelarna. Färska blad, till exempel sockerbetsblast, innehåller ~95% vatten, ~4% fibrer och endast ~1-2% protein. Vi skulle helt enkelt bli mätta långt innan vi har ätit tillräckligt med blad för att tillgodose vårt dagliga proteinbehov.

En möjlig lösning är att framställa ett bladproteinkoncentrat (*leaf protein concentrate*, LPC) där merparten av de andra komponenterna avlägsnas, därmed ökas den relativa proteinhalten. Ett sådant koncentrat skulle inte bara vara ett näringsrikt livsmedel, utan det skulle också kunna vara en funktionell ingrediens, till exempel genom att utnyttja dess skumstabiliserande förmåga. I denna avhandling påvisades att LPC från flera olika sorters gröna blad kunde stabilisera skum och skulle på sikt kunna ersätta t.ex. äggvita i många livsmedelsapplikationer.

Som en viktig del av avhandlingsarbetet utvecklades en extraktionssprocess för att utvinna vattenlösliga proteiner från olika sorters gröna blad, inte bara sockerbetsblast. Nio olika sorters grön bladbiomassa utvärderades som råmaterial för den här processen och från majoriteten av dessa grödor kunde proteiner utvinnas. Metodutvecklingen syftade inte till att hitta det bästa möjliga sättet att producera LPC på, utan till att hitta ett sätt som är lämpligt för flera olika sorters grön bladbiomassa. I en industriell utvinningsprocess behövs ett mångsidigt extraktionsprotokoll, eftersom

tillgången på olika sorters grön biomassa varierar betydligt över året, särskilt i Sverige och i andra nordiska länder. Utvinningsmetoden som användes gav dock inte tillräckligt hög proteinutvinningsgrad för att en sådan uppskalad process skulle vara ekonomiskt lönsam - än. Därför behövs ytterligare processutveckling, såväl som utvinning av fler av de potentiellt värdefulla ämnen, exempelvis antioxidanter, som finns i den gröna bladbiomassan.

Genom att använda gröna blad från jordbruket som en råvara i ett bioraffinaderi, där proteiner såväl som andra ämnen utvinns, skulle biomassan tillföras ytterligare ekonomiskt värde. Det skulle också kunna vara en del av omställningen till ett mer hållbart produktionssystem för livsmedel. Resurser i form av vatten, jordbruksmark, energi och gödsel går åt till att producera hela grödan och dessa resurser går till spillo om växtbiomassan inte tas tillvara på bästa möjliga sätt. Med andra ord, proteiner från gröna blad i vår kost kan vara en del av vår väg mot en hållbar framtid!

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# I

# The underutilised side streams of broccoli and kale – Valorisation via proteins and phenols

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**Abstract**

As the world's population is growing, simultaneously as availability of water, arable land and fertilisers are decreasing, an increase in the utilisation of biomass is essential in order to reach sustainable food production. In agricultural primary production an extensive part of the total biomass produced ends up in side streams, which today are of low value. Studies including the whole food supply chain, as well as studies on a global level are generally lacking. This knowledge gap is hindering the work towards sustainable food production. Broccoli (*Brassica oleracea Italica* group) and kale (*Brassica oleracea Sabellica* group) are examples of crops where at most 10% and 50% of the total plant is harvested, respectively, for further processing into consumable products. The side streams are mainly composed of stems and leaves, which are potential sources of contents beneficial to health, such as dietary fibres and bioactive phenolic compounds, as well as proteins of high nutritional value and functionality. These substances all have potential as high value side products, which could be used as food supplements or ingredients. One proposed approach to reach a more sustainable primary production system is to use side stream products in a biorefinery concept, where proteins, dietary fibres and phenolic compounds are the main targets. This paper aims to be a first step in evaluating the feasibility of broccoli and kale side streams in such a biorefinery process. This requires knowledge of what compounds are present, and in which quantities, in the leaves and stems of the two different crops. It can be concluded, based on pilot studies and previously reported data, that kale and broccoli side streams are candidates in further studies on usage in a biorefinery process. Ethical perspectives of these uses of broccoli and kale have not been investigated thoroughly.

**Keywords:** biorefinery, primary production, food, climate impact

**Definitions:** *Food waste:* Losses at retail and household level, *Food loss:* Losses during processing and transport, *Field waste:* Side streams in the primary production (Gustavsson, 2011).

## Introduction

At present, we are facing two major challenges: the climate change and a growing world population needed to be sustainably fed. In 2018, the Intergovernmental Panel on Climate Change (IPCC) highlighted evidence for the need to limit the total temperature increase on Earth to 1.5 °C in order to reduce the consequences of global warming (Masson-Delmotte *et al.*, 2018). Numbers from 2016 estimated 815 million people on Earth to be undernourished (FAO *et al.*, 2017). Simultaneously, ⅓ of all produced food is wasted (Gustavsson *et al.*, 2011), corresponding to the nutrition needed to feed 1.9 billion people (Kummu *et al.*, 2012). These statements in combination address the necessity of a global commitment in food security. Both FAO and IPCC have listed increased agricultural productivity and decreased food waste as priority actions to fight food insecurity and climate change (FAO, 2014; Masson-Delmotte *et al.*, 2018).

The waste of food is not only a dissipation of nutrition, but also other limited resources. Throughout the food supply chain e.g. water, fertilisers, farmland, and energy are invested, resulting in greenhouse gas (GHG) emissions (Kummu *et al.*, 2012; Bryngelsson *et al.*, 2016; Röös *et al.*, 2018). The FAO has estimated that 1.7 billion tonnes of food waste is produced every year throughout the chain, which requires 0.9 million hectares of farmland, 3.49 GT $CO_2$e, and 306 $km^3$ of drinkable water (FAO, 2014).

A more complete use of the agriculturally produced biomass would contribute to an increased productivity with less field waste, and thereby the IPCC climate goals will be approached. To efficiently use the total biomass, reducing waste both from the field and further down the production chain, the concept of biorefinery might be useful in increasing the productivity of agriculture and adding value to waste streams.

In Sweden, broccoli (*Brassica oleracea Italica* group) production occupied 362 hectares in 2016, and the production of kale (*Brassica oleracea Sabellica* group) used 89 hectares in 2017, with approximate yields of 8-9 tonnes per hectare (Persson, 2018).The corresponding data for EU is found in Figure 1. Despite the fact that these crops are considered important food crops, only 20–50% of the field produce are harvested and used for food in Sweden (unpublished data). The unharvested parts are mainly the stems, and in the case of broccoli all leaves, and for kale the lowermost leaves (unpublished data). Both broccoli and kale contain considerable amounts of compounds beneficiary to health, and most of those compounds are also present in high levels in the underutilised parts of the plant (Vilar et al., 2007; Drabińska et al., 2018). Some examples of valuable compounds with reported levels are presented in Figure 1. This makes broccoli and kale interesting subjects when investigating the potential of agricultural side streams as raw material in biorefineries for added value ingredients, including using suitable plant parts for processed food ingredients, e.g. mixing dried ingredients in bread.

**Potential of side streams in the primary production of broccoli and kale**

In the primary production of many vegetables, including broccoli and kale, only a small fraction of the produced biomass is reaching the consumer. This is illustrated in Figure 1, where the florets constitute only a smaller fraction of the total biomass. In a pilot study on field production of broccoli, approximately 80% of the produced biomass was stem and leaves, parts which are not used for food applications in Sweden (unpublished data). This is in accordance with the reported values of 90% for greenhouse produced broccoli (Liu *et al.*, 2018). An additional part of the biomass is wasted during processing, with 45–50% of the harvested broccoli florets discarded (Campas-Baypoli *et al.*, 2009). Based on values from Persson (2018) (see Figure 1), approximately 11 600 tonnes of broccoli side streams were generated in 2017 in Sweden alone. Iceberg lettuce is another example of a vegetable with a high level of waste. In the primary production more than 60% of the produced biomass is left as field waste, and additionally 12% of the harvested lettuce is lost on the way to the consumer (Strid *et al.*, 2014).

Biorefineries have been proposed as a way of recovering high value products from low value material (Rönnlund *et al.*, 2014; Johansson *et al.*, 2015). In this concept also plant food from side streams processed to food ingredients, by e.g. drying or blanching, can be included. In addition, agricultural side streams, such as leaves and stems from broccoli and kale cultivation, have high potential as feed stock for biorefinery processes targeting proteins, phenolic compounds, and dietary fibres, which can be used as ingredients and additives in the food and medical industry. Proteins have been extracted in pilot scale processes from green biomass, mainly grasses (Edwards *et al.*, 1975; Pouvreau *et al.*, 2014; Stødkilde *et al.*, 2018). The leafy side streams from sugar beet production (Martin *et al.*, 2019) and cauliflower have been evaluated for their potential use for different products (Xu *et al.*, 2017). High amounts of phenolic compounds, known for their antioxidant properties, can be extracted from by-products of fruit and vegetable production, including broccoli (Peschel *et al.*, 2006). Such extracts are useful both for food and cosmetic applications. Phenolic compounds have been extracted on pilot scale from e.g. apple pomace (Virot *et al.*, 2010; Pingret *et al.*, 2012) and mango leaves (Fernández-Ponce *et al.*, 2016).

The potential of using green leaves as a nutritious protein source was described several decades ago (Pirie, 1966). One of the main proteins in green leaves is RuBisCO, an enzyme active in the first step of photosynthesis. RuBisCO is present in high concentrations in all green plants, with 15–30% of the total leaf protein being RuBisCO (Evans, 1989). Leaf protein isolates have a high nutritive value containing all the essential amino acids (Pouvreau *et al.*, 2014), and have been shown to have properties of interest in food applications. The proteins have been shown to stabilise foams (Pouvreau *et al.*, 2014) and emulsions (Martin *et al.*, 2019), and work as gelling agents (Hojilla-Evangelista *et al.*, 2017).

Green leafy vegetables, including broccoli leaves, contain high amounts of phenolic compounds (Lin and Harnly, 2010; Bhandari and Kwak, 2015). Phenolic compounds are a diverse group of substances having antioxidative capacity (Shen *et al.*, 2017), which have been shown to be beneficial for human health, mainly through the interaction with the gut microbiota (Perez-Jimenez *et al.*, 2009; Selma *et al.*, 2009). Furthermore, the phenolic compounds may also lower the risk for developing cancer (Kyle *et al.*, 2010) and cardiovascular disease (Williamson, 2017), and can improve general vascular health (Wang *et al.*, 2011). Thus, side streams from broccoli cultivation can be used as a raw material for recovery of vegetable antioxidants (Aires *et al.*, 2017), with comparative antioxidative activity to that of synthetic antioxidants used in food products (Balasundram *et al.*, 2006).

Broccoli and kale are both vegetables known for their high levels of dietary fibre (Vilar *et al.*, 2007; Schäfer *et al.*, 2017). Examples of dietary fibre is cellulose, hemicellulose, pectin and inulin, all carbohydrates intrinsic in plants and with associated health benefits (Stephen *et al.*, 2017). A high intake of dietary fibre is associated with lower mortality from cardiovascular disease, coronary heart disease, and cancer (Kim and Je, 2016) and will also impact gut microbiota (Yang *et al.*, 2013). The average daily intake of dietary fibres in most Western countries is below recommendations (Stephen *et al.*, 2017). As a way of increasing the daily intake, food products can be enriched with dietary fibre, with increased health beneficial properties as a result (Elleuch *et al.*, 2011). Broccoli leaf powder has been proposed for use in gluten free sponge cake to increase the content of dietary fibre (Drabińska *et al.*, 2018). Dietary fibre ingredients can also be used to improve functional properties, in e.g. meat, dairy, and wheat flour-based products (Yang *et al.*, 2017).

**Discussion**

The world population is increasing, reaching 9.8 billion in 2050 (United Nations, 2017), and as prime farmland is becoming scarce, responsible land use is required (Garnett, 2011). A responsible use of farmland would in general include a more optimised agriculture, and a more efficient use of the produced biomass. The issue of land use is not only a question of food production, but also relevant for deforestation, biodiversity, and GHG emissions. Efficient land use decreases the need of converting land areas into farmland, thereby protecting wild life and biodiversity (Kummu *et al.*, 2012). Both avoiding deforestation and enabling revegetation of farmland have a positive effect on GHG emissions, since agricultural land in many cases has a significantly lower $CO_2$ uptake than the surrounding vegetation (Bryngelsson *et al.*, 2016).

It is estimated that the global yearly food waste has potential to feed 1.9 billion people (Kummu *et al.*, 2012), a number excluding the vast amounts of field waste in the primary production. One possible approach for a more efficient utilisation of biomass, and the resources invested during production, is

| | | Broccoli and cauliflower | Kale and other brassicas |
|---|---|---|---|
| Harvested production[a] [1000 t] | EU: | 2 435.14 | 11 393.39 |
| | Swe: | 10.30 (2.9)[b] | 0.7 |
| Area[a] [1000 ha] | EU: | 138.45 | 2.22 |
| | Swe: | 0.76 (0.3)[b] | 0.09 |

| | Broccoli | Kale |
|---|---|---|
| Protein [g/100g DW] | 29[c] | 11-25[d] |
| Phenolic compounds [mg GAE/100g] | 740.1mg[g] (DW) | 385.9mg[f] (FW) |
| Dietary fibre [g/100 g DW] | 36[e] (Stem) | 8.0-17.7[d] (Leaves) |

a. Data from Eurostat, b. Persson 2018, c.Drabińska et al., 2018, d. Vilar et al., 2007, e. Schäfer et al., 2017, f. Korus & Lisiewska, 2011, g. Bhandari & Kwak, 2014

*Figure 1. The picture illustrates the different sections (florets, stem with roots, and leaves) of a mature broccoli plant and shows the large variation in biomass. The upper table presents data for production of broccoli and cauliflower, and for kale and five other brassicas, with the corresponding value for the production of broccoli in Sweden in parentheses. The lower table shows examples of valuable substances in each crop, and in which amounts they can be found. DW: Dry weight, FW: Fresh Weight, GAE: Gallic acid equivalents.*

valorisation of the field waste. Especially green biomass has a large potential for refinement into nutritious and functional food ingredients or products, either through advanced biorefinery fractionation targeting valuable compounds or by more straightforward processing. Such processes would require further research and extensive knowledge of the process, and developed countries with available resources should invest and share their findings to help less industrialised countries reaching the climate goals. Through a biorefinery process cosmetically defect plants not fulfilling quality criteria, or overgrown plants unsuitable for direct consumption can be used. In some cases this might even be preferable, since the levels of dietary fibre and phenolic compounds depend on the part and maturity of the plant (Korus, 2011; Schäfer *et al.*, 2017). A more complete use of the biomass by valuing a crop not only for its main outcome, e.g. the broccoli florets, but also for the potential of side streams in other applications, would enable an increased primary productivity, without further hampering the environment.

To meet the temperature limits of 1.5 °C, a reduction of GHG emission with 65–80% is required from food and agricultural production in Western Europe (Bryngelsson *et al.*, 2016). A reduction of meat consumption with 50% in Western Europe, replacing it with more sustainable protein sources, is among the suggestions having the highest positive impact on the climate (Bryngelsson *et al.*, 2016; Röös *et al.*, 2018). In Sweden such a change would reduce the use of farmland by 23%, and the climate impact of Swedish food consumption by 20% (Röös *et al.*, 2018). To enable this, novel and sustainable protein sources, preferably of vegetal origin, are needed. Protein from green biomass has potential as a sustainable option, with high nutritive value and functional properties of interest in food applications (Pouvreau *et al.*, 2014). It could be argued that the consumers would prefer leaf proteins from familiar crops, such as broccoli and kale, to other proposed protein sources, e.g. insects.

As stated previously kale and broccoli contain high levels of health promoting compounds, including proteins, dietary fibre, and phenolic compounds. Fractionation of those compounds would give valuable ingredients useful in the manufacturing of healthier food options. Dietary fibre supplements added to regular food products can improve consumers' health (Kim and Je, 2016) leading to a healthier diet without considerable effort. Natural phenolic compounds with antioxidative effects comparable to synthetic antioxidants used in food production can be extracted from broccoli (Balasundram et al., 2006), reducing the need of synthetic ingredients and moving towards more natural food. Side stream based ingredients would have a double value, including the valuable compound, as well as the added value of the striving for a sustainable and high-producing agriculture.

Bryngelsson *et al* (2016) estimate only a 1-3% decrease of GHG emissions by a 50% reduction of food waste. However, these calculations do not include the impact of reducing or utilising the field waste available. This is a reoccurring issue in the research around food waste. Most studies on waste in food production are made in few countries and usually only including the later parts of the food supply chain, i.e. do not include field waste (Xue *et al.*, 2017). The limited data available on the total amounts of waste has the consequence that it is difficult to reach a consensus about the situation in the global food production (Parfitt *et al.*, 2010), and what effects a presumed reduction would give. In line with the goal of a maximum temperature rise of 1.5 °C, the potential uses of field waste in food production needs to be evaluated. Furthermore, consumer aspects of the suggested changes in food production, utilizing green plant material side streams for high value food products need investigations as studies in this field do practically not exist.

**Conclusion**

In this paper we have focused on possible utilisation of side streams from two horticultural products: broccoli and kale, although the concept of using side streams for food purposes is applicable for many other crops. Up to this date, kale and broccoli have not been reported in the literature, as a raw material for protein extraction processes, despite their potential for such uses. Some studies are available, describing extraction of phenolic compounds and dietary fibre from part of their side streams, i.e. leaves. However, side streams in broccoli and kale production are a readily available and inexpensive resource with great potential for valorisation. The use of kale and broccoli side streams in a biorefinery process would enable using the total produced biomass, resulting in a sustainable and resource efficient production of food ingredients, nutrients and compounds for the food industry. These finding are in line

with the work towards a sustainable food production for an increasing world population, thereby contributing to reducing the size of the undernourished population in the world, as well as promoting an ethical consumption in the Western world.

## References


Aires, A., Carvalho, R. and Saavedra, M. J. (2017). Reuse potential of vegetable wastes (broccoli, green bean and tomato) for the recovery of antioxidant phenolic acids and flavonoids. *International Journal of Food Science & Technology*, 52(1), pp 98–107.

Balasundram, N., Sundram, K. and Samman, S. (2006). Phenolic compounds in plants and agri-industrial by-products: Antioxidant activity, occurrence, and potential uses. *Food Chemistry*, 99(1), pp 191–203.

Bhandari, S. R. and Kwak, J.-H. (2015). Chemical Composition and Antioxidant Activity in Different Tissues of Brassica Vegetables. *Molecules*, 20(1), pp 1228–1243.

Bryngelsson, D., Wirsenius, S., Hedenus, F. and Sonesson, U. (2016). How can the EU climate targets be met? A combined analysis of technological and demand-side changes in food and agriculture. *Food Policy*, 59, pp 152–164.

Campas-Baypoli, O. N., Sanchez-Machado, D. I., Bueno-Solano, C., Nunez-Gastelum, J. A., Reyes-Moreno, C. and Lopez-Cervantes, J. (2009). Biochemical composition and physicochemical properties of broccoli flours. *International Journal of Food Sciences and Nutrition*, 60, pp 163–173.

Drabińska, N., Ciska, E., Szmatowicz, B. and Krupa-Kozak, U. (2018). Broccoli by-products improve the nutraceutical potential of gluten-free mini sponge cakes. *Food Chemistry*, 267, pp 170–177 (1st Food Chemistry Conference: Shaping the future of food quality, health and safety).

Edwards, R. H., Miller, R. E., De Fremery, D., Knuckles, B. E., Bickoff, E. M. and Kohler, G. O. (1975). Pilot plant production of an edible white fraction leaf protein concentrate from alfalfa. *Journal of Agricultural and Food Chemistry*, 23(4), pp 620–626.

Elleuch, M., Bedigian, D., Roiseux, O., Besbes, S., Blecker, C. and Attia, H. (2011). Dietary fibre and fibre-rich by-products of food processing: Characterisation, technological functionality and commercial applications: A review. *Food Chemistry*, 124(2), pp 411–421.

Evans, J. R. (1989). Photosynthesis and nitrogen relationships in leaves of C3 plants. *Oecologia*, 78(1), pp 9–19.

FAO (2014). *Mitigation of food wastage – societal costs and benefits*. Rome: Food and Agriculture organization of the United Nation (FAO). ISBN 978-92-5-108511-0.

FAO, IFAD, UNICEF, WFP and WHO (2017). *The State of Food Security and Nutrition in the World 2017. Building resilience for peace and food security*. Rome: Food and Agriculture organization of the United Nation (FAO).

Fernández-Ponce, M. T., Parjikolaei, B. R., Lari, H. N., Casas, L., Mantell, C. and Martínez de la Ossa, E. J. (2016). Pilot-plant scale extraction of phenolic compounds from mango leaves using different green techniques: Kinetic and scale up study. *Chemical Engineering Journal*, 299, pp 420–430.

Garnett, T. (2011). Where are the best opportunities for reducing greenhouse gas emissions in the food system (including the food chain)? *Food Policy*, 36, pp S23–S32 (The challenge of global food sustainability).

Gustavsson, J., Cederberg, C. and Sonesson, U. (2011). *Global food losses and food waste: extent, causes and prevention ; study conducted for the International Congress Save Food! at Interpack 2011, [16 - 17 May], Düsseldorf, Germany*. Rome: Food and Agriculture Organization of the United Nations. ISBN 978-92-5-107205-9.

Hojilla-Evangelista, M. P., Selling, G. W., Hatfield, R. and Digman, M. (2017). Extraction, composition, and functional properties of dried alfalfa (Medicago sativa L.) leaf protein. *Journal of the Science of Food and Agriculture*, 97(3), pp 882–888.

Johansson, E., Prade, T., Angelidaki, I., Svensson, S.-E., Newson, W. R., Gunnarsson, I. B. and Hovmalm, H. P. (2015). Economically Viable Components from Jerusalem Artichoke (Helianthus tuberosus L.) in a Biorefinery Concept. *International Journal of Molecular Sciences*, 16(4), pp 8997–9016.

Kim, Y. and Je, Y. (2016). Dietary fibre intake and mortality from cardiovascular disease and all cancers: A meta-analysis of prospective cohort studies. *Archives of Cardiovascular Diseases*, 109(1), pp 39–54.

Korus, A. (2011). Level of vitamin C, polyphenols, and antioxidant and enzymatic activity in three varieties of kale (Brassica oleracea l. var. Acephala) at different stages of Maturity. *International Journal of Food Properties*, 14(5), pp 1069–1080.

Kummu, M., de Moel, H., Porkka, M., Siebert, S., Varis, O. and Ward, P. J. (2012). Lost food, wasted resources: Global food supply chain losses and their impacts on freshwater, cropland, and fertiliser use. *Science of the Total Environment*, 438, pp 477–489.

Kyle, J. A. M., Sharp, L., Little, J., Duthie, G. G. and McNeill, G. (2010). Dietary flavonoid intake and colorectal cancer: a case–control study. *British Journal of Nutrition*, 103(3), pp 429–436.

Lin, L.-Z. and Harnly, J. M. (2010). Phenolic Component Profiles of Mustard Greens, Yu Choy, and 15 Other Brassica Vegetables. *Journal of agricultural and food chemistry*, 58(11), pp 6850–6857.

Liu, M., Zhang, L., Ser, S. L., Cumming, J. R. and Ku, K.-M. (2018). Comparative phytonutrient analysis of broccoli by-products: The potentials for broccoli by-product utilization. *Molecules*, 23(4), p 18.

Martin, A. H., Castellani, O., de Jong, G. A., Bovetto, L. and Schmitt, C. (2019). Comparison of the functional properties of RuBisCO protein isolate extracted from sugar beet leaves with commercial whey protein and soy protein isolates. *Journal of the Science of Food and Agriculture*, 99(4), pp 1568–1576.

Masson-Delmotte, V. P, P. Zhai, H.-O. Pörter, D. Roberts, J. Skea, P.R. Shukla, A. Pirani, W. Moufouma-Okia, C. Péan, R. Pidcock, S. Connors, J.B.R. Matthews, Y. Chen, X. Zhou, M.I. Gomis, E. Lonnoy, T. Maycock, M. Tignor and T. Waterfields (2018). *Global Warming of 1.5°C. An IPCC Special Report on the impacts of global warming of 1.5°C above pre-industrial levels and related global greenhouse gas emission pathways, in the context of strengthening the global response to the threat of climate change, sustainable development, and efforts to eradicate poverty*. In press: The Intergovernmental Panel on Climate Change (IPCC).

Parfitt, J., Barthel, M. and Macnaughton, S. (2010). Food waste within food supply chains: quantification and potential for change to 2050. *Philosophical Transactions of the Royal Society B-Biological Sciences*, 365(1554), pp 3065–3081.

Perez-Jimenez, J., Serrano, J., Tabernero, M., Arranz, S., Diaz-Rubio, M. E., Garcia-Diz, L., Goni, I. and Saura-Calixto, F. (2009). Bioavailability of Phenolic Antioxidants Associated with Dietary Fiber: Plasma Antioxidant Capacity After Acute and Long-Term Intake in Humans. *Plant Foods for Human Nutrition*, 64(2), pp 102–107.

Persson, J. (2018). *Trädgårdsproduktion 2017 (The 2017 Horticultural Census)* [online]. Jordbruksverket. (JO 33 SM 1801).

Peschel, W., Sánchez-Rabaneda, F., Diekmann, W., Plescher, A., Gartzía, I., Jiménez, D., Lamuela-Raventós, R., Buxaderas, S. and Codina, C. (2006). An industrial approach in the search of natural antioxidants from vegetable and fruit wastes. *Food Chemistry*, 97(1), pp 137–150.

Pingret, D., Fabiano-Tixier, A.-S., Bourvellec, C. L., Renard, C. M. G. C. and Chemat, F. (2012). Lab and pilot-scale ultrasound-assisted water extraction of polyphenols from apple pomace. *Journal of Food Engineering*, 111(1), pp 73–81.

Pirie, N. W. (1966). Leaf Protein as a Human Food. *Science*, 152(3730), pp 1701–1705.

Pouvreau, L., Smit, B. and Velde, F. van de (2014). Securing Food Proteins: From by-Products to Functional Ingredient. *Gums and Stabilisers for the Food Industry 17*. pp 46–51.

Rönnlund, I., Pursula, T., Bröckl, M., Hakala, L., Luoma, P., Aho, M., Pathan, A. and Pallesen, B. E. (2014). *Creating value from bioresources:*

*Innovation in Nordic Bioeconomy* [online]. Oslo: Nordic Innovation. (2014:01).
Röös, E., Carlsson, G., Ferawati, F., Hefni, M., Stephan, A., Tidåker, P. and Witthöft, C. (2018). Less meat, more legumes: prospects and challenges in the transition toward sustainable diets in Sweden. *Renewable Agriculture and Food Systems*, pp 1–14.
Schäfer, J., Stanojlovic, L., Trierweiler, B. and Bunzel, M. (2017). Storage related changes of cell wall based dietary fiber components of broccoli (Brassica oleracea var. italica) stems. *Food Research International*, 93, pp 43–51.
Selma, M. V., Espín, J. C. and Tomás-Barberán, F. A. (2009). Interaction between Phenolics and Gut Microbiota: Role in Human Health. *Journal of Agricultural and Food Chemistry*, 57(15), pp 6485–6501.
Shen, T., Han, X.-Z., Wang, X.-N., Fan, P.-H., Ren, D.-M. and Lou, H.-X. (2017). Protective Effects of Dietary Polyphenols in Human Diseases and Mechanisms of Action. In: Al-Gubory, K. H. and Laher, I. (Eds) *Nutritional Antioxidant Therapies: Treatments and Perspectives*. pp 307–345. Cham: Springer International Publishing. ISBN 978-3-319-67623-4.
Stephen, A. M., Champ, M. M.-J., Cloran, S. J., Fleith, M., van Lieshout, L., Mejborn, H. and Burley, V. J. (2017). Dietary fibre in Europe: current state of knowledge on definitions, sources, recommendations, intakes and relationships to health. *Nutrition Research Reviews*, 30(02), pp 149–190.
Strid, I., Eriksson, M., Andersson, S. and Olsson, M. (2014). *Svinn av isbergssallat i primärproduktionen och grossistledet i Sverige*. Jordbruksverket. (2014:06).
Stødkilde, L., Damborg, V. K., Jørgensen, H., Laerke, H. N. and Jensen, S. K. (2018). White clover fractions as protein source for monogastrics: dry matter digestibility and protein digestibility-corrected amino acid scores. *Journal of the Science of Food and Agriculture*, 98(7), pp 2557–2563.
United Nations (2017). *World Population Prospects: The 2017 Revisiom, Key Findings and Advance Tables.* [online]. United Nations, Department of Economic and Social Affairs, Population Division. (Working paper No. ESA/P/WP/248).
Vilar, M., Cartea, M. E., Padilla, G., Soengas, P. and Velasco, P. (2007). The potential of kales as a promising vegetable crop. *Euphytica*, 159(1), p 153.
Virot, M., Tomao, V., Le Bourvellec, C., Renard, C. M. C. G. and Chemat, F. (2010). Towards the industrial production of antioxidants from food processing by-products with ultrasound-assisted extraction. *Ultrasonics Sonochemistry*, 17(6), pp 1066–1074 (Sonochemistry: Scale up and industrial development).
Wang, S., Melnyk, J. P., Tsao, R. and Marcone, M. F. (2011). How natural dietary antioxidants in fruits, vegetables and legumes promote vascular health. *Food Research International*, 44(1), pp 14–22.
Williamson, G. (2017). The role of polyphenols in modern nutrition. *Nutrition Bulletin*, 42(3), pp 226–235.
Xu, Y., Li, Y., Bao, T., Zheng, X., Chen, W. and Wang, J. (2017). A recyclable protein resource derived from cauliflower by-products: Potential biological activities of protein hydrolysates. *Food Chemistry*, 221, pp 114–122.
Xue, L., Liu, G., Parfitt, J., Liu, X., Van Herpen, E., Stenmarck, Å., O'Connor, C., Östergren, K. and Cheng, S. (2017). Missing Food, Missing Data? A Critical Review of Global Food Losses and Food Waste Data. *Environmental Science & Technology*, 51(12), pp 6618–6633.
Yang, J., Martinez, I., Walter, J., Keshavarzian, A. and Rose, D. J. (2013). In vitro characterization of the impact of selected dietary fibers on fecal microbiota composition and short chain fatty acid production. *Anaerobe*, 23, pp 74–81.
Yang, Y., Ma, S., Wang, X. and Zheng, X. *Modification and Application of Dietary Fiber in Foods*. [online] (2017) (Journal of Chemistry). Available from: https://www.hindawi.com/journals/jchem/2017/9340427/. [Accessed 2019-02-15].

# II

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*Article*

# Protein Fractionation of Green Leaves as an Underutilized Food Source—Protein Yield and the Effect of Process Parameters

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**Abstract:** Green biomass has potential as a sustainable protein source for human consumption, due to its abundance and favorable properties of its main protein, RuBisCO. Here, protein fractionation outcomes of green leafy biomass from nine crops were evaluated using a standard protocol with three major steps: juicing, thermal precipitation, and acid precipitation. Successful protein fractionation, with a freeze-dried, resolubilized white protein isolate containing RuBisCO as the final fraction, was achieved for seven of the crops, although the amount and quality of the resulting fractions differed considerably between crops. Biomass structure was negatively correlated with successful fractionation of proteins from biomass to green juice. The proteins in carrot and cabbage leaves were strongly associated with particles in the green juice, resulting in unsuccessful fractionation. Differences in thermal stability were correlated with relatedness of the biomass types, e.g., *Beta vulgaris* varieties showed similar performance in thermal precipitation. The optimal pH values identified for acid precipitation of soluble leaf proteins were lower than the theoretical value for RuBisCO for all biomass types, but with clear differences between biomass types. These findings reveal the challenges in using one standard fractionation protocol for production of food proteins from all types of green biomass and indicate that a general fractionation procedure where parameters are easily adjusted based on biomass type should instead be developed.

**Keywords:** leaf protein extraction; RuBisCO; green biorefinery; leaf protein concentrate; white protein precipitation; thermal protein precipitation

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## 1. Introduction

In Europe and beyond, consumer preferences are shifting to increased consumption of plant-based instead of animal-based protein [1,2]. This shift has resulted in a multitude of novel products appearing on supermarket shelves. However, these novel food products, like protein-based animal feed products currently in use, are largely based on soy protein [3], the majority of which is grown in America and Asia [4]. Thus, replacement of soy with locally produced plant protein would contribute to food independence for the country of production, while a reduction in transportation could lead to increased sustainability [5]. Valorization of green leaves by protein extraction has been a concept since the 1940s [6], but has gained increasing attention in recent decades as an additional plant protein source for food and feed [7,8]. Green leaves are a major source of biomass worldwide and also one of the largest side-streams from modern agricultural and horticultural production. For example, only 20–50% of total plant biomass of broccoli is currently harvested and used for food [9], and similar percentages can be expected for crops such as carrot, beetroot, sugarbeet, and cabbage. Increased and diversified use of the residual side-streams would increase sustainability and profitability in crop production.

Fresh green leaves consist of 1.6–8.2% protein, with large variation between species [8]. The major protein in all green leaves is the enzyme ribulose-1,5-bisphosphate

carboxylase/oxygenase (RuBisCO), considered the most abundant protein in the world [10]. RuBisCO catalyzes uptake of $CO_2$ during photosynthesis and is therefore present in high amounts in all photosynthetic organisms. RuBisCO is an interesting target protein as a source for novel protein-rich foods, as it has a highly desirable amino acid composition for human consumption and functional properties resembling those of soy and whey protein [11–13].

Protein fractionation can be utilized to create value-added products from leaf proteins, resulting in (i) a green protein fraction, mainly consisting of membrane-bound proteins and chlorophyll-related proteins, and (ii) a "non-green" protein fraction of water-soluble proteins, mainly RuBisCO [14]. This fraction is commonly referred to as "white protein", a convention followed in this paper. The white protein fraction has been shown to be beneficial for humans, with high levels of essential amino acids [13,15,16]. It has also been shown to have functional properties useful in food applications, e.g., foaming, emulsification, and gelation [11–13,16–18].

Most published studies on protein fractionation from green biomass for food applications have applied a relatively gentle extraction process, to maintain the desirable functional properties of the proteins [18]. This process generally consists of three main steps (Figure 1): (i) pressing liquid from fresh or frozen green leaves to obtain a green juice (GJ); (ii) separating the green and white protein fractions by exploiting differences in thermal sensitivity of the proteins, to create white juice (WJ) and a green protein fraction [11,13,18–21]; and (iii) concentrating the white protein fraction in the WJ further, by heating at 80 °C [19], acid precipitation at pH between 3.5 and 4.5 [13,16,22–24], chromatography [20], or filtration [11,17].

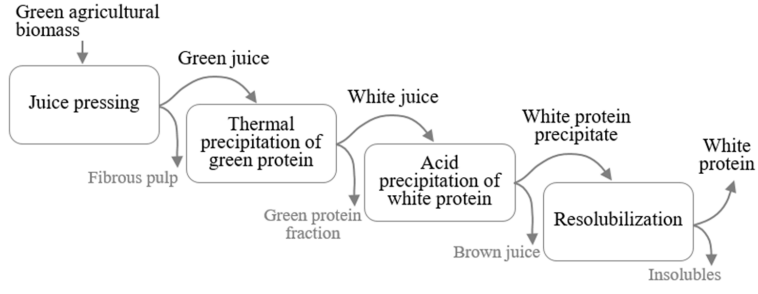


**Figure 1.** Main steps in a general process for producing white protein isolate from green agricultural biomass.

Industrial-scale protein fractionation of green leaves for food would require a year-round process where a range of different biomass types are used in a similar methodological system. In a Nordic context, biomass availability will clearly vary between seasons and a biorefinery would benefit from having multiple raw materials available. However, previous studies have mainly focused on green biomass of three crops (lucerne, sugarbeet, and spinach) for protein fractionation [11,13,15–18,21,23–32], with only a few studies focusing on other biomass sources (e.g., beetroot, broccoli, cauliflower, or cabbage leaves [33]). Thus, there is limited information available on how different biomass sources perform as raw material in protein extraction and how the extraction process may need to be adapted for individual sources.

The aim of the present study was to evaluate the role of green biomass source in protein fractionation and to characterize the background and underlying reasons for any differences between biomass types. In characterization, the emphasis was on protein precipitation temperatures suitable pH for white protein precipitation and on the flow of water, dry matter, and nitrogen through the extraction process. An overarching goal was to establish a basis for industrial use of green agricultural waste/side-streams for protein valorization into

food ingredients, additives, and products. The starting hypothesis was that any green leafy biomass can be used as raw material in a general protein extraction process.

## 2. Materials and Methods

### *2.1. Collection of Biomass*

To evaluate similarities and differences between a wide array of green biomass sources of possible use in a Nordic set-up for protein fractionation, nine different crops were selected (Table 1). Fresh leafy harvest residues from broccoli, cabbage, kale, carrot, beetroot, and sugarbeet were collected from fields in southern Sweden at the time of harvest. The mangold residues were over-mature and not suitable as a food ingredient, mainly due to cosmetic reasons, but did have a value as a green biomass. Lucerne (first cut) was collected from a field on campus in Alnarp, Sweden. Fresh baby spinach was included in the study as a model crop, as it has previously been shown to perform well in a protein extraction process, it was purchased from a supermarket. After collection, all biomass was rinsed with tapwater, frozen, and stored at −20 °C until processing. Samples for dry matter and nitrogen determination were collected prior to freezing, the details are described in Section 2.4. Further processing was done following the procedure described in 2.2, based on process parameters developed in Section 2.3.

**Table 1.** The nine types of agricultural green biomass evaluated in this study and their nitrogen (N) content on a dry matter (DM) basis.

| Biomass Source | | Collection Date | %N | %DM |
|---|---|---|---|---|
| Broccoli * | *Brassica oleracea,* var. *italica* | 2 Oct 2017 | 3.2 | 13.3 |
| Cabbage * | *Brassica oleracea,* var. *capitata* | 30 Aug 2017 | 2.1 | 11.0 |
| Kale * | *Brassica oleracea,* var. *sabellica* | 23 Oct 2017 | 3.0 | 13.3 |
| Mangold | *Beta vulgaris,* subsp. *vulgaris,* var. *cicla* | 30 Aug 2017 | 2.1 | 8.4 |
| Beetroot * | *Beta vulgaris,* subsp. *vulgaris,* var. *Red hawk* | 13 Sept 2017 | 3.2 | 9.9 |
| Sugarbeet * | *Beta vulgaris,* subsp. *vulgaris,* var. *Lombok* | 12 Oct 2018 | 3.0 | 13.0 |
| Carrot * | *Daucus carota* subsp. *sativus* | 28 June 2018 | 2.1 | 17.7 |
| Lucerne | *Medicago sativa* | 25 May 2018 | 2.8 | 20.9 |
| Spinach | *Spinacia oleracea* | Retail ** | 4.8 | 10.6 |

* True harvest residues; ** Italian produce.

### *2.2. Protein Fractionation from Green Leaves*

To enable direct comparisons of different green leaf sources during protein fractionation, the same protein fractionation procedure was applied for all crops. The methodology selected was mainly based on literature data [18,24,28,31], although with some modifications. In principle, the fractionation procedure comprised three steps; screw pressing, thermal precipitation, and acid precipitation (see Sections 2.2.1–2.2.3). To investigate the background and reasons for differences in biomass behavior during protein fractionation, additional steps were included (see Section 2.3). A Sankey chart of the full process is shown in Figure 2.

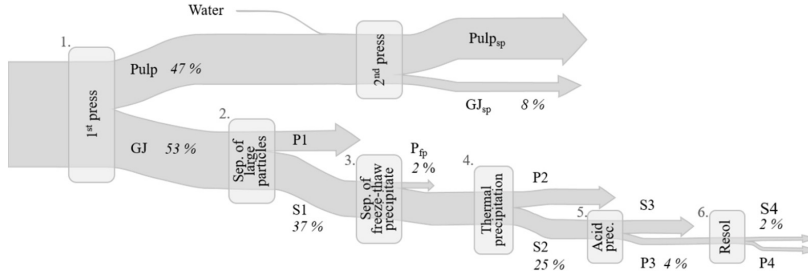


**Figure 2.** Sankey chart of the full protein extraction process, including all additional steps, with values for mangold as an example. Percentages shown indicate nitrogen flow through the process steps 1–6 (% of initial biomass nitrogen). GJ: green juice, S: supernatant, P: pellet, sp: second press, fp: freeze precipitate.

### 2.2.1. Juice Pressing

Frozen leaves of the selected crops were thawed and divided into three 300 g portions, representing process triplicates. Thawed, but still cold, leaves were juiced in a twin-screw press (Angelia 5500, Angel Co. Ltd., Busan Korea), and the green juice (GJ) obtained was centrifuged (11,800 RCF, 10 min, 4 °C), producing a particle-free supernatant (S1) containing water-soluble proteins, and a pellet with particles (P1). The extruded fibrous pulp was mixed with MilliQ water corresponding to 50% (or 100% for carrot) of its mass after sampling and re-fed to the juicer. As in the first juicing, the second press resulted in a green juice ($GJ_{sp}$), which was collected and centrifuged into an $S1_{sp}$ and $P1_{sp}$ fraction. Portions of all samples were freeze-dried for dry matter determination and analysis of nitrogen content. The additional S1 juices were stored at −20 °C until further processing.

### 2.2.2. Thermal Precipitation

Subsamples of thawed S1 juice (40 mL) from each crop and process replicate were transferred to triplicate 50 mL conical tubes and centrifuged (3200 RCF, 10 min, 4 °C) to remove particles resulting from protein precipitation during freezing ($P_{fp}$). The supernatant in each tube was transferred to a new tube and thermally treated in a water bath at 55 °C for 25 min. The thermally treated samples were immediately cooled on ice and centrifuged (3200 RCF, 10 min, 4 °C), resulting in a supernatant (S2) containing the white protein fraction, and a pellet (P2) containing the green protein fraction. The supernatants were frozen and stored at −20 °C until further processing. Aliquots of the S2 supernatant and the pellets ($P_{fp}$ and P2) were freeze-dried and analyzed for dry matter and nitrogen content, while the remaining S2 supernatant was retained for further processing.

### 2.2.3. Acid Precipitation and Resolubilization of the White Protein Fraction

Part of the S2 fraction of each crop and their process replicates were pH-adjusted to 4.5 by drop-wise addition of 1 M HCl, and the solution was divided into (A) a sample with total volume 1.5 mL for monitoring precipitation yield, and (B) a sample with volume 8.0 mL intended for resolubilization of the precipitated white protein (see Figure 2). The precipitated particles in both sample sets were separated by centrifugation (1150 RCF, 10 min, 4 °C), and the supernatant (S3) was separated from the pellet (P3). The S3 and P3 from sample A were weighed and frozen. The P3 from sample B was dispersed in 1 mL MilliQ water and the pH of the solution was adjusted to 7 through drop-wise addition of 0.1 M NaOH. Thereafter the sample was stirred at room temperature for 30 min, followed by gentle centrifugation (260 RCF, 5 min, 4 °C) to remove insoluble particles. The supernatant (S4) was poured off and the pellet (P4) was washed by addition of 1 mL MilliQ water, mixing, and another centrifugation step. The supernatant was then pooled with the first S4. To assess unintended protein precipitation due to freezing, a 1.5

mL sample of S2 from each crop and process replicate was treated following the same procedure as described for sample A, but without pH adjustment. The supernatant and pellet from this test are referred to as $S_{fp2}$ and $P_{fp2}$. All samples produced (S3 and P3 from sample A, $S_{fp2}$, $P_{fp2}$, S4, and P4) were freeze-dried and the dry matter and nitrogen content were determined.

### *2.3. Thermal and Acid Precipitation Tests to Determine Differences between Biomass Sources*

#### 2.3.1. Thermal Precipitation

The S1 fraction from the different crops and their processing replicates were thawed on ice and 100 µL aliquots were transferred to 300 µL micro-Eppendorf tubes. For each crop and processing replicate, three tubes per temperature were placed in a water bath at 40, 45, 50, 55, 60, 65, 70, or 80 °C for 10 min, and placed on ice immediately afterwards. A reference sample corresponding to 0 °C was left on ice. The samples were centrifuged (1900 RCF, 10 min, 4 °C) to separate the precipitated protein from the soluble proteins. The protein concentration in the supernatant of each sample was analyzed in triplicate using a bicinchoninic acid protein assay kit (Pierce BCA protein assay, Thermo Scientific, USA) in 96-well format according to the manufacturer's instructions, using a Multiskan GO spectrophotometer (ThermoFisher Scientific, Vantaa, Finland).

#### 2.3.2. Acid Precipitation

Part of the S2 fraction was filtered through a 0.45 µm syringe filter to remove particles. The filtrate was diluted in MilliQ water to reach a protein concentration of around 1 mg/mL ($W_{prot}/V$), transferred to a disposable capillary zeta cell (Malvern Instruments, Malvern, UK), and analyzed with a Zetasizer nano ZS (Malvern Instruments, Malvern, UK) coupled with an autotitration unit (MPT-2, Malvern). The pH of the solution was changed in steps of 0.5 units using 0.1 and 0.01 M HCl, and particle size and their zeta potential (ZP) were measured at each step. Duplicate measurements were made for two process replicates, and at each pH value triplicate particle size and ZP measurements were averaged.

#### 2.3.3. SDS-PAGE Analysis

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) analysis was performed using an analysis kit with pre-cast gradient mini-gels (Invitrogen Novex Bolt, 4–12%, Bis-Tris Plus, Thermo Fisher Scientific, Waltham, MA, USA), and a protein ladder (Invitrogen SeeBlue®). A voltage of 145 V was applied during 35 min for separation. The protein bands were stained for 15 min (GelCode™ Blue Safe protein stain, Thermo Scientific, USA), and the gels were washed overnight. For SDS-PAGE analysis of freeze-dried protein concentrates, the material was dissolved in MilliQ water to reach a concentration of 4 mg dry material/mL and the samples were mixed at room temperature for 10 min before analysis. In SDS-PAGE analysis, the RuBisCO subunits are found at ~55 kDa and ~14 kDa [10].

### *2.4. Dry Matter and Nitrogen Determinations*

Fresh biomass samples of 3 or 5 g were oven-dried in duplicate or triplicate at 110 or 130 °C. All other samples were freeze-dried. All samples were weighed before and after drying, and the dry matter content was calculated. Nitrogen (N) determination was carried out on dried samples through applying the Dumas method on a Flash 2000 NC Analyzer (Thermo Fisher Scientific, Waltham, MA, USA) in duplicate. In this study the N content is presented, rather than protein content, since the conversion factor (N content to protein content) was not known for the materials used.

### *2.5. Yield Calculations*

Process yield of total N and of total dry and wet matter, for each specific process step and for the full process, was calculated as:

$$\text{Yield}_{X,F} = \frac{X_{F,out}}{X_{F,in}} \quad (1)$$

where X is total N, total dry matter, or total wet matter, and F is the process step, or steps, for which the yield is calculated. The yields were calculated for each process replicate separately and the mean values ± standard deviation are presented, with the numbers of replicates as stated in the method descriptions above.

### *2.6. Calculation of Theoretical pI*

The theoretical isoelectric point (pI) for spinach RuBisCO was determined using the online pI calculation tool ProtParam on the Expasy server (web.expasy.org/cgi-bin/protparam/protparam, accession data 5 june 2020) [34]. The amino acid sequences were taken from the UniProt database under the entries P00870 (small subunit) and P00875 (large subunit) (www.uniprot.org, accession date 5 June 2020).

### *2.7. Statistical evaluation*

Flows of N, dry matter, and total mass through the process for the different crops were compared using general linear model analysis of variance (ANOVA) with Kenward-Roger's method and a significance level of $p < 0.05$, followed by Tukey post-hoc test. All statistical analyses were performed in RStudio version 1.4.1106 [35], using the function packages lme4, emmeans, lmerTest, multcomp, and multcompView. The Kenward-Roger´s method was chosen due to the complex correlation structures of the data [36].

## 3. Results and Discussion

### *3.1. Effect of Biomass Source on Protein Fractionation*

Protein fractionation with the selected method was successful for seven out of nine of the green biomass sources evaluated, as demonstrated by the N yield from the original biomass (BM) to re-dissolved white protein (S4) (Table 2a, Supplementary Figure S2). For carrot and cabbage protein fractionation was not successful, as shown by the low N yield and lack of RuBisCO in S4 from these crops (Figure 3). Thus, the origin of green leafy biomass had a large impact on the protein fractionation outcome, contradicting the hypothesis that any green leafy biomass can be used as raw material in a general protein extraction process. Other studies have achieved successful protein extraction from, e.g., cabbage, by applying different extraction methods [33].

The protein fractionation results also differed significantly for the biomass types that were successfully fractionated (Table 2a), confirming that biomass source had a large impact on the outcome of the fractionation process. Presence of RuBisCO was detected in all biomass types, in the white juices (Figure 4, Supplementary Figure S1), and in the white protein fraction of the substrates for which the process was successful (Figure 3).

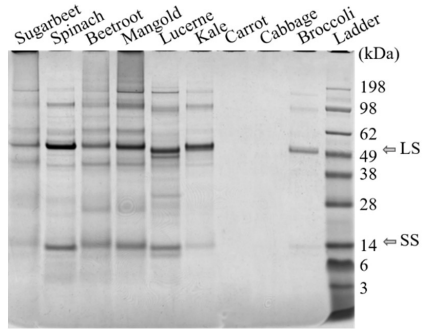


**Figure 3.** Results of SDS-PAGE analysis of freeze-dried white protein isolates (S4, see Figure 2) dissolved in water. The RuBisCO subunits are at ~55 kDa and ~14 kDa. LS: large subunit, SS: small subunit.

Of the nine biomass types evaluated, mangold leaves resulted in the highest N yield after the full process, with 1.9% of initial N in the biomass recovered in the white extract (S4) (Table 2a). The corresponding value for lucerne, the crop with the second highest level, was 1.5%. The highest yield of soluble N from the particle-free green juice (S1) to the final white protein (S4) (Supplementary Table S1) was seen for sugarbeet (7.5%). Previous studies evaluating white protein extracts from green biomass often report higher yields than those obtained in the present study. However, comparison of N yields between studies was hampered by the fact that i) extraction methods generally differ between studies, ii) a limited number of crop biomass types (mainly sugarbeet, lucerne, and spinach) have been extensively studied in this regard, and iii) different combinations of methods and fractions are used for the calculations. Thus, results are rarely comparable in practice. In a previous study, juicing of sugarbeet leaves followed by acid and heat precipitation of the white protein fraction (a procedure partly comparable to steps 1 and 5 in Figure 2) resulted in N yield of ~25% [16]. In another study using sugarbeet leaves, heat precipitation of the green protein fraction and ultracentrifugation to concentrate the white protein (steps 1 and 4 in Figure 2) resulted in N yield of ~12% [11]. Thus, the fraction used to determine the N yield in those two studies was the most similar to our S2 fraction (WJ), but not our final resolubilized extract S4 (Table 2a). The N yield in S2 in the present study was 24.6% for mangold, 20.0% for lucerne, and 11.7% for sugarbeet, which is comparable to previous findings [11,16].

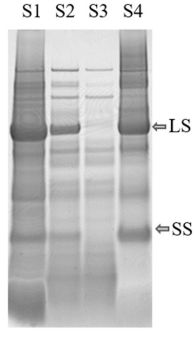


**Figure 4.** Protein content in mangold leaves, as determined by SDS-PAGE analysis of supernatants S1-S4 from the extraction process (see Figure 2). LS: large subunit, SS: small subunit of RuBisCO.

Mangold and lucerne not only had the highest N yields in this study, but also the highest N content in the white protein isolate (S4), with values of 4.0% and 3.6%,

respectively (Table 2c). Higher N content has been reported in other studies fractionating white protein from sugarbeet, e.g., 9.6% and 7.6% [16,28], and from lucerne, e.g., 11.2%, 10.7%, 11.8%, and 14.8% [11,15,24,37]. As noted above for N yield, comparisons between studies are difficult, although a product mostly corresponding to fraction P3 in this study is often used as an end-point in other studies. The N content in P3 was between 5.1 and 12.5% for the different crops evaluated, corresponding well with previous findings.

To understand and characterize the background and reasons for differences in protein fractionation between the nine green biomass types studied, in this study additional steps were included in the standard fractionation method (as outlined in Figure 2).

**Table 2.** (a) Yield (%) of nitrogen (N) relative to N content in initial biomass (BM) in the different process steps (see Figure 2). Mean of three process replicates (unless otherwise indicated by superscript numbers). (b) Yield (%) of N relative to the ingoing material for each process step. (c) N content (%) on a dry matter basis (average of replicates) of the flows in the extraction process. Different letters (A–D) indicate significant differences ($p < 0.05$) in yield. Superscript numbers indicate numbers of replicates if not triplicates. GJ: green juice, $GJ_{sp}$: green juice from the second press, S1: particle-free green juice, $P_{fp}$: pellet with freeze-thaw precipitate, S2: supernatant after thermal treatment, P3: acid-precipitated white protein, S4: resolubilized protein fraction, P4: insoluble fraction.

| **(a)** | **Process Step** | | | | | |
|---|---|---|---|---|---|---|
| **Yield of N (%)** | **BM Pressing** | **Second BM Pressing** | **Separation of Particles** | **Thermal Precipitation** | **Acid Precipitation** | **Full Process** |
| Biomass | BM to GJ | BM to $GJ_{sp}$ | BM to S1 | BM to S2 | BM to P3 | BM to S4 |
| Broccoli | 28.2 ± 2.0 $^{BC}$ | 7.2 ± 1.5 $^{BC}$ | 14.8 ± 1.4 $^{BC}$ | 15.5± 2.2 $^{B}$ | 2.9 ± 0.7 $^{CD}$ | 0.4± 0.0 $^{A}$ |
| Cabbage | 37.4 ± 5.4 $^{C}$ | 7.9 ± 2.0 $^{BC}$ | 9.3 ± 2.4 $^{AB}$ | 14.4± 3.1 $^{AB}$ | 0.2 ± 0.1 $^{AB}$ | 0.2± 0.1 $^{A}$ |
| Kale | 30.3 ± 3.3 $^{BC}$ | 5.0 ± 0.2 $^{AB}$ | 19.3 ± 0.7 $^{CD}$ | 18.7± 0.7 $^{BC}$ | 2.1 ± 0.2 $^{ABCD}$ | 0.5± 0.1 $^{AB}$ |
| Mangold | 53.1 ± 1.0 $^{D}$ | 8.4 ± 0.6 $^{C}$ | 36.9 ± 1.3 $^{E}$ | 24.6± 4.6 $^{C}$ | 3.7 ± 1.6 $^{D}$ | 1.9± 1.1 $^{B}$ |
| Beetroot | 35.5 ± 2.1 $^{C}$ | 6.3 ± 0.4 $^{ABC}$ | 21.9 ± 2.1 $^{D}$ | 17.5± 0.3 $^{BC}$ | 3.8 ± 0.1 $^{D}$ | 1.0± 0.5 $^{AB}$ |
| Sugarbeet | 14.6 ± 4.0 $^{A}$ | 3.7 ± 0.6 $^{A}$ | 12.1 ± 1.5 $^{AB}$ | 11.72± 2.4 $^{AB}$ | 1.2$^{2}$ ± 0.3 $^{ABC}$ | 0.9$^{2}$± 0 $^{AB}$ |
| Carrot | 20.9 ± 2.7 $^{AB}$ | 5.3 ± 0.4 $^{AB}$ | 6.0 ± 0.5 $^{A}$ | 7.2± 0.4 $^{A}$ | 0.2 ± 0.2 $^{A}$ | 0.1± 0.1 $^{A}$ |
| Lucerne | 52.1 ± 5.4 $^{D}$ | 15.1 ± 0.9 $^{D}$ | 42.9 ± 4.4 $^{E}$ | 20.0± 0.9 $^{BC}$ | 3.3$^{1}$ $^{BCD}$ | 1.5$^{1}$ $^{AB}$ |
| Spinach | 27.6 ± 5.0 $^{BC}$ | 8.9 ± 1.3 $^{C}$ | 25.1 ± 0.4 $^{D}$ | 18.0± 3.8 $^{BC}$ | 3.0$^{2}$ ± 0.1 $^{CD}$ | 1.0$^{2}$± 0.4 $^{AB}$ |

| **(b)** | **Process step** | | | |
|---|---|---|---|---|
| **Yield of N (%)** | **Separation of particles** | **Separation of freeze-thaw precipitate** | **Thermal precipitation** | **Acid precipitation** |
| Biomass | GJ to S1 | S1 to $P_{fp}$ | S1 to S2 | S2 to P3 |
| Broccoli | 52.6 ± 4.2 $^{AB}$ | 12.6± 1.9 $^{A}$ | 84.2± 0.5 $^{CD}$ | 18.8± 1.7 $^{BC}$ |
| Cabbage | 24.9 ± 5.9 $^{A}$ | 42.2± 14.1 $^{B}$ | 89.9± 1.8 $^{DE}$ | 1.2± 1.1 $^{A}$ |
| Kale | 64.1 ± 6.4 $^{BC}$ | 6.5± 1.4 $^{A}$ | 84.3± 2.5 $^{CD}$ | 11.4± 1.2 $^{B}$ |
| Mangold | 69.6 ± 3.6 $^{BC}$ | 4.6± 3.9 $^{A}$ | 52.0± 6.0 $^{A}$ | 15.2± 5.4 $^{BC}$ |
| Beetroot | 61.6 ± 3.5 $^{BC}$ | 6.0± 6.0 $^{A}$ | 84.9± 16.1 $^{CD}$ | 21.6± 0.3 $^{C}$ |
| Sugarbeet | 79.7 ± 31.4 $^{BC}$ | 1.2± 0.2 $^{A}$ | 61.4$^{2}$± 13.2 $^{ABC}$ | 10.5$^{2}$± 0.5 $^{AB}$ |
| Carrot | 29.2 ± 5.1 $^{A}$ | 1.8± 0.1 $^{A}$ | 110.6± 8.0 $^{E}$ | 2.8± 2.4 $^{A}$ |
| Lucerne | 82.5 ± 8.5 $^{BC}$ | 7.7± 1.5 $^{A}$ | 52.2± 4.4 $^{AB}$ | 16.1$^{1}$ $^{BC}$ |
| Spinach | 93.3 ± 17.9 $^{C}$ | 7.1± 0.7 $^{A}$ | 79.5± 10.1 $^{BCD}$ | 16.9$^{2}$± 4.0 $^{BC}$ |

| **(c)** | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **% N** | **Flow in the process** | | | | | | | | | | |
| **Biomass** | **BM *** | **GJ** | **Pulp** | **$GJ_{sp}$** | **$Pulp_{sp}$** | **S1** | **$P_{fp}$** | **S2** | **P3** | **S4** | **P4** |
| Broccoli | 3.2 | 2.1± 0.1 | 1.9± 0.2 | 2.4± 0.1 | 1.9± 0.2 | 1.7± 0.1 | 5.8± 0.2 | 2.0± 0.2 | 7.9± 0.2 | 1.8± 0.4 | 13.6± 0.2 |
| Cabbage | 2.1 | 1.7± 0.2 | 1.2± 0.1 | 1.5± 0.3 | 1.3± 0.2 | 0.5± 0.1 | 8.2± 0.3 | 0.8± 0.2 | 1.2± 0.0 | 0.9± 0.1 | 0.7± 0.5 |
| Kale | 3.0 | 2.7± 0.2 | 1.5± 0.2 | 2.9± 0.1 | 2.2± 0.1 | 2.2± 0.0 | 1.7± 0.1 | 2.3± 0.1 | 7.9± 0.4 | 2.6± 0.4 | 12.3± 1.2 |
| Mangold | 2.1 | 2.3± 0.1 | 2.4± 0.4 | 2.6± 0.2 | 1.4± 0.1 | 1.9± 0.2 | 4.3± 0.3 | 1.6± 0.3 | 7.2± 0.6 | 3.6± 0.9 | 13.7± 0.4 |
| Beetroot | 3.2 | 3.0± 0.0 | 3.3± 0.2 | 3.6± 0.1 | 1.6± 0.2 | 2.6± 0.1 | 5.5± 0.3 | 2.2± 0.1 | 9.2± 0.7 | 3.3± 0.7 | 14.0± 0.3 |
| Sugarbeet | 3.0 | 1.1± 0.2 | 1.3± 0.3 | 1.6± 0.2 | 2.6± 0.5 | 1.0± 0.1 | 5.7± 0.4 | 1.1± 0.1 | 2.6$^{2}$± 1.5 | 2.3$^{2}$± 0.9 | 0.5$^{2}$± 0.1 |
| Carrot | 2.5 | 1.5± 0.1 | 2.1± 0.0 | 2.3± 0.1 | 3.2± 0.3 | 0.6± 0.1 | 8.0± 0.2 | 0.6± 0.0 | 1.1± 0.4 | 0.6± 0.1 | |
| Lucerne | 2.8 | 3.8± 0.3 | 2.0± 0.1 | 4.1± 0.1 | 5.1± 0.2 | 4.3± 0.1 | 8.0± 0.0 | 3.2± 0.1 | 7.2$^{1}$ | 4.0$^{1}$ | 12.9$^{1}$ |
| Spinach | 5.1 | 4.4± 0.3 | 4.9± 0.2 | 4.1± 0.1 | 1.5± 0.3 | 4.2± 0.3 | 2.9± 0.5 | 4.0± 0.2 | 8.1± 1.8 | 3.3± 0.5 | 14.1± 0.5 |

* Biomass N composition was assumed to be the same in all process replicates. Standard deviations for biomass % N are found in Table 1.

### *3.2. Differences in Protein Fractionation between Biomass Types*

#### 3.2.1. Juice Pressing

Juice pressing resulted in large variation between the crops in terms of N, total mass, wet mass, dry matter, and N yield, etc., from the biomass to GJ (Table 2), even for crops of the same species, e.g., for the *Beta vulgaris* varieties the N yield ranged from 15% for sugarbeet to 53% for mangold (Table 2, Supplementary Table S1). Variation in N yield between the biomass types evaluated in this study may be partly explained by structural differences in the biomass of various crops in terms of cell wall strength and water content. Previous studies have shown a significant negative correlation between maturity and protein extractability in other crops, e.g., clover, timothy, and chicory [38], so differences in maturity and cell wall thickness might also partly explain the differences between crops in this study. Therefore, factors such as biomass structural features, crop maturity, and cell wall thickness should be considered in industrial applications involving green biomass protein fractionation, especially when using crops, such as lucerne, which may be harvested several times during the season.

Higher total N yields were achieved from all biomass types when an additional juicing step was included (i.e., adding water to the extruded pulp and re-feeding it to the screw press), although the recovery varied (Table 2a). The effect was most prominent for lucerne, where the second press resulted in an N yield of 15% from BM to $GJ_{sp}$. This increased the total N yield from juicing of lucerne to 67% (GJ + $GJ_{sp}$), which is similar to previous findings of 47% and 50% N yield in GJ for lucerne [24,25] and 69% for sugarbeet [18]. A second press caused further cell disruption, which released soluble proteins, and the added water carried the protein into the $GJ_{sp}$, which is critical when aiming for high N yield [25]. Overall, lucerne gave the highest N yield and carrot and sugarbeet the lowest after the two presses, with all cases showing substantially increased total N extraction from BM after the second pressing. Thus, a second juice pressing was beneficial for all biomass types evaluated, increasing the efficiency of the protein fractionation process.

Solid particles in the GJ were removed through centrifugation, resulting in a substantially particle-free green juice (S1) that was still green in color for all biomass types. This step was added to examine how water-soluble white proteins were separated in the following thermal precipitation step, in which any particles present in the GJ would be separated together with the green protein fraction. The centrifugation step was identified as the main reason for the unsuccessful protein fractionation of carrot and cabbage, resulting in low white protein yield. Less than 30% of the protein content in GJ was found in S1 after the centrifugation step for these two crops. For all other crops the transfer rate from GJ to S1 exceeded 50%, which corresponds to results reported for lucerne in a previous study [25]. The highest GJ to S1 recovery rates were found for spinach, lucerne (both ~80%), and sugarbeet (>90%). The low rates found for cabbage and carrot indicated poor cell breakage, high content of proteins bound to, or contained in, solid particles/organelles, or high content of insoluble proteins. This issue needs to be resolved for crops such as cabbage and carrot before they are suitable green biomass substrates for protein fractionation.

#### 3.2.2. Green Protein Fractionation and White Juice Production

There were differences in thermal coagulation behavior of GJ from the different green biomass types, as indicated by the optimal temperature for precipitating the green proteins while simultaneously keeping the white protein in the WJ (Figure 5). The RuBisCO subunits (around 55 kDa and 14 kDa) and most other proteins in all biomass types were generally unaffected by heating for 25 min at temperatures of up to 50 °C, while protein bands started to disappear (precipitate together with the green proteins) at 50–55 °C and disappeared (precipitated fully) at 60–65 °C (Figures 5 and 6). The reason for the weak reappearance of the RuBisCO large subunit at 80 °C for some biomass types (e.g., broccoli and sugarbeet; Figure 6) is unclear but could possibly be a result of the two

subunits dissociating at higher temperature, and a difference in the solubility of the two subunits at these temperatures. The clearest difference between the biomass types with regard to precipitation temperature of RuBisCO was the upper temperature for full precipitation. RuBisCO was still present to some extent at 55 °C in all biomass types evaluated (Figure 5, Supplementary Figure S1), but at 65 °C RuBisCO was still only clearly present in the spinach sample. Similarities in response to thermal treatment (e.g., changes in protein composition and concentration) were seen for crops of the same family, e.g., the three varieties of *Beta vulgaris* (sugarbeet, beetroot, and mangold) and the two successfully extracted *Brassica* species (broccoli and kale). The *Beta vulgaris* varieties showed relatively low protein thermal stability, with a clear drop in protein concentration at 55 °C (Figure 5) and a noticeable decrease in the intensity of the RuBisCO bands at temperatures above 50 °C (Supplementary Figure S1). A similar concentration drop at 55 °C was observed for kale and broccoli (Figure 5), but the band intensity was intact at temperatures up to 55 °C (Supplementary Figure S1), indicating greater thermal stability of RuBisCO in *Brassica* biomass. Previous studies have determined the denaturation temperature of RuBisCO from lucerne to be between 61.85 °C and 66.85 °C, depending on the environment [39], and that of RuBisCO from spinach to be 64.9 °C [20]. Thus, differences in denaturation and precipitation of RuBisCO in the biomass types examined in this study might be explained by the environment of the protein, with the S1 matrix affecting the stability and thermosensitivity of RuBisCO and other proteins present. For successful thermal protein precipitation, the optimal temperature may have to be determined for each biomass type to be used in an industrial process.

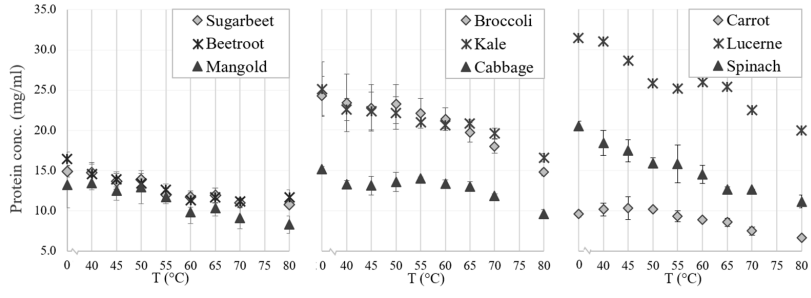


**Figure 5.** Effects of thermal treatment temperature on protein concentration (analyzed with the bicinchoninic acid method) in particle-free supernatant (fraction S1) from crop biomass.

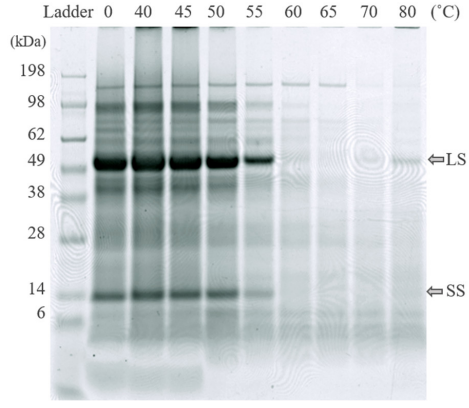


**Figure 6.** Effects of thermal treatment temperature on protein composition (analyzed using SDS-PAGE) in particle-free supernatant (fraction S1) from sugarbeet biomass. LS: large subunit, SS: small subunit of RuBisCO.

Effects of duration of thermal treatment on protein precipitation from S1 of the different biomass types were not assessed in detail, but initial experiments indicated that thermal treatment duration may be important (results not shown). Previous studies have reported that lucerne GJ treated at 50 °C for 5 min is still green, but after 20 min the supernatant is clear [26], and that heating sugarbeet GJ at 55 °C for 5 min gives higher white protein yield than heating for 10 min [28]. Further studies are required to identify the optimal time and temperature settings for each of the nine biomass types evaluated here.

The N yield obtained from thermal precipitation (relative amount of N in S1 transferred to fractions S2 and P2) differed significantly between the biomass types. The N yield from S1 to S2 exceeded 80% for all biomass types except lucerne, mangold, and sugarbeet, for which the yield was considerably lower (52–61%) (Table 2b). These differences likely reflect variation in thermal sensitivity of the multiple proteins in S1 of the different biomass types.

### 3.2.3. Acid Precipitation of the White Protein Fraction

The N yield from acid precipitation at pH 4.5 (S2 to P3) varied between 10.5% and 21.5% for most biomass types, but the values for cabbage and carrot were considerably lower (1.2% and 2.8%, respectively) (Table 2b). The N content of the acid-precipitated protein in the resulting pellet (P3) was around 8% for all crops except cabbage, carrot, and sugarbeet, for which the value was between 1.1% and 2.6% (Table 2c). Low N yield and N content of cabbage and carrot after precipitation was expected, based on the low yields already observed during particle separation of GJ to obtain S1 (i.e., low BM to S1 value; Table 2a). For the other biomass types, variations in precipitated protein amount may be the result of differences in the most suitable pH for precipitation. Previous studies have reported pH 4 or lower as optimal for precipitation of proteins in sugarbeet [23,24] and pH 3.5 as optimal for lucerne proteins [37].

Particle size measurements and pI determinations confirmed that proteins from the different biomass types responded differently to variations in pH (Figure 7). Differences were even seen between varieties, e.g., the particle size of beetroot aggregates increased drastically at pH 4.5 and those of sugarbeet at pH 3.5. Data on pI values and on solubility of the full S2 fraction have not been reported previously, but the pH for minimum solubility of the final white protein concentrates has been reported to be e.g., 3.5 for soy bean leaves [40], 4 for spinach [31], and 5 for sugarbeet [11].

For all crops evaluated except cabbage, protein aggregates were formed during titration with acid and the particles generally increased in size at pH values just below 5

and decreased in size at values below 2.5 (Figure 7). Precipitation, causing the formation of particles, occurs when the charge on amino acids reaches a net zero state; the isoelectric point (pI) [41]. The pI for the S2 fractions from the different crops ranged between 2.2 and 4.3, according to the zeta potential measurements (Figure 7). The pI for spinach S2 was 4.3, which is considerably lower than the theoretical pI value for pure spinach RuBisCO of 6.03. This discrepancy is most likely related to the presence of other proteins and compounds in the solution, which would have a large effect on the net charge.

The minimum solubility, and also the largest aggregates of the proteins, were expected around the pI, but for several of the crops the particle size increased before pI was reached during titration. This indicates that some proteins present, e.g., RuBisCO, started to precipitate earlier in the titration, i.e., at higher pH. For example, precipitation of RuBisCO from mangold clearly occurred at pH 4.5 (see Figure 4), since RuBisCO was not present in S3 but was present in S2, even though the maximum particle size (measured by dynamic light scattering) occurred close to pH 2.5 (Figure 7), which was the pI of the overall solution.

To achieve sufficient separation of white proteins, the particle size did not necessarily need to reach the maximum. For all biomass types studied here, aggregation was initiated at pH 4.5 (the pH used for precipitation of the white proteins). When acid precipitation is chosen as a method for separating white proteins in a biorefinery set-up, the same pH could be used for different biomass types, but the yields would most likely benefit from individual adjustments.

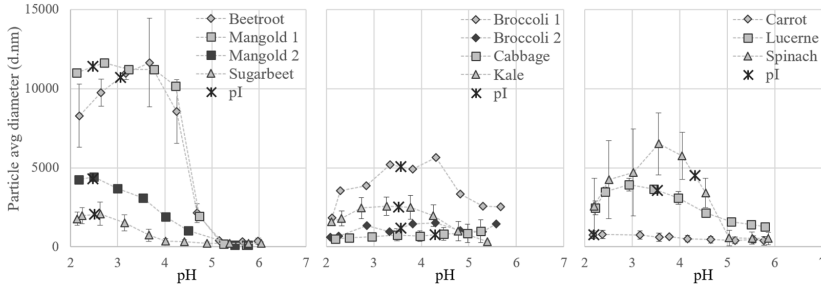


**Figure 7.** Particle size of white protein in thermally treated supernatant (fraction S2) from the different crops at different pH values during titration. Isoelectric point (pI) of S2 is marked on the respective curve.

### 3.2.4. Resolubilization of the White Protein Fraction

The N content in the resulting resolubilized, neutralized, and freeze-dried white protein concentrate (S4) varied for the different biomass types evaluated. It was highest for lucerne (4%) and lowest for carrot and cabbage (0.6% and 0.9%, respectively) (Table 2c). Again, the low levels for carrot and cabbage were the result of incomplete protein fractionation in earlier steps of the process. The other biomass types resulted in white protein concentrates with N content ranging from 2.3% to 3.6%. Higher N content could be achieved by adding a washing step for the acid-precipitated protein (P3) prior to resolubilization, as this would remove some of the co-precipitated compounds present. Several factors are known to have an impact on resolubilization of the white protein fraction, including pH, treatment temperature, use of enzymes, enzyme inhibitors, and adsorptive resins that influence the impact of phytochemicals on protein resolubilization [22,40,42,43]. The variation in protein resolubilization of the P3 fraction between biomass types might thus be the result of differences in phytochemicals binding to the proteins. Use of enzymes or enzyme inhibitors might result in more similar white protein yield for different biomass types.

### *3.3. Use of Green Biomass in Industrial Protein Fractionation for Food Ingredients, Additives, and Products*

The long-term goal behind the present study was a desire to set up an industrial process for protein fractionation of green biomass that could contribute high- value food ingredients, additives and products. Such a process would require green biomass, from a range of different sources, to be available throughout the year. Additionally, such an upscaling would require a protocol suitable for a large range of green biomass types, to allow for maximum facility utilization throughout the year. Therefore, a general protocol based on literature methods [18,24,28,31] was used in this study. However, the present study clearly revealed differences in protein fractionation between green biomass of different origin, suggesting a need for a crop-specific (and eventually also growth stage-specific) fractionation procedure in order to achieve successful production of high-value proteins suitable for food. This study identified important steps to consider for industrial protein fractionation of each of the biomass types, although individual biomass-based settings in an industrial production plant may result in higher production costs. A year-round availability of green biomass, of similar growth stage, would also require large storage facilities for e.g., frozen [44] or silage green biomass. Such a storage will also highly affect the costs (storage of frozen biomass) or destroy the proteins (silage).

Analysis of the protein composition by SDS-PAGE (Figures 3 and 4) showed a high content of RuBisCO in the initial particle-free green juice (S1), the thermally treated S1 (S2), and the final resolubilized protein (S4), while the supernatant remaining after acid precipitation (S3) contained negligible amounts, indicating successful RuBisCO separation. From a food perspective, the RuBisCO-rich white protein concentrate is highly interesting, as it is known to be highly nutritious and have promising functional food properties [11–13]. However, the green protein fraction (P1 combined with P2) and other fractions obtained from the protein fractionation might also be relevant co-products, not least for further fractionation of interesting compounds. Phenolic compounds were present to various degrees in all fractions (data not shown), and these might be of interest as antioxidant-enriched products or for further fractionation before use in food and biomedical applications [45,46]. Fractionation of additional high-value compounds from the green biomass may contribute positively to the economy for the whole process [46].

## 4. Conclusions

Green biomass source substantially influenced the outcome, when a general protein fractionation procedure was applied to extract high-quality protein for use by the food industry. White protein concentrate rich in RuBisCO was extractable from a majority of nine green leafy biomass types subjected to general fractionation, although with considerable variation in protein yield and quality. Biomass type affected protein yield all fractionation steps, i.e., juicing, thermal precipitation, acid precipitation, and resolubilization. Factors such as biomass structural features, crop maturity, and cell wall thickness probably affected the outcome of the juicing step, with stronger structures needing harsher treatment. Protein from the different biomass sources associated to particles in the green juice to varying degrees, with a strong association of proteins for carrot and cabbage leaves, resulting in low yield of high-quality food protein. Modification of the protein fractionation procedure is required to release such protein in these biomass types.

The biomass matrix influenced the thermosensitivity of RuBisCO and other proteins present in the biomass and was important for the thermal precipitation step. Similarities in thermosensitivity were seen for biomass of related origin, e.g., proteins from different varieties of *Beta vulgaris* were generally more sensitive to heat than proteins from, e.g., lucerne. For biomass of sugarbeet, lucerne, kale, and broccoli, pH < 4.5 was most suitable for acid precipitation, while for mangold, beetroot and spinach pH of 4.5 was most

suitable. This indicates that differences in the protein environment influenced acid precipitation behavior, as RuBiSCO theoretically precipitates at around pH 6.

In a biorefinery context, use of a general procedure, but with some parameters modified for each new biomass type, would improve the final outcome compared with using a general procedure for all green leafy biomass.

**Supplementary Materials:** The following are available online at www.mdpi.com/article/10.3390/foods10112533/s1, Supplementary Figure S1: SDS-PAGE analyses of thermally treated particle free green juice (S2), Supplementary Figure S2. Visualization of N yields in some of the process steps for the different biomass types. Supplementary Table S1: Yields (in %) of total mass (m), dry matter (DM), and nitrogen (N), in the white protein extraction process.

**Author Contributions:** Conceptualization, A.-L.N., W.R.N., and E.J.; methodology, A.-L.N.; software, A.-L.N.; validation, A.-L.N., W.R.N., and E.J.; formal analysis, A.-L.N.; investigation, A.-L.N.; resources, E.J.; data curation, A.-L.N.; writing—original draft preparation, A.-L.N.; writing—review and editing, A.-L.N., W.R.N., and E.J.; visualization, A.-L.N.; supervision, W.R.N. and E.J.; project administration, E.J.; funding acquisition, E.J. All authors have read and agreed to the published version of the manuscript.

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## References

1. Barclays. Carving up the alternative meat market. Available online: https://www.investmentbank.barclays.com/our-insights/carving-up-the-alternative-meat-market.html (accessed on 6 July 2020).
2. Smart Protein Project. *Plant-Based Foods in Europe: How big is the Market?* 2020. *Smart Protein Plant-based Food Sector Report by Smart Protein Project, European Union's Horizon 2020 research and innovation programme (No 862957)*
3. Furu, M. *Marknadsanalys och Potential för Växtbaserade Proteiner*. 2020. Available online: https://www.lrf.se/globalassets/dokument/mitt-lrf/nyheter/2020/marknadsanalys-och-potential-for-vaxtbaserade-proteiner.pdf (accessed on 27 Mar 2020).
4. USDA. *Oilseeds: World Markets and Trade*; Monthly report July 2021; United States Department of Agriculture: Economics, Statistics and Market Information System: Washington, DC, USA, 2021. Available online: https://usda.library.cornell.edu/concern/publications/tx31qh68h?locale=en (accessed on 16 Aug 2021).
5. Sozer, N.; Poutanen, K. Plant protein ingredients for future foods. *Agro FOOD Ind. Hi-Tech* **2015**, *26*, 56–59.
6. Pirie, N.W. The direct use of leaf protein in human nutrition. *Chem. Ind.* **1942**, *61*, 45–48.
7. Di Stefano, E.; Agyei, D.; Njoku, E.N.; Udenigwe, C.C. Plant RuBisCo: An underutilized protein for food applications. *J. Am. Oil Chem. Soc.* **2018**, *95*, 1063–1074, doi:10.1002/aocs.12104.
8. Van de Velde, F.; Alting, A.; Pouvreau, L. From waste product to food ingredient: The extraction of abundant plant protein RuBisCo. *New Food* **2011**, *14*, 10–13.
9. Berndtsson, E.; Nynäs, A.-L.; Newson, W.R.; Langton, M.; Andersson, R.; Johansson, E.; Olsson, M.E. The underutilised side streams of broccoli and kale–valorisation via proteins and phenols. In *Sustainable Governance and Management of Food Systems: Ethical Perspectives*; Wageningen Academic Publishers: Wageningen, Netherlands, 2019; pp. 74–81.
10. Andersson, I.; Backlund, A. Structure and function of Rubisco. *Plant Physiol. Biochem.* **2008**, *46*, 275–291, doi:10.1016/j.plaphy.2008.01.001.
11. Martin, A.H.; Castellani, O.; de Jong, G.A.H.; Bovetto, L.; Schmitt, C. Comparison of the functional properties of RuBisCO protein isolate extracted from sugar beet leaves with commercial whey protein and soy protein isolates. *J. Sci. Food Agric.* **2019**, *99*, 1568–1576, doi:10.1002/jsfa.9335.

12. Lamsal, B.P.; Koegel, R.G.; Gunasekaran, S. Some physicochemical and functional properties of alfalfa soluble leaf proteins. *LWT—Food Sci. Technol.* **2007**, *40*, 1520–1526, doi:10.1016/j.lwt.2006.11.010.
13. Hojilla-Evangelista, M.P.; Selling, G.W.; Hatfield, R.; Digman, M. Extraction, composition, and functional properties of dried alfalfa (Medicago sativa L.) leaf protein. *J. Sci. Food Agric.* **2016**, *97*, 882–888, doi:10.1002/jsfa.7810.
14. Patel, M.; Berry, J.O. Rubisco gene expression in C4 plants. *J. Exp. Bot.* **2008**, *59*, 1625–1634, doi:10.1093/jxb/erm368.
15. Wang, J.; Kinsella, J.E. Composition of alfalfa leaf protein isolates. *J. Food Sci.* **1975**, *40*, 1156–1161, doi:10.1111/j.1365-2621.1975.tb01041.x.
16. Merodio, C.; Sabater, B. Preparation and properties of a white protein fraction in high yield from sugar beet (Beta vulgaris L.) leaves. *J. Sci. Food Agric.* **1987**, *44*, 237–243, doi:10.1002/jsfa.2740440305.
17. Knuckles, B.E.; Kohler, G.O. Functional properties of edible protein concentrates from alfalfa. *J. Agric. Food Chem.* **1982**, *30*, 748–752, doi:10.1021/jf00112a030.
18. Tamayo Tenorio, A.; Gieteling, J.; de Jong, G.A.; Boom, R.M.; van der Goot, A.J. Recovery of protein from green leaves: Overview of crucial steps for utilisation. *Food Chem.* **2016**, *203*, 402–408, doi:10.1016/j.foodchem.2016.02.092.
19. Edwards, R.H.; Miller, R.E.; De Fremery, D.; Knuckles, B.E.; Bickoff, E.M.; Kohler, G.O. Pilot plant production of an edible white fraction leaf protein concentrate from alfalfa. *J. Agric. Food Chem.* **1975**, *23*, 620–626, doi:10.1021/jf60200a046.
20. Martin, A.H.; Nieuwland, M.; de Jong, G.A.H. Characterization of heat-set gels from RuBisCO in comparison to those from other proteins. *J. Agric. Food Chem.* **2014**, *62*, 10783–10791, doi:10.1021/jf502905g.
21. Sheen, S.J. Comparison of chemical and functional properties of soluble leaf proteins from four plant species. *J. Agric. Food Chem.* **1991**, *39*, 681–685, doi:10.1021/jf00004a011.
22. Amer, B.; Juul, L.; Møller, A.; Møller, H.; Dalsgaard, T. Improved solubility of proteins from white and red clover–Inhibition of redox enzymes. *Int. J. Food Sci. Technol.* **2020**, doi:10.1111/ijfs.14632.
23. Bray, W.J.; Humphries, C. Preparation of white leaf protein concentrate using a polyanionic flocculant. *J. Sci. Food Agric.* **1979**, *30*, 171–176, doi:10.1002/jsfa.2740300212.
24. Fiorentini, R.; Galoppini, C. Pilot plant production of an edible alfalfa protein concentrate. *J. Food Sci.* **1981**, *46*, 1514–1517, doi:10.1111/j.1365-2621.1981.tb04209.x.
25. Colas, D.; Doumeng, C.; Pontalier, P.; Rigal, L. Green crop fractionation by twin-screw extrusion: Influence of the screw profile on alfalfa (Medicago sativa) dehydration and protein extraction. *Chem. Eng. Process. : Process Intensif.* **2013**, *72*, 1–9, doi:10.1016/j.cep.2013.05.017.
26. De Fremery, D.; Miller, R.E.; Edwards, R.H.; Knuckles, B.E.; Bickoff, E.M.; Kohler, G.O. Centrifugal separation of white and green protein fractions from alfalfa juice following controlled heating. *J. Agric. Food Chem.* **1973**, *21*, 886–889, doi:10.1021/jf60189a020.
27. Hernández, A.; Hernández, T.; Martinez, C. Production and chemical composition of alfalfa protein concentrate obtained by freezing. *Anim. Feed Sci. Technol.* **1998**, *72*, 169–174, doi:10.1016/S0377-8401(97)00167-3.
28. Jwanny, E.W.; Montanari, L.; Fantozzi, P. Protein production for human use from Sugarbeet: Byproducts. *Bioresour. Technol.* **1993**, *43*, 67–70, doi:10.1016/0960-8524(93)90085-P.
29. Kiskini, A.; Vissers, A.; Vincken, J.-P.; Gruppen, H.; Wierenga, P.A. Effect of plant age on the quantity and quality of proteins extracted from sugar beet (Beta vulgaris L.) leaves. *J. Agric. Food Chem.* **2016**, *64*, 8305–8314, doi:10.1021/acs.jafc.6b03095.
30. Lamsal, B.P.; Koegel, R.G.; Boettcher, M.E. Separation of protein fractions in alfalfa juice: Effects of some pre-treatment methods. *Trans. ASAE* **2003**, *46*, 715–720, doi:10.13031/2013.13572.
31. Merodio, C.; Martin, M.; Sabater, B. Improved separation of green and soluble leaf proteins by pH shift. *J. Agric. Food Chem.* **1983**, *31*, 957–959, doi:10.1021/jf00119a009.
32. Zhang, W.; Grimi, N.; Jaffrin, M.Y.; Ding, L. Leaf protein concentration of alfalfa juice by membrane technology. *J. Membr. Sci.* **2015**, *489*, 183–193, doi:10.1016/j.memsci.2015.03.092.
33. Sedlar, T.; Čakarević, J.; Tomić, J.; Popović, L. Vegetable by-products as new sources of functional proteins. *Plant Foods Hum. Nutr.* **2021**, *76*, 31–36.
34. Gasteiger, E.; Hoogland, C.; Gattiker, A.; Wilkins, M.R.; Appel, R.D.; Bairoch, A. Protein identification and analysis tools on the ExPASy server. In *The proteomics protocols handbook*; Walker, J.M., Ed.; Humana Press: Totowa, NJ, USA, 2005; pp. 571–607.
35. RStudio Team. *RStudio: Integrated Development for R. RStudio*; RStudio, PBC: Boston, MA, USA, 2020.
36. Kuznetsova, A.; Brockhoff, P.B.; Christensen, R.H. lmerTest package: Tests in linear mixed effects models. *J. Stat. Softw.* **2017**, *82*, 1–26.
37. Miller, R.E.; de Fremery, D.; Bickoff, E.M.; Kohler, G.O. Soluble protein concentrate from alfalfa by low-temperature acid precipitation. *J. Agric. Food Chem.* **1975**, *23*, 1177–1179, doi:10.1021/jf60202a037.
38. Santamaría-Fernández, M.; Ytting, N.K.; Lübeck, M. Influence of the development stage of perennial forage crops for the recovery yields of extractable proteins using lactic acid fermentation. *J. Clean. Prod.* **2019**, *218*, 1055–1064, doi:10.1016/j.jclepro.2019.01.292.
39. Béghin, V.; Bizot, H.; Audebrand, M.; Lefebvre, J.; Libouga, D.G.; Douillard, R. Differential scanning calorimetric studies of the effects of ions and pH on ribulose 1, 5-bisphosphate carboxylase/oxygenase. *Int. J. Biol. Macromol.* **1993**, *15*, 195–200, doi:10.1016/0141-8130(93)90037-M.
40. Betschart, A.; Kinsella, J.E. Extractability and solubility of leaf protein. *J. Agric. Food Chem.* **1973**, *21*, 60–65, doi:10.1021/jf60185a019.

41. Bhatia, S.; Dahiya, R. Chapter 4—Concepts and Techniques of Plant Tissue Culture Science. In *Modern Applications of Plant Biotechnology in Pharmaceutical Sciences*; Bhatia, S., Sharma, K., Dahiya, R., Bera, T., Eds.; Academic Press: Boston, MA, USA, 2015; pp. 121–156.
42. Kobbi, S.; Bougatef, A.; Balti, R.; Mickael, C.; Fertin, B.; Chaabouni, S.; Dhulster, P.; Nedjar, N. Purification and Recovery of RuBisCO Protein from Alfalfa Green Juice: Antioxidative Properties of Generated Protein Hydrolysate. *Waste Biomass Valorization* **2017**, *8*, 493–504, doi:10.1007/s12649-016-9589-y.
43. Firdaous, L.; Fertin, B.; Khelissa, O.; Dhainaut, M.; Nedjar, N.; Chataigné, G.; Ouhoud, L.; Lutin, F.; Dhulster, P. Adsorptive removal of polyphenols from an alfalfa white proteins concentrate: Adsorbent screening, adsorption kinetics and equilibrium study. *Sep. Purif. Technol.* **2017**, *178*, 29–39, doi:10.1016/j.seppur.2017.01.009.
44. Tenorio, A.T.; Schreuders, F.; Zisopoulos, F.; Boom, R.; Van der Goot, A. Processing concepts for the use of green leaves as raw materials for the food industry. *J. Clean. Prod.* **2017**, *164*, 736–748.
45. Muneer, F.; Hovmalm, H.P.; Svensson, S.-E.; Newson, W.R.; Johansson, E.; Prade, T. Economic viability of protein concentrate production from green biomass of intermediate crops: A pre-feasibility study. *J. Clean. Prod.* **2021**, *294*, 126304.
46. Prade, T.; Muneer, F.; Berndtsson, E.; Nynäs, A.-L.; Svensson, S.-E.; Newson, W.R.; Johansson, E. Protein fractionation of broccoli (Brassica oleracea, var Italica) and kale (Brassica oleracea, var. Sabellica) leaves—A pre-feasibility assessment and evaluation of fraction phenol and fibre content. *Food Bioprod. Process.* **2021**; accepted for publication.

Supplementary Materials

For the paper: Protein fractionation of green leaves as an underutilized food source – protein yield and the effect of process parameters

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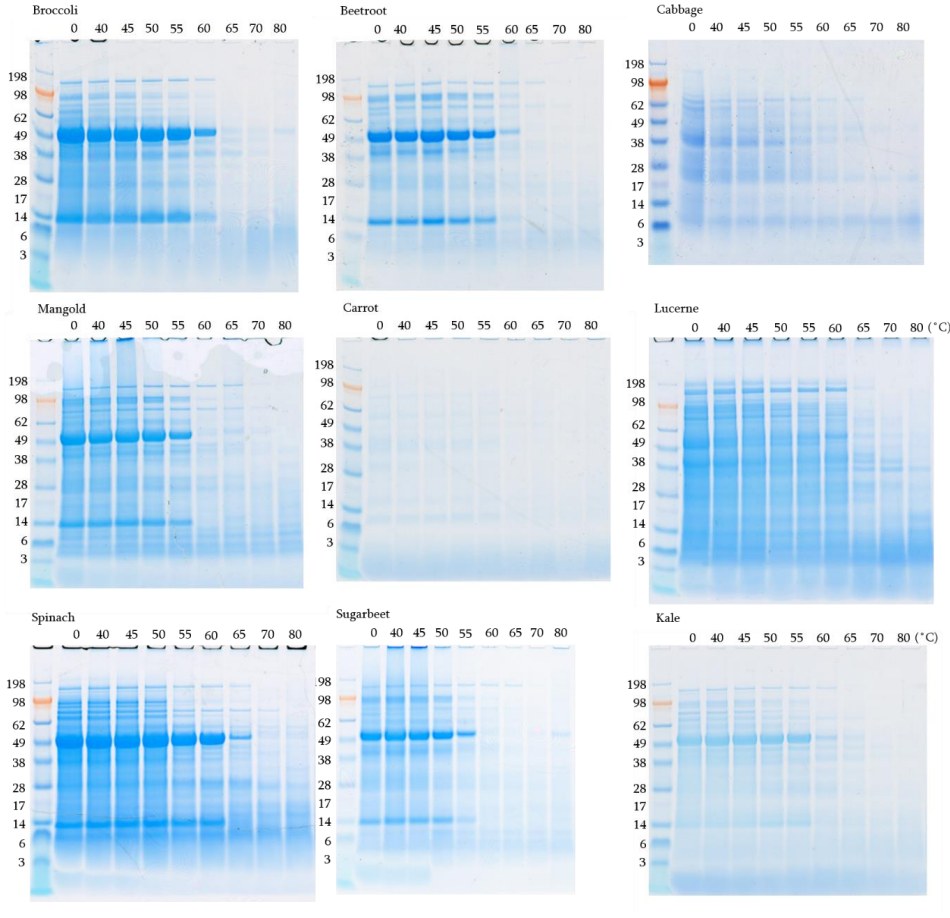
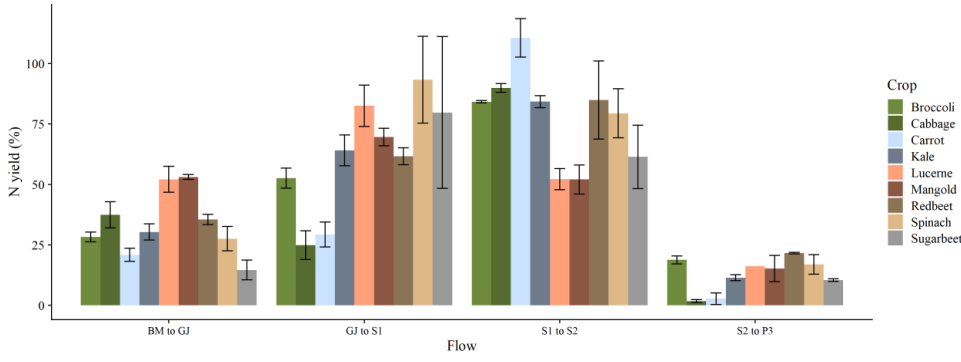
**Supplementary Figure S1.** SDS-PAGE analysis of particle free green juice (S2) thermally treated at different temperatures.



**Supplementary Figure S2.** Visualization of N yields in some of the process steps for the different biomass types.



**Supplementary Table S1.** Yields (in %) of total mass (m), dry matter (DM), and nitrogen (N), in the white protein extraction process. The italic values are the standard deviations for the replicate treatments, and the superscripted values are the number of replicates if not three. BM: Biomass, GJ: Green juice, $GJ_{sp}$: GJ from second press, S1: particle free GJ, $P_{fp}$: Freeze-precipitated proteins from frozen and thawed S1, S2: Supernatant of thermally treated GJ, $P_{fp}2$: Freeze-precipitated proteins from frozen and thawed S2, P3: Acid precipitated white proteins, S4: Resolubilized and freeze-dried white proteins, tot. green frac.: Green particles and thermally precipitated green protein.

| | | Pressing of juice | | | | Separation of green particles | | Freeze precipitated protein | | Thermal precipitation of green protein | | | | Freeze precipitated protein | | Acid precipitation of white protein | | | | Yield of white protein | | | | | | Yield of green protein | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | BM to GJ | | BM to GJ | | GJ to S1 | | S1 to $P_{fp}$ | | GJ to S2 | | S1 to S2 | | S2 to $P_{fp}2$ | | S2 to P3 | | BM to P3 | | S1 to S4 | | S2 to S4 | | BM to S4 | | BM to tot. green frac. | | GJ to tot green frac. | |
| Broccoli | m | 65.9 | *1.7* | 6.3 | *0.9* | 90.5 | *1.2* | | | 82.7 | *2.5* | 92.6 | *1.7* | | | 2.1 | *0.4* | 1.2 | *0.2* | | | | | | | 7.0 | *0.6* | 10.7 | *1.2* |
| | D | 43.0 | *2.2* | 9.6 | *1.7* | 63.4 | *3.1* | 3.8 | *0.8* | 59.0 | *2.3* | 96.8 | *0.9* | 4.2 | *0.8* | 4.6 | *0.8* | 1.2 | *0.3* | 2.9 | *0.7* | 3.1 | *0.7* | 0.8 | *0.2* | 14.0 | *1.2* | 32.5 | *1.0* |
| | N | 28.2 | *2.0* | 7.2 | *1.5* | 52.6 | *4.2* | 12.6 | *1.9* | 54.8 | *4.5* | 84.2 | *0.5* | | | 18.8 | *1.7* | 2.9 | *0.7* | 2.9 | *0.2* | 2.8 | *0.1* | 0.4 | *0.0* | 13.1 | *1.7* | 46.4 | *3.6* |
| Cabbage | m | 63.5 | *2.4* | 9.6 | *1.9* | 93.4 | *0.7* | | | 90.1 | *2.3* | 97.5 | *1.8* | | | 1.2 | *0.2* | 0.7 | *0.1* | | | | | | | 4.4 | *0.3* | 6.9 | *0.7* |
| | D | 44.9 | *1.3* | 11.0 | *0.5* | 78.6 | *3.6* | 2.7 | *0.1* | 79.2 | *3.9* | 103.4 | *1.7* | 1.5 | *0.4* | 1.5 | *0.5* | 0.5 | *0.1* | 1.7 | *0.6* | 1.7 | *0.6* | 0.6 | *0.2* | 9.6 | *1.0* | 21.3 | *1.5* |
| | N | 37.4 | *5.4* | 7.9 | *2.0* | 24.9 | *5.9* | 42.2 | *14.1* | 38.4 | *5.1* | 89.9 | *1.8* | | | 1.2 | *1.1* | 0.2 | *0.1* | 2.8 | *1.3* | 1.6 | *0.2* | 0.2 | *0.1* | 16.5 | *0.7* | 44.5 | *5.7* |
| Kale | m | 71.1 | *0.3* | 4.0 | *0.5* | 92.1 | *0.3* | | | 87.6 | *0.7* | 96.5 | *1.2* | | | 1.6 | *0.1* | 1.0 | *0.0* | | | | | | | 6.0 | *0.3* | 8.5 | *0.5* |
| | D | 34.8 | *1.6* | 5.3 | *0.2* | 77.1 | *3.9* | 3.3 | *0.5* | 71.8 | *2.2* | 95.5 | *2.7* | 2.6 | *0.3* | 3.3 | *0.4* | 0.8 | *0.1* | 2.3 | *0.5* | 2.5 | *0.6* | 0.6 | *0.2* | 8.1 | *0.2* | 23.4 | *0.4* |
| | N | 30.3 | *3.3* | 5.0 | *0.2* | 64.1 | *6.4* | 6.5 | *1.4* | 62.0 | *4.4* | 84.3 | *2.5* | | | 11.4 | *1.2* | 2.1 | *0.2* | 2.6 | *0.4* | 2.7 | *0.5* | 0.5 | *0.1* | 12.9 | *0.7* | 42.6 | *2.5* |
| Mangol | m | 72.5 | *2.0* | 5.4 | *1.3* | 95.0 | *0.6* | | | 88.2 | *0.8* | 93.3 | *1.3* | | | 1.8 | *0.8* | 1.1 | *0.6* | | | | | | | 4.3 | *0.4* | 6.0 | *0.6* |
| | D | 50.0 | *3.2* | 6.9 | *0.8* | 84.0 | *2.2* | 1.5 | *1.2* | 67.7 | *5.8* | 81.8 | *5.4* | 1.4 | *0.8* | 3.3 | *1.5* | 1.1 | *0.5* | 2.6 | *1.2* | 3.2 | *1.3* | 1.1 | *0.4* | 10.1 | *1.0* | 20.1 | *0.9* |
| | N | 53.1 | *1.0* | 8.4 | *0.6* | 69.6 | *3.6* | 4.6 | *3.9* | 46.4 | *9.5* | 52.0 | *6.0* | | | 15.2 | *5.4* | 3.7 | *1.6* | 5.1 | *2.8* | 7.5 | *3.3* | 1.9 | *1.1* | 23.5 | *0.8* | 44.2 | *0.7* |
| Redbeet | m | 74.1 | *1.2* | 5.1 | *0.7* | 95.1 | *0.4* | | | 89.8 | *1.6* | 95.7 | *1.5* | | | 2.4 | *0.5* | 1.6 | *0.3* | | | | | | | 4.2 | *0.4* | 5.6 | *0.5* |
| | D | 37.6 | *2.4* | 5.6 | *0.3* | 71.9 | *6.2* | 2.0 | *1.9* | 68.1 | *2.7* | 99.1 | *7.3* | 1.0 | *0.6* | 5.2 | *0.6* | 1.3 | *0.1* | 3.7 | *1.5* | 3.8 | *1.3* | 1.0 | *0.3* | 9.0 | *1.2* | 23.7 | *1.7* |
| | N | 35.5 | *2.1* | 6.3 | *0.4* | 61.6 | *3.5* | 6.0 | *6.0* | 49.4 | *3.0* | 84.9 | *16.1* | | | 21.6 | *0.3* | 3.8 | *0.1* | 4.9 | *2.7* | 6.0 | *2.9* | 1.0 | *0.5* | 14.3 | *1.9* | 40.0 | *3.0* |
| Sugarbee | m | 69.5 | *1.3* | 6.7 | *0.6* | 95.5 | *0.6* | | | 90.0 | *1.9* | 95.0 | *2.5* | | | 1.7 | *0.7* | 1.1 | *0.4* | | | | | | | 3.7 | *0.3* | 5.4 | *0.4* |
| | D | 39.7 | *5.6* | 7.2 | *0.5* | 93.9 | *9.3* | 0.6 | *0.3* | 83.6 | *13.0* | 89.6 | *10.5* | 2.5 | *1.8* | 4.1 | *1.7* | 1.3 | *0.4* | 2.9 | *0.8* | 3.3 | *1.4* | 1.1 | *0.3* | 6.7 | *0.6* | 17.0 | *1.2* |
| | N | 14.6 | *4.0* | 3.7[2] | *0.6* | 79.7[2] | *31.4* | 1.2[2] | *0.2* | 73.5 | *5.4* | 61.4[2] | *13.2* | | | 10.5[2] | *0.5* | 1.2[2] | *0.3* | 7.5[2] | *1.3* | 7.8[2] | *1.3* | 0.9[2] | *0.0* | 7.1[2] | *1.4* | 49.6[2] | *5.2* |
| Carrot | m | 67.7 | *0.7* | 3.4 | *0.8* | 87.5 | *0.1* | | | 86.0 | *1.3* | 98.6 | *1.4* | | | 1.0 | *0.1* | 0.6 | *0.1* | | | | | | | 8.3 | *0.0* | 12.3 | *0.2* |
| | D | 36.1 | *2.2* | 5.8 | *0.6* | 73.9 | *3.5* | 0.6 | *0.0* | 78.3 | *4.0* | 106.6 | *0.6* | 1.0 | *0.2* | 1.4 | *0.8* | 0.4 | *0.2* | 1.4 | *0.8* | 1.3 | *0.8* | 0.4 | *0.2* | 11.9 | *0.3* | 33.0 | *1.1* |
| | N | 20.9 | *2.7* | 5.3 | *0.4* | 29.2 | *5.1* | 1.8 | *0.1* | 35.0 | *5.3* | 110.6 | *8.0* | | | 2.8 | *2.4* | 0.2 | *0.2* | 1.6 | *0.8* | 1.3 | *0.7* | 0.1 | *0.1* | 22.8 | *0.7* | 110.3 | *13.2* |
| Lucerne | m | 62.8 | *1.6* | 5.6 | *0.5* | 88.5 | *1.3* | | | 64.8 | *0.5* | 76.6[2] | *0.2* | | | 3.8[1] | - | 1.5[1] | - | | | | | | | 11.0[2] | *0.3* | 17.7[2] | *0.7* |
| | D | 37.3 | *1.1* | 10.2 | *0.6* | 74.1 | *6.3* | 5.9 | *0.9* | 46.8 | *0.5* | 70.0[2] | *5.2* | 5.7[1] | - | 7.0[1] | - | 1.2[1] | - | 3.9[1] | - | 5.6[1] | - | 1.0[1] | - | 14.1[2] | *0.7* | 38.1[2] | *0.6* |
| | N | 52.1 | *5.4* | 15.1 | *0.9* | 82.5 | *8.5* | 7.7 | *1.5* | 40.0 | *3.0* | 52.2[2] | *4.4* | | | 16.1[1] | - | 3.3[1] | - | 3.7[1] | - | 7.2[1] | - | 1.5[1] | - | 17.6[2] | *1.0* | 35.1[2] | *2.1* |
| Spinach | m | 54.9 | *8.4* | 8.7 | *5.4* | 94.6 | *1.0* | | | 83.2 | *3.8* | 90.2[2] | *3.4* | | | 3.6[2] | *1.0* | 1.5 | *0.2* | | | | | | | 4.1[2] | *0.2* | 8.2 | *1.4* |
| | D | 31.5 | *4.3* | 10.9 | *1.4* | 98.0 | *21.6* | 3.9 | *0.5* | 67.0 | *9.6* | 81.3[2] | *9.0* | 0.3[1] | - | 8.5[2] | *3.6* | 1.9[2] | *0.5* | 5.8[2] | *3.7* | 7.8[2] | *5.6* | 1.7[2] | *1.0* | 6.2[2] | *0.6* | 18.3 | *2.2* |
| | N | 27.6 | *5.0* | 8.9 | *1.3* | 93.3 | *17.9* | 7.1 | *0.7* | 59.3 | *15.0* | 79.5[2] | *10.1* | | | 16.9[2] | *4.0* | 3.0[2] | *0.1* | 4.2[2] | *1.8* | 6.2[2] | *3.8* | 1.0[2] | *0.4* | 6.8[2] | *0.8* | 22.4 | *1.7* |

# IV

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# Protein fractionation of broccoli (*Brassica oleracea*, var. Italica) and kale (*Brassica oleracea*, var. Sabellica) residual leaves — A pre-feasibility assessment and evaluation of fraction phenol and fibre content

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Leaf protein
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ABSTRACT

This pre-feasibility study evaluates the use of residual leafy green biomass from broccoli (*Brassica oleracea*, var. Italica) and kale (*Brassica oleracea*, var. Sabellica) as feedstock for protein fractionation and potential application of the fractions in food and feed products. The protein concentration, protein recovery potential and the content of phenols and dietary fibre in these biomass sources and fractions were investigated. Field produce and side-stream analysis showed that among broccoli and kale side-streams the potentially suitable leaves for protein fractionation constitute up to 16 and 1.9 t/ha (DM content), respectively. Fractionation demonstrated that between 34–42 and 25–34 kg total protein could be extracted per t DM of broccoli and kale residue leaves, respectively. The amount of protein was generally high in green protein fraction (GPF) and the white protein concentrate (WPC) of both crops, although significantly higher in broccoli compared to kale. The recovery of bound and free phenolic compounds was up to 18% in the GPF of both crops, while only 0.4% ended up in the WPC. The economic assessment showed that the feedstock and processing costs of producing GPF and WPC, as well as of the combined protein fraction (CPF) 1.9–6.0 and 1.3–3.9 times higher than expected revenues for broccoli and kale, respectively, indicating that the production of protein fractions is not economically feasible with the current production scheme. However, potentially higher revenues may be obtained if value-added products such as fractionated phenols and dietary fibre components are also included and investigated in future production schemes. The pathway investigated, that included a direct drying and milling of leaf biomass showed a low processing cost and thereby the most favourable economic alternative, with approx. 7–30% profit for kale, while for broccoli revenues covered only 44–47% of the costs due to the extra harvest cost of the broccoli leaves.



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## 1. Introduction

Fruits and vegetables are an essential part of the human diet, with a high content of health promoting compounds and a significant correlation between their intake and human health has been proven (Liu, 2003). The consumption of cruciferous vegetables has been associated with health benefits, and are suggested to have both anticancer and antioxidant properties (Liu et al., 2018; Melchini and Traka, 2010). Broccoli (*Brassica oleracea*, var. Italica) and kale (*Brassica oleracea*, var. Sabellica) are two commonly consumed vegetables, offering a high nutritive and dietetic value with their suitable content of proteins, bioactive compounds (e.g. polyphenols and glucosinolates), vitamins, minerals and dietary fibre (Campas-Baypoli et al., 2009; Lisiewska et al., 2008). However, during harvesting, sorting and processing of these two crops, a significant portion of the plant is not utilized, which is either discarded in the field or in the processing facility. Thus, for broccoli, the leaves, stalks and stems (together ca. 70% of the plant) are left on the fields after the harvest of the heads/florets (Liu et al., 2018; Zhang et al., 2017). Similarly, during harvesting and factory sorting of kale leaves, up to 50% of the kale plant is discarded in the form of green residues (leaves, stalks and stems), which is ploughed back into the field as green fertilizer (Berndtsson et al., 2019). Such a waste of valuable resources is both a loss of nutritious green biomass, and of investments in the form of limited resources such as water, fertilizer, farmland and energy, which contributes to greenhouse gas emissions (Röös et al., 2020).

Recent developments in bio-refining technologies to valorize agro-industrial side-streams into added-value products create opportunities for a climate-smart and sustainable use of the above described underutilized biomass. The fractionation of plant proteins into valuable, bioactive compound-rich food products from green leaves is a possible pathway to improved use of the leafy green crop residues (Berndtsson, 2019; Berndtsson et al., 2020). Interest in plant proteins from fractionation of green biomass, especially leaves, for food and feed uses is currently growing by: (i) a demand for plant-protein based food products from the increasing number of flexitarians, vegetarians and vegans, (ii) ethical and environmental issues regarding meat production (Pojić et al., 2018; Rosenfeld and Burrow, 2017), (iii) an interest to reduce food waste in field production and the whole production chain, (iv) a wish to contribute added-value to agricultural side-streams (Berndtsson et al., 2020, 2019) and (v) an increased desire to produce proteins for feed locally, reducing the dependency on imported feed meals (e.g. soy protein import to Europe) (de Visser et al., 2014). This interest is reflected in several ongoing projects targeting green biorefining including at Aarhus University in Foulum, Denmak (dca.au.dk/en/current-news/news/show/artikel/indvielse-af-bioraffineringsanlaeg-paa-au-foulum/), at Töreboda, Sweden under the EU GreenValleys project (vgregion.se/f/naturbruk/utveckling-och-innovation/pagaende-projekt/green-valleys—testpilot-for-gron-bioraffinering) the project Biorefinery Glas in Ireland (biorefinery-glas.eu/) and new commercial scale ventures in Denmark (dlf.com/about-dlf/news-and-press-releases/article/danish-cooperatives-join-forces-on-green-protein?Action=1&PID=1905) all apparently focussed on protein for animal feed. Other projects such as the GreenProteinProject headed by Wageningen University in Netherlands (greenproteinproject.eu) and the PlantProteinFactory at the Swedish University of Agricultural Sciences in Alnarp, Sweden (vinnova.se/en/p/plantproteinfactory-step-2) use a hybrid food/feed approach. Projects aimed at green biomass from several crops, such as alfalfa (Colas et al., 2013) and sugar beet leaves (Tenorio et al., 2016), have been evaluated as source for protein concentrate/isolate production for food and feed applications. Similar to other green biomasses, the underutilized leaves obtained as residue from broccoli and kale production could be a potential source for plant protein production using a biorefinery/fractionation approach.

In addition to proteins, the residual leaves from broccoli and kale contain bioactive compounds and fibre that can be of value for fractionation into food and feed ingredients. Biochemical analyses of broccoli side-streams have shown that the composition of bioactive compounds (e.g. polyphenols and glucosinolates), vitamins, dietary fibre and minerals in leaves resembles that found in the florets (Berndtsson et al., 2020; Zhang et al., 2017). Owing to their attractive nutritional profile, broccoli leaves have been studied as a food ingredient in pasta (Angiolillo et al., 2019), bread (Ranawana et al., 2016), green tea (Campas-Baypoli et al., 2009; Dominguez-Perles et al., 2011) and as functional food ingredient for delivery of specific compounds (Shi et al., 2020), thereby providing added value to food. In kale leaves, a high content of glucosinolates, polyphenols, vitamin C and minerals has been demonstrated (Biegańska-Marecik et al., 2017; Lisiewska et al., 2008). However, studies on the composition and content of bioactive compounds found in kale leaves rejected from the factory sorting process are still lacking (Berndtsson et al., 2019). Since most rejected kale leaves in the factory sorting process are discarded only due to their poor aesthetic appeal to consumers and retailer packaging demands, it is fair to assume that they possess a similar nutritional profile compared to marketed leaves. Therefore, alternative protein and bioactive compound-rich feed and food products from residue leaves of broccoli and kale would not only contribute with consumer-desired products but also increase value for such side-streams. An increased understanding on protein recovery and chemical compositions of different fractions produced from broccoli and kale residual leaves is needed for their commercial application. In addition, economic feasibility studies on the production of proteins for food and feed using broccoli and kale residual leaves in a biorefinery/fractionation concept are still lacking.

In this study, the use of broccoli and kale leaf residue for the extraction of proteins, fibre and phenolic compounds for potential use in food and feed products was evaluated. To our knowledge, this is the first study comparing phenolic and dietary fibre contents in different fractions after fractionation of broccoli and kale leaf residues. To understand such an opportunity, a complete analysis of total proteins, phenolics and dietary fibre was performed to estimate their content in residual leaves and in different fractions produced during a protein extraction process. Based on the amount of different compounds in broccoli and kale leaves, a prefeasibility assessment was carried out on an up-scaled fractionation process of multiple value-added products, evaluating the economic viability of protein extraction and its use in food and feed.

## 2. Materials and methods

### 2.1. *Determination of amount of field residues*

For broccoli, the amount of field residues was determined on August 29, 2018, at a commercial farm in north-western Skåne, Sweden, according to Strid et al. (2014). For this purpose, three squares (1.5 m × 1.5 m) were randomly placed in the field and 10 broccoli plants in each square were cut 2 cm above the ground, weighed, and then divided into different fractions (heads, leaves and stalks), which were individually weighed. The mean weight per 2.25 $m^2$ square for the different fractions and for the whole plants was calculated.

The amount of residual leaves from kale was determined in October 2020, at a commercial farm, Viklunda farm, in north-western Skåne, Sweden. On commercial harvesting and sorting of kale, plants were cut 40 cm above the ground and brought to a sorting facility, with the remaining stems left unharvested in the fields. Thereafter, kale plants were divided into three fractions; (i) leaves that could be sold, (ii) leaves rejected for sale on the fresh market, and (iii) residual stem remaining after all leaves were picked from the top stem in the manual sorting operation. For determination of the residual leaves, kale plants were randomly picked from an ongoing sorting process, weighed and divided into the above described fractions.

### 2.2. Plant material

For lab analysis of protein content, bound and free phenolic compounds and dietary fibre, leaves from broccoli (*Brassica oleracea*, Italica group) and kale (*Brassica oleracea*, Sabellica group) were collected from six commercial production fields, in north-western Skåne, Sweden (56°24′38.5″N 12°39′34.5″E). The broccoli and kale plants were collected during the autumn of 2017 and 2018, within 24 h after the last harvest of the main produce (2 and 23 October 2017, and 30 October 2018 for broccoli, 23 October and 6 December 2017, and 12 November and 11 December 2018 for kale) to minimise deterioration of the leaves. Plants of broccoli and kale were cut approximately 2 cm above ground (excluding most woody part of the stems). Leaves already laying on the ground were not collected. Plants collected in 2017 and 2018 were only used for lab analysis.

The plant samples were washed to remove dirt and thereafter the leaves were collected and the other parts were discarded. Leaves were stored at −80 °C until further analysis. Dry matter content was measured by weighting the frozen samples before and after lyophilisation. Prior to analyses of protein content, dietary fibre and bioactive compounds such as bound and free phenolics, the samples were lyophilised.

### 2.3. Fractionation of the leaf biomass

The fractionation procedure to obtain a green protein fraction (GPF) and a white protein concentrate (WPC) from leaf biomass is depicted in Fig. 1 as pathway B. Similarly, Fig. 1 shows the fractionation procedure to obtain a combined protein fraction (CPF) as pathway C. Both fractionation procedures have been used previously for intermediate crops (Muneer et al., 2021). In the present study, analysis and characterization of proteins, phenols and fibre, was carried out on different fractions obtained along the fractionation pathway to produce GPF and WPC (Fig. 1). The full protein fractionation procedure is fully described in Nynäs et al. (2021). In short, a green juice (GJ) was separated from the leaf pulp (P) through screw pressing of green residue leaves. From GJ, the GPF was thermally precipitated at 55 °C and collected through centrifugation. The WPC was thereafter obtained from the supernatant (white juice — WJ) through acid precipitation (pH 4.5) and collected through centrifugation leaving a supernatant (brown juice — BJ).

#### 2.3.1. Determination of dry matter and protein content

Dry matter and nitrogen/protein content were evaluated for the P, GJ, WJ, BJ, GPF and WPC. For dry matter content evaluation, ~30 ml of each of the juices and ~30 g of each of the protein fractions were weighed before and after lyophilisation. The nitrogen content was analysed on dried samples, in triplicate, using the Dumas method on a Flash 2000 NC Analyser (Thermo Scientific, USA). The protein content was estimated by applying a nitrogen conversion factor of 5.6 (Mariotti et al., 2008).

#### 2.3.2. Determination of total free and bound phenolics content

The amount of total free and bound phenolics was evaluated in triplicate for each of the P, GJ, WJ, BJ, GPF and WPC fractions of broccoli and kale leaves, following the extraction procedure of Dinelli et al. (2009). All samples were lyophilised and milled prior to analysis.

Thus, for free phenolic acids extraction, 1 ml 80% ethanol was added to 50 mg (DM) of sample, vortexed for 10 s and thereafter, ultrasonically treated (Bandelin sonorex digitec, Germany) at 35 kHz for 10 min at room temperature (RT), followed by centrifugation (2500 RCF, 5 min). The resulting supernatant was transferred to a new tube, and the pellet re-extracted using the same procedure. The supernatants were pooled and thereafter evaporated using a SpeedVac SVC 100 (Savant, USA) for 60 min. The samples were cooled in a freezer (−20 °C), reconstituted in cold solution (0.5 ml of 50% ethanol and 2% acetic acid (v/v)) and stored in the freezer for further analysis.

Extraction of bound phenolics was subsequently carried out using alkali and acidic procedures on the remaining pellets after extraction of free phenolic acids. The pellet was dispersed in 1.2 ml water and vortexed, followed by addition of 0.5 ml of 10 M NaOH. The samples were then stored at room temperature overnight (16 h). Thereafter, the samples were centrifuged (16.2k RCF, 20 min), and the supernatants transferred to new tubes before further extraction three times with 0.6 ml ethyl acetate followed by centrifugation (16.2k RCF, 20 min). The ethyl acetate layer (top) was removed by pipette, and the three supernatants were pooled and thereafter evaporated by use of $N_2$, cooled, reconstituted and frozen as described above until analysis.

The pellets remaining after alkali hydrolysis were acidified by the addition of 0.2 ml 37% HCl and heated in a heating block at 85 °C in an oven for 30 min. Thereafter, the samples were cooled to RT, gently shaken using a vortex and the pH adjusted to below 2 using 37% HCl. The tubes were centrifuged (16.2k RCF, 20 min) and the supernatants were transferred to new tubes. The supernatants were further extracted and stored as described for the alkali extracted samples.

The phenolic content of the samples produced as described above was determined according to Singleton and Rossi (1965), with some modifications (Dewanto et al., 2002; Gao et al., 2000). A standard solution of gallic acid (2 mg/ml in methanol) was used for making a six-point standard curve (10, 20, 50, 100, and 200 μg/ml diluted in 5% ethanol). The prepared extracts were diluted with Millipore water to get readouts within the standard range. A total of 12 μl of extract or standard solution was mixed with 50 μl of Millipore water directly in a 96-well plate, and 12 μl of Folin–Ciocalteau reagent (Sigma-Aldrich, Sweden) was added to the wells. After 6 min of incubation 125 μl of 7% (w/v) $Na_2CO_3$ was added. The samples were incubated for 75 min and the absorbance measured at 765 nm with a spectrophotometer (ThermoFisher Multiskan GO, USA). An empty well was used as a blank. The concentration of phenolic compounds in the samples was expressed as mg gallic acid equivalents based on the standard curve.

#### 2.3.3. Determination of fibre content

Total content of dietary fibre was analysed in lyophilised and milled samples of the P, GJ, WJ, BJ, GPF and WPC by the ISO/IEC 17025:2005 SWEDAC 1977 accredited laboratory Eurofins Food & Feed Testing Sweden (Lidköping, Sweden) using the standard method (AOAC 991.43).

### 2.4. Economic assessment

A cost-benefit analysis was conducted on the use of broccoli and kale leaves for the valorisation of leaf proteins for food and feed applications. Calculations were carried out as a step-by-step assessment that included all necessary machinery operations in the field, transport, storage and processing in a theoretical protein extraction plant based on the nec-

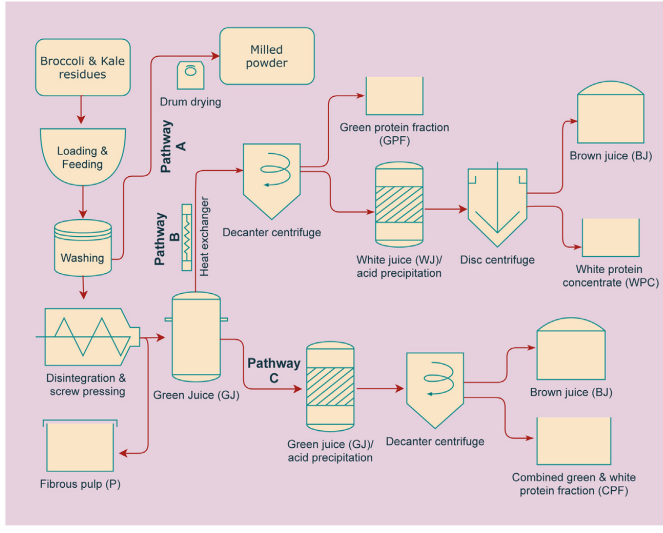


**Fig. 1 – Overview of proposed use of broccoli and kale residual leaves as dried and milled biomass (pathway A), and material flow in protein extraction pathways (B and C), with different fractions and side products.**

essary operations described below. Results of this type of pre-feasibility study usually have an error margin of up to ±30% (Bals and Dale, 2011). To present also the variation of data in the results, a low and a high range analysis for cost and revenue structure for each fractionation pathway was employed.

#### 2.4.1. *Feedstock supply*

The amount of available broccoli and kale leaf biomass was estimated based on typical wet yields of marketable product (broccoli florets and kale leaves), corresponding total above-ground biomass wet yields and typical proportions between marketable product and leaves suitable for protein extraction. Data used for the further economic assessment is presented in the results section. A conversion factor of 1 SEK = 0.0938 € was applied.

For the cost assessment in the case of broccoli, data from both conventional and organic cultivation systems was considered. The harvest of broccoli leaves was assumed to be added as an additional manual harvest operation. Labour and machinery costs were considered for harvest and transport operations (Table 1). Transport of the leaves to the protein processing plant was accounted for assuming a distance of 150 km. To avoid degradation and assure compliance with regulations regarding the microbial safety of food and feed products, broccoli leaf biomass was assumed to be transported without cooling to the processing plant within 4 h after harvest.

For kale, costs based on the already occurring sorting practice in the sorting facility at the farm was estimated. Instead of only sorting kale leaves into marketable and non-marketable leaves, the non-marketable fraction would be further sorted into leaves suitable for protein extraction and leaves to be discarded. This distinction was assumed to be done based on a visual judgement and would result in slightly damaged and discoloured leaves to be used for protein extraction, while heavily damaged leaves and leaves with microbiological defects would be discarded, which could be used in a biogas plant. The useful feedstock was considered to have no additional costs for harvest, only for transport with the same assumptions as for broccoli leaves.

#### 2.4.2. *Protein extraction pathways*

Three production pathways were evaluated in this study: (A) milled biomass, (B) production of green protein fraction (GPF) and white protein concentrate (WPC) and (C) total recoverable combined protein fraction (CPF, both green and white proteins) (Fig. 1). All three pathways assume a processing capacity of 100 t/h. In a previous study, economic assessment has been carried out on application of pathways B and C, respectively, on intermediate crops (Muneer et al., 2021). In the present study, the same setup was followed, however additional data on fibre and phenolic contents in different protein fractions is presented for the crops investigate here. However, since it is unknown if the presence of phenolic compounds in different protein fractions have a positive or negative health effect, their economic value has not been considered. Fibre was considered to be part of the final product and fibre content was used to compare to other products on the market.

For the economic assessment of pathway A, broccoli and kale leaves were assumed to be dried in a drum dryer to a moisture content of approx. 6%, and then milled to a fine powder with an assumed long shelf-life. Initial moisture content of broccoli was assumed to be 88 and 74% for the low and high case, respectively, and 86 and 77% for kale.

For the economic assessment of pathways B and C (Fig. 1), the production of the different fractions follows the same procedure as previously have been described for intermediate crops (Muneer et al., 2021). Thus, in the protein extraction plant, the leaf biomass is directly fed to a washing basin to remove contaminants, e.g. soil particles. From the washing step, the biomass is fed into a screw-press designed to disrupt the cell wall structure and to separate the material into a P and GJ fraction. The P is ensiled for later use, for example to biogas production or used as cattle feed. In pathway B, the GJ is heated to 55 °C to coagulate and precipitate the GPF. In a decanter centrifuge the GPF is separated from the WJ, which is transferred

**Table 1 – Working time requirements and related costs for harvest of broccoli leaves based on Ascard et al. (2008).**

| Parameter | Unit | Harvest: labour | | Harvest: machinery | | Transport: labour & machinery[a] | |
|---|---|---|---|---|---|---|---|
| | | Low | High | Low | High | Low | High |
| Work | [h/ha] | 67 | 75 | 13 | 15 | | |
| Cost | [€/ha] | 1257 | 1407 | 146 | 169 | 169 | 253 |

[a] Estimated at approx. 2.8 €-ct/kg, which corresponds to a transport of 150 km in a full truck (Ascard et al., 2008).

to a tank for further extraction of WPC. The GPF collected in this step is dried to a green powder using a drum dryer. The pH of the WJ is adjusted to approximately pH 4.5 to precipitate the white protein fraction, which is separated using a disk centrifuge. This WPC is later dried to obtain a white protein powder. The clarified BJ produced in this process is stored for later use e.g. in biogas production. In pathway C, to obtain a CPF, the pH of the GJ is adjusted to approximately pH 4.5 to precipitate both green and white proteins. The precipitated CPF is then separated using a decanter centrifuge and the BJ fraction obtained in this process is stored for use in biogas production.

Economic data on an extraction process with mechanical screw-pressing for fraction separation were used as presented by Bals and Dale (2011) (Table 2). However, the processes differ somewhat, e.g. the Bals and Dale process includes additional milling for further cell disruption of the switchgrass feedstock used in the study and a secondary pressing step, both of which are energy and capital intensive (Bals and Dale, 2011). Milling was considered not necessary as broccoli and kale leaves are less fibrous compared to switchgrass. A cost reduction of 31 and 39% for capital and operational cost was suggested by Bals and Dale (2011). Simulating the CPF pathway (C), a simpler process with direct protein precipitation and no milling was assumed. To not overestimate the cost of the avoided milling step, a 20% cost reduction was assumed here. Protein fractions were dried before sale as products to an average moisture content of 6%.

#### 2.4.3. *Final products*

The fine powder produced through pathway A, is assumed to be suitable for a product that could be used in food industry either as a bulk food additive or as a niche health product. As economic revenue differs extremely between these two markets, milled biomass from broccoli and kale leaves is assessed for both applications.

For the production pathway B, WPC powder is intended as a product for human consumption, e.g. as food ingredient in the food industry. The DM protein content (and yield) depends strongly on precipitation conditions and typically ranges between approx. 0–30% (Bals et al., 2012). In this study, a protein content in the WPC of 29% and 16% for broccoli and kale-derived white protein, respectively, was assumed, following the results of the lab analyses. This protein concentration was assumed to be increased to 85% in the final product assuming additional purification steps (Edwards et al., 1975; Tenorio et al., 2016). The product is an off-white powder dried to a moisture content of 4–8% resulting in a long shelf-life. A protein profile suitable for human consumption was assumed. Monetary valuation considered only the nutritional value, with no functional value attached to the proteins.

Both green protein fractions (from production pathways B and C) were assumed to be refined into a green powder intended for use as feed or feed ingredient. Based on lab analyses, the protein content in the protein precipitates was 24–26% for products from both broccoli and kale. The final product is a green powder dried to a moisture content of 4–8% assumed to result in a long shelf-life. Although a protein profile suitable for use as animal feed for both monogastric animals and ruminants was assumed, the economic assessment was carried out for the use as horse feed, specifically as high-protein horse feed additive. However, similar products available on the market have a considerably lower protein content, 11–17% (Appendix Table A1). The kale product had a fibre content of 16%, whereas the broccoli product had a lower fibre content, 11%, which compares to a fibre content in commercial products that ranges 7–27%.

Fibre pulp from production pathways B and C is ensiled at a moisture content of 30% and intended for use as cattle feed. Protein content is approx. 4.3 and 3.0% wet basis for broccoli and kale, respectively, and a protein profile suitable for use as animal feed for ruminants (Dolores Megías et al., 2014; Yi et al., 2015) was assumed.

Brown juice from production pathways B and C is a residue product with potential use as biogas substrate. However, due to the low dry matter content (approx. 6–7%), transport costs are high. Treatment to increase DM content needs to be balanced against product value. Depending on the transport distance, this by-product can be a cost or produce revenues. Therefore, revenues from this by-product have not been included in the economic assessment. The estimations of revenue from the different fractions were carried out based on market reviews for the corresponding applications (Table 3).

## 3. Results and discussion

### 3.1. *Field produce and side-streams*

Broccoli harvest following Nordic routines means that only florets of 10–15 cm in diameter and with a weight of approx. 300 g are harvested, although several harvests per year occur in the same field, which allows for continued growth and harvest. The present study showed that field production of broccoli in Southern Sweden resulted in a high variability in the size of the broccoli heads (140–300 g) and in the total biomass of broccoli heads (13–21%; including those being too small to be marketed) within the same field of production. A total of 43–87% of the biomass was leaves and stems suitable to be used as side-streams for fractionation into different products. This corresponds with previous studies on Swedish broccoli production systems, reporting above ground broccoli biomass yield in the field of 49–160 t wet weight per hectare, of which only 10–33 t per hectare are marketable, leaving 32–138 t of harvest residues (Fink et al., 1999). Additional side-streams are produced during processing, corresponding to 45–50% of the initial broccoli head weights (Campas-Baypoli et al., 2009). In the present study, broccoli leaves constituted 43–78% of the wet weight of the broccoli plants and 64–84% of the crop residues after removal of the broccoli heads. Another

**Table 2 – Cost given as range per t of initial feedstock for protein extraction and drying for final product formulation.**

| Fraction | Crop | Operational cost [€/t] | Investment cost[a] [€/t] | Technology used | References |
|---|---|---|---|---|---|
| *Milling* (pathway A) | Broccoli and kale | 6.6–8.1 | 2.2–2.7 | Disc mill | Bals and Dale (2011) |
| *Extraction* | | | | | |
| White and green protein (pathway B) | Broccoli and kale | 18.7–23.5 | 8.0–9.6 | Mech. separation | Bals and Dale (2011) |
| Total recoverable green protein (pathway C) | Broccoli and kale | 15.0–18.8 | 6.4–7.7 | Mech. separation | Bals and Dale (2011) |
| *Drying* | | | | | |
| Milled biomass | Broccoli | 12.1– 31.9 | 5.5–12.8 | Mechanical dewatering & thermal drying | Own estimation[b] |
| | Kale | 12.5–32.5 | 5.6–13.0 | | |
| White protein | Broccoli | 0.6–3.8 | 0.3–1.5 | Spray drying | Own estimation[b] |
| | Kale | 0.7–4.7 | 0.3–1.9 | | |
| Green protein fraction | Broccoli | 1.9–6.8 | 0.9–2.7 | Drum drying | Own estimation[b] |
| | Kale | 2.1–7.3 | 0.9–2.9 | | |
| Total recoverable combined protein fraction | Broccoli | 4.6–16.2 | 2.1–6.5 | Drum drying | Own estimation[b] |
| | Kale | 4.1–14.5 | 1.9–5.8 | | |

[a] For the drying processes estimated as 40 and 45% of high and low operational costs, respectively.
[b] Estimated based on the energy consumption of 3–7 MJ/kg evaporated water (Baker and McKenzie, 2005) and energy prices of 1.0–1.8 €-ct/MJ (SCB, 2019).

**Table 3 – Product revenues per kilogram protein as assumed for the economic assessment.**

| Product | Application | Chosen value [€/kg] (market range) |
|---|---|---|
| Green protein, GPF | Horse feed | 8.5 (6.6–10.4) |
| White protein, WPC | Food for human consumption | 11.2 (8.6–13.8)[a] |
| Total green protein, CPF | Horse feed | 8.5 (6.6–10.4) |
| Fibre pulp; P | Feed for ruminants | 0.21 (0.14–0.28)[b] |
| Milled broccoli leaves | Health product (protein value only) | 1.7 (1.3–2.1)[c] |
| Milled kale leaves | Health product (protein value only) | 2.1 (1.6–2.6)[c] |

GPF = green protein fraction; WPC = white protein concentrate; CPF = combined protein fraction; P = pulp.
[a] Range as analysed on Alibaba.com (8 June 2019) for plant-based protein; when a default price of 1 US$ $kg^{-1}$ product was given as the lower price range, this was corrected by assuming the lower price limit being at 50% or the upper price limit of the same product.
[b] Assumed to have the same value as that of untreated ley crop biomass used as ruminant feed.
[c] Based on a protein content of 11 and 14% in the final product from broccoli and kale, respectively, and the protein value of white protein.

study has reported leaf shares of 74–85% of the wet weight of greenhouse-grown broccoli (Domínguez-Perles et al., 2010). Dry matter (DM) content of leaf biomass varied between 12.5–25.7% in the present study and an average DM content of 15% was assumed for the economic assessment. The economic feasibility study here is focusing on using the leaves as a suitable side-stream as broccoli stems were determined less suitable, being hard and fibrous and thereby difficult to process in a plant protein factory. Based on above mentioned yield related parameters for Southern Sweden, a total yield of 3.8–16.0 t DM per hectare of broccoli leaves was selected as a basis for the pre-feasibility calculations. If not used as a side-stream, broccoli residues are normally ploughed into the soil as green fertiliser. Broccoli florets are normally harvested by hand and leaves as a side-stream can also be harvested by hand, simultaneously with the last floret harvest. Another option would be to harvest the top leaves with the top stem, mechanically, after the manual harvest of the last florets. Here, our pre-feasibility study was based on a simultaneous hand harvesting of leaf residues with the final harvest of the florets.

The kale harvest includes manual cutting and collection of the top, which is transported to the facility for sorting and packaging of the marketable leaves. The rejected leaves correspond to ca. 16% of the whole kale plant, which means that a mean weight of ca. 1.6 kg/kale plant and on average 30,000 plants/ha per, will result in ca. 7.7 t/ha of rejected residue leaves for protein fractionation. Based on the experience of kale producers (personal communication), approx. 50% of the weight of the kale plant is marketable leaves while ca. 10–20% are residual leaves and ca. 30–40% are stem parts. Thus, in the economic assessment carried out here, these assumptions were used. These results correspond well with results from Fink et al. (1999) on the Swedish production system for kale with a total aboveground biomass yield of 21–65 t wet weight per hectare, of which 10–26 t per hectare are marketable, leaving 10–49 t of harvest residues per hectare. Dry matter content of leaf biomass varied between 14.0–22.8% in the present study and an average DM content of 15% was assumed for the economic assessment. Based on the above mentioned parameters for Southern Sweden, a total yield of 0.32–1.95 t DM per hectare of kale leaves was selected as a basis for the pre-feasibility calculations. Within the current harvesting system, discarded kale leaves, which can be used for extraction of added-value compounds, can be collected simultaneously as marketable kale leaves are collected, and thereby no extra harvest operation is required.

### 3.2. Composition of fractions

#### 3.2.1. Dry matter, protein content and nitrogen recovery

Dry matter (DM) content varied for both crops and in the different fractions (Table 4). Generally, higher DM content was observed in kale than in broccoli, and higher DM content in kale stems than in kale leaves. Furthermore, for both broccoli and kale the highest DM content was obtained in the P (277 and 313 g $kg^{-1}$), and rather high values were found in the GPF (195 and 183 g $kg^{-1}$), while generally low values were found in the GJ, WJ, BJ and WPC (65–84 g $kg^{-1}$), respectively.

Interestingly, a high protein content was found in all the fractions obtained, although with the highest content in the GPF and WPC in both crops (Table 4). Corresponding to the dry matter content, the protein content in the various fractions varied similarly for the two crops evaluated. However, the protein content was consistently lower in all fractions for kale compared to broccoli, which also corresponds to previous reports on total amino acid contents in the crops with significantly lower values for kale than for broccoli (Campas-Baypoli et al., 2009; Lisiewska et al., 2008). Inconsistent with the previous findings, leaves of kale showed higher protein content than those of broccoli in the present study. However, the values for leaves are based on a single measurement. Then, a larger amount of leaves of each crop was processed into the different fractions from which three separate samples were taken for analyses. Thus, the discrepancies in the protein content between the raw material and the fractions might be the result of a single sample being analysed from the raw material. Broccoli is known as a high-protein vegetable (Kmiecik et al., 2010), which is not the case for kale, but both crops have an excellent amino acid profile (Campas-Baypoli et al., 2009; Lisiewska et al., 2008). The dominating protein in all green biomass is RuBisCO, that catalyses the uptake of $CO_2$ in photosynthesis, which is considered to be the most abundant protein in the world (Andersson and Backlund, 2008). RuBisCO should have the same amino acid profile independent of crop background (Udenigwe et al., 2017), and previous studies have indicated alanine, glycine, glutamate and leucine to be the major amino acids (Udenigwe et al. (2017). However, different green biomasses have been shown to contain varying amino acid profiles, due to the fact that other proteins are present in the green biomass. In broccoli and kale parts, the dominant amino acids are aspartic acid, glutamic acid and proline (Campas-Baypoli et al., 2009; Lisiewska et al., 2008). Studies reporting amino acid composition in various fractions are scarce, although high levels of essential amino acids have been reported for the WPC (Hojilla-Evangelista et al., 2017; Kaszás et al., 2020; Merodio and Sabater, 1988; Wang and Kinsella, 1975). Recent results (unpublished) from our lab on hemp and red clover biomass, have indicated an increased accumulation in the relative content of essential amino acids in the P, GPF and WPC (ca. 55% essential amino acids in each), in comparison to the dry biomass (48–49% essential amino acids), while the WJ and BJ were low in relative content of essential amino acids (15–35%).

Nitrogen recovery from the original leafy green biomass to the different fractions was similar for the two crops evaluated. Thus, more than 50% of the N in the green biomass ended up in the P, around 30% ended up in the GPF, 15% in the BJ and only around 2% in the WPC (Table 5). The fact that broccoli and kale behaved similarly when it comes to protein content and N recovery in various fractions after fractionation, does not necessarily mean that this also is the case for other green biomasses. A recent study has in fact shown the opposite, i.e. that the fractionation process must be optimized in relation to different green biomass to obtain reasonable protein content in the WPC (Nynäs et al., 2021). Furthermore, what fractionation processes are being used and type of WPC product compared is also of relevance when evaluating protein content in various fractions as discussed by Nynäs et al. (2021).

From the present study, it is clear that the GPF and WPC both have a generally high protein content (Table 4) and a valuable amino acid composition, which makes them suitable as food and feed sources. In addition, the P and the GJ hold a considerable content of proteins and a good amino acid profile. Therefore, P and GJ should also be considered and further analysed as sources for food and feed products in a protein factory concept. However, the proteins in the P are known to be captured in cell wall components, and as insoluble proteins retained in fibrous scaffold (Damborg et al., 2020). In this study, more than 50% of the N in the green biomass ended up in the P and the protein content in the P was actually 20–50% higher per kg DW as compared to unprocessed plant biomass, which makes the P an attractive feed material for ruminants. For the BJ, previous studies have indicated it contains mainly non-protein components, small peptides and free amino acids, separated during the extraction process (Damborg et al., 2020; Santamaría-Fernández et al., 2017). However, results from Nynäs et al. (2021) indicated the presence of proteins in the BJ, verified by SDS-PAGE. Here, BJ was reported to contain proteins, although measurements were carried out on nitrogen content and then converted to protein by the use of a conversion factor. Thus, the protein content value presented includes non-protein nitrogen and the actual protein content of the BJ requires further investigation.

Based on the results of the analyses presented in Table 4, assumptions were made on the amount of protein to become available in the final products (Table 5). This follows a low/high approach that represents the variation in the lab analyses. For the combined green protein fraction, some of the protein that could be precipitated in a heat treatment as in pathway B would be precipitated in the direct acid treatment of pathway C. The additional amount of protein compared to the GPF was estimated to be 15 and 20% for the low and high case, respectively.

#### 3.2.2. Phenolics

Strikingly, phenolic compounds are clearly present in all the fractions and with equal levels for both the crops. The measured content of the free and bound phenolics of the broccoli and kale biomass corresponded well with previous studies (Berndtsson et al., 2020; Goupy et al., 1990; Liu et al., 2018; Olsen et al., 2009).

The highest contents are found in the juices (GJ, WJ and BJ) and in the WPC (Table 4) for both crops and for both bound and free phenolic compounds. Highest recovery of the phenolic compounds was found in the juices (GJ, WJ, and BJ), although also a relatively high recovery was found in the P (Table 4). Recovery was similar for bound and free phenolics and in both crops, with 33–43% of the phenolics ending up in the P (somewhat higher values for kale than broccoli), 50–66% in the juices, with higher values in the GJ than in the WJ and BJ (larger differences for broccoli than for kale), 4–18% in the GPF (larger values for broccoli than kale), and 0.3–0.4% in the WPC (Table 4).

Previous studies evaluating the health benefits of phenolics have shown that a human diet rich in phenolics contributes to

**Table 4 – Average content of analysed compounds; dry matter, protein (average of triplicates, except for stems and leaves, measured as N content and thereafter transferred to protein content using a conversion factor of 5.6), total phenolic content, free phenolic content, and fibre (n = 1), and nitrogen, phenolics and fibre recovery from the original biomass (leaves), in the different fractions in the process. Numbers give the mean value and the standard deviation (in parentheses), where analysis was based on triplicates. N = not analysed.**

| Crop | Component | Unit | Leaves | P | GJ | GPF | WJ | BJ | WPC |
|---|---|---|---|---|---|---|---|---|---|
| Broccoli | Dry matter content | [g/kg] | 125 | 277 (±43) | 84 (±2) | 195 (±5) | 65 (±1) | 65 (±0) | 81 (±14) |
| | Protein content | [g/kg DM] | 120 | 142 (±11) | 164 (±0) | 272 (±10,2) | 110 (±0,7) | 110 (±0) | 304 (±132) |
| | Bound phenolics content | [mg GAE/g DM] | 8.2 (±0.4) | 4 (±0.1) | 10.2 (±0.6) | 6.0 (±0.9) | 11.2 (±1.3) | 11.6 (±0.6) | 13.6 (±0.3) |
| | Free phenolics content | [$Fe^{2+}$ μmol/g DM] | 108.4 (±7.2) | 40.1 (±1.1) | 114.3 (±4.7) | 57 (±6.6) | 128.7 (±4.8) | 135.3 (±2.7) | 153.1 (±4.9) |
| | Dietary fibre content | [g/kg DM] | 352 | 431 | 63 | 116 | 47 | 30 | N |
| | Nitrogen recovery from biomass | [%] | – | 56 (±20) | 44 (±2) | 29 (±1) | 16 (±2) | 15 (±2) | 0.31 (±0.17) |
| | Bound phenolics recovery from biomass | [%] | – | 36 (±0.7) | 64 (±1.3) | 18 (±1.9) | 45 (±1.6) | 45 (±0.02) | 0.4 (±0.007) |
| | Free phenolics recovery | [%] | – | 34 (±0.6) | 66 (±90.9) | 16 (±1.5) | 50 (±0.5) | 50 (±0.01) | 0.4 (±0.01) |
| | Fibre recovery from biomass | [%] | – | 91 | 9.2 | 5.9 | 3.2 | 3.2 | 0 |
| Kale | Dry matter content | [g/kg] | 143 | 313 (±23) | 76 (±1) | 183 (±6) | 68 (±2) | 68 (±1) | 88 (±8) |
| | Protein content | [g/kg DM] | 150 | 99 (±4.5) | 120 (±3.1) | 258 (±1.9) | 89 (±0.3) | 88 (±0) | 167 (±4.6) |
| | Bound phenolics content | [mg GAE/g DM] | 7.7 (±0.2) | 4.3 (±0.1) | 10.6 (±0.4) | 4.7 (±0.2) | 11 (±0.1) | 10.7 (±0.1) | 15.7 (±0.7) |
| | Free phenolics content | [$Fe^{2+}$ μmol/g DM] | 87.8 (±1.7) | 47.4 (±0.5) | 115.2 (±2.7) | 42.3 (±2.2) | 141.7 (±1.1) | 141.9 (±3.2) | 166.3 (±8.8) |
| | Dietary fibre content | [g/kg DM] | 407 | 602 | 49 | 171 | N | N | N |
| | Nitrogen recovery from biomass | [%] | – | 61 (±2) | 39 (±2) | 18 (±1) | 21 (±1) | 21 (±1) | 0.20 (±0.11) |
| | Bound phenolics recovery from biomass | [%] | – | 43 (±0.9) | 57 (±0.9) | 6.2 (±0.2) | 51 (±0.06) | 51 (±0.003) | 0.4 (±0.02) |
| | Free phenolics recovery from biomass | [%] | – | 43 (±0.3) | 57 (±0.6) | 4.4 (±0.2) | 53 (±0.03) | 52 (±0.01) | 0.3 (±0.02) |
| | Fibre recovery from biomass | [%] | – | 96 | 4.3 | 4.3 | N | N | N |

GAE = gallic acid equivalent; P = pulp, GJ = green juice, GPF = green protein fraction, WJ = white juice, BJ = brown juice, WPC = white protein concentrate, CPF = combined protein fraction.

**Table 5 – Recoverable protein in the different fractions relative to the initial amount of protein in the leaf biomass as used in the economic assessment.**

| Parameter | Unit | Broccoli | | Kale | |
|---|---|---|---|---|---|
| | | Low | High | Low | High |
| White protein fraction (WPC) | [%] | 0.11 | 0.41 | 0.09 | 0.31 |
| Green protein fraction (GPF) | [%] | 28.0 | 29.3 | 16.5 | 19.0 |
| Combined green protein (CPF) | [%] | 32.2 | 35.2 | 19.0 | 22.8 |
| Brown juice (BJ) | [%] | 13.7 | 17.0 | 20.1 | 21.8 |
| Fibre pulp (P) | [%] | 54.5 | 57.9 | 59.0 | 63.1 |

improved cardiovascular health (Wang et al., 2011), decreased risk of developing some forms of cancer (Kyle et al., 2010) and a decreased mortality due to cancer (Ivey et al., 2015) or by cardiovascular diseases (Manach et al., 2005; Williamson, 2017). Furthermore, phenolic compounds have been suggested to have a positive impact on the gut microbiota in humans (Selma et al., 2009), and flavonoids, such as quercetin and kaempferol, have shown some possible positive impact on ruminant health by reducing inflammation (Olagaray and Bradford, 2019). Also, positive impact on human health has been reported from the intake of phenolic compounds of vegetable origin when compared to synthetic antioxidants added to food (Peschel et al., 2006). Due to all the positive benefits from consumption of plant based phenolics, the content of phenolics reported here in the different fractions are highly relevant if some fractions are to be used for food purposes as e.g. as nutritional additives. Another opportunity is to carry the fractionation process further and extract the phenolics from the rich fractions for further use as plant phenolic concentrates.

The present study did not evaluate the composition of the specific phenolic compounds in the different fractions. Thus, for further studies, this will be an important topic in order to understand where and in what amount beneficial phenolic compounds are present in the different fractions. The current results indicate that there might be a difference in the composition between the P and the juices and protein fractions. Phenolics found in the P might be such types that are more thoroughly bound to dietary fibre. Earlier studies have indicated human health benefits from combined phenolic-dietary fibre complexes (Saura-Calixto, 2011). Phenolics soluble in the GJ seem to mainly continue through the process in the juice fractions and phenolics found in the protein fractions (GP and WPC) might be bound to the proteins. Earlier studies have shown that there are high levels of kaempferol and quercetin in kale leaves (Olsen et al., 2009; Schmidt et al., 2010), two compounds that might have different health benefits (Martinez et al., 2017). The fact that the phenolics are found together with dietary fibre (Saura-Calixto, 2011) or protein (Foegeding et al., 2017) could have an impact on both bioavailability and on extractability, as the co-occurrence of these groups of compounds are often needed. Such issues require further study.

#### 3.2.3. *Fibre*

The broccoli leaves in this study contained 35 g dietary fibre/100 g DW, which is in line with earlier studies (Berndtsson et al., 2020). Kale leaves contained higher levels of dietary fibre compared to the broccoli leaves, with 41 g/100 g DW, and this content was similar to what has been found in previous studies (Thavarajah et al., 2019).

The highest fibre content (>90%) was clearly seen in the P fraction for both crops and second highest level in the GPF (Table 4). Dietary fibre as a supplement in food and feed is of interest because of the suggested health benefits, improving human gastrointestinal and cardiovascular health (Kim and Je, 2016), e.g. lowering blood cholesterol levels (Surampudi et al., 2016). Furthermore, fibre improves the gastrointestinal health and the immune system in animals (Jha et al., 2019). However, for animals the dietary fibre might also be considered as an anti-nutritional factor, as it increases satiety (Jha et al., 2019) which could reduce total caloric intake. Dietary fibre also positively influences the bioavailability of phenolic compounds by entrapping them, leading to more phenolic compounds reaching the gut microbiota (Edwards et al., 2017).

To further estimate the value or possible health benefits of fibre from the broccoli and kale fractions, the proportions of soluble and insoluble dietary fibre, as well as the composition of dietary fibre needs to be evaluated. Also, a larger data set is required, since the current data set is minimal and serves to demonstrate the presence of interesting opportunities in these kinds of biomasses.

#### 3.2.4. *Anti-nutritional components*

In this study, a chemical analysis to identify potential anti-nutritional components was not performed, although literature indicates that the presence of such components needs to be evaluated before any fractions can be used for food and feed purposes. The total content and distribution of anti-nutritional compounds may vary according to genera and species of plants used for protein extraction, although major anti-nutritional factors commonly found in green leafy vegetables are nitrates, oxalates, phytates, tannins and saponins (Gupta and Wagle, 1988; Natesh et al., 2017; Satheesh and Workneh Fanta, 2020). Presence of such anti-nutritional compounds may have a direct or indirect impact on the health of an ingesting human or animal (Natesh et al., 2017). In general, the amount of anti-nutritional compounds e.g. nitrates, oxalates, phytates and tannins, are relatively low in kale and broccoli as compared to other leafy vegetables such as spinach (Natesh et al., 2017). However, during fractionation anti-nutritional compounds can possibly be accumulated in specific fractions, resulting in some of the fractions being less useful or even harmful for food and feed purposes. Our preliminary results indicate accumulation of nitrates and nitrites in all of the juice fractions. Therefore, it would be highly relevant to further evaluate the accumulation of these compounds in the different fractions and to improve the separation processes in future work.

### 3.3. *Economic evaluation*

Economic assessment evaluating the use of broccoli and kale leaves as milled biomass (pathway A) and extraction of white and green protein following pathways B and C showed large differences in both costs and revenues for the investigated range of low and high yields in field production and pro-

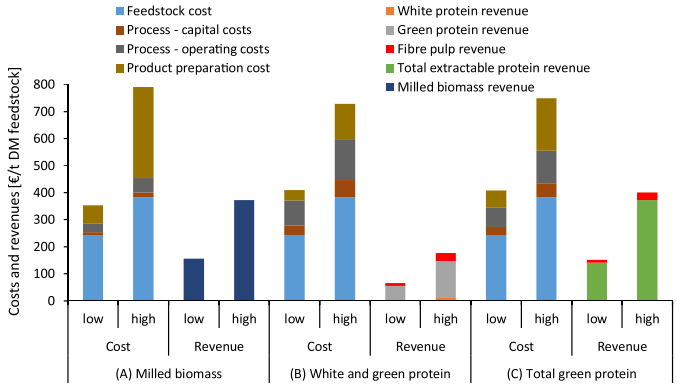


**Fig. 2 – Cost and revenues [€/t DM of feedstock] of broccoli leaf-derived products in the three production pathways given as low–high range. 'Process' refers to extraction of proteins and production formulation refers to drying or ensiling for the different product fractions. Revenue for milled biomass refer to use as a bulk food additive, revenues from application in health products is presented in the text.**

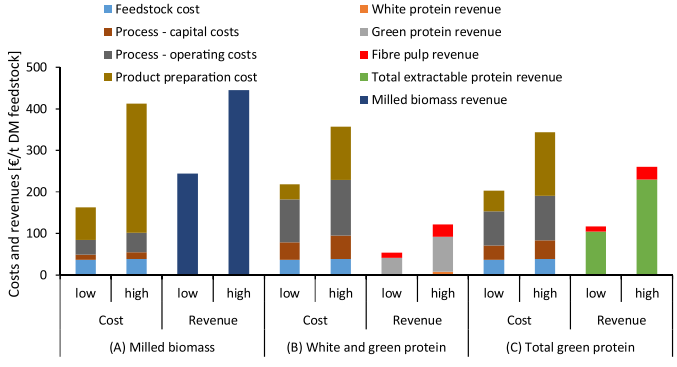


**Fig. 3 – Cost and revenues [€/t DM of feedstock] of kale leaf-derived products in the three production pathways given as low–high range. 'Process' refers to extraction of proteins and 'production formulation' refers to drying or ensiling for the different product fractions. Revenue for milled biomass refers to use as a bulk food additive, revenues from application in health products are presented in the text.**

tein extraction combined with variability in the process data (Figs. 2 and 3).

### 3.3.1. Costs

3.3.1.1. *Broccoli.* Feedstock costs ranged between 240–380 €/t DM and represented the largest cost for production of protein products from broccoli leaves (Fig. 2). Feedstock costs were the same for all three production pathways with 48–69% of the total cost. Process capital costs, process operating costs and product preparation corresponded to 2–9, 7–22 and 9–43% of the total costs, respectively (Fig. 2). Capital and operating process costs in the less intense processing of the milled biomass pathway (A) were approx. 2–3 and 7–9% of the total cost, respectively. Due to a large amount of material requiring drying, product preparation in the milled biomass pathway corresponded to a higher share of total cost of 1 9–43% compared to the 9–26% in the production of white and green protein fractions. Processing of white and green protein that included an additional step for white protein precipitation, was 25% more expensive per t of feedstock compared to production of the green CPF. Product preparation of white and green protein had a 32–39% lower cost due to the lower amount of product to be dried per t of feedstock.

3.3.1.2. *Kale.* Feedstock cost of kale leaves were approx. 40 €/t DM (Fig. 3), which was considerably lower than the feedstock costs for broccoli leaves. Feedstock costs were the same for all three production pathways and represented 9–22% of the total cost, which was much lower compared to the broccoli leaf feedstock. The much smaller absolute cost is a consequence of that the leaves were available from the sorting facility without further harvest costs. Process capital costs, process operating costs and product preparation corresponded to 4–19, 12–47 and 17–75% of the total costs. Similar to the broccoli case, the less intense processing in the milled biomass pathway (A) resulted in a considerably lower range of relative capital and operating process costs of 4–7 and 12–22%, respectively. Again, due to a large amount of material requiring drying, product preparation in the milled biomass pathway showed a much higher relative cost of 49–75%. Compared to the CPF production pathway for broccoli leaves, product preparation costs per t of feedstock for production of white and green protein fractions were 16–27% lower. Similar to the broccoli

case, this can be explained by the lower extraction efficiency for white protein extraction and corresponding lower drying requirements.

#### 3.3.2. Revenues

Revenues from milled biomass marketed as a health food product (pathway A) ranged from approx. 160–370 and 240–440 €/t DM of feedstock for broccoli (Fig. 2) and kale (Fig. 3) leaves, respectively. For the assessment, value was attributed only to the protein content and not to any health effect of the fibre or phenolic content of the biomass. However, if health effects based on the phenolic content can be substantiated, as has been shown with similar products, e.g. wheatgrass (Rana et al., 2011) or pulse shoots (Ghumman et al., 2017), the value and therefore the pricing of the product could be increased. Even without this health claim, milled biomass products from broccoli and kale leaves show an approx. 70–180 and 90–210 times higher protein price, respectively, compared to the protein value assumed here and based on our market analysis.

Revenues from the production of white and green protein (pathway B), ranged from approx. 50 to 180 €/t DM of feedstock for both broccoli (Fig. 2) and kale (Fig. 3) leaves. Here, the proportion of revenue originating from the WPC was extremely low, 2–6%, for both broccoli and kale. This was based on lab experiments that aimed at extracting protein with a high functional value (e.g. foaming properties). Here, the revenues from the GPF represented 69–84% of the total revenues. The P contribution to revenues ranged between 5–25%.

Revenues from the production of total recoverable CPF (pathway C) ranged from approx. 120 to 400 €/t DM of feedstock for both broccoli (Fig. 2) and kale (Fig. 3) leaves. Here, the proportion of revenue originating from the CPF varied little and was 88–94% of the total revenues, for both broccoli and kale leaves. Revenues from use as horse feed varied mainly due to a large price variability of the Swedish market (Appendix Table A1). The P contributed the remaining approx. 11–12% of revenue. Early technological assessments and economic estimates of leaf protein concentrates as presented in the 1970s–80 s, e.g. using alfalfa for chicken feed production (Enochian, 1980; Vosloh, 1976), predicted good profitability. A more recent study on plant protein concentrates from alfalfa employing a process comparable to the CPF process of the present study has found similar discrepancies between feedstock cost and corresponding revenues, at higher yields of total recoverable combined protein but lower protein value (Sinclair and MacManus, 2009). Similar to the CPF production from broccoli presented here, Hermansen et al. (2017) found feedstock costs for purpose-grown grass-clover leys corresponding to 76–83% of the resulting revenues when the green protein concentrates were valorised as pig feed and fibrous pulp as feed for ruminants.

### 3.4. *Economic feasibility*

#### 3.4.1. Broccoli

For the milled biomass and total green protein production pathways, revenues in the high case were similar to the cost in the low case, but much lower than the costs in the high case, indicating that a more detailed assessment is required for evaluation if there is a potential to develop these pathways commercially. The focus of a more detailed assessment should be on reducing the feedstock costs and improving the product quality enabling a better value assessment and market placement. The extraction of WPC is not an economically feasible option under the investigated conditions. This is mainly due to the extremely small fractions of protein that was recovered.

None of the investigated production pathways were economically viable without an adjustment of the current practices of harvesting broccoli florets as the additional harvest operations for recovering broccoli leaves were costly. The potential to reduce feedstock supply costs for additionally harvested broccoli leaves is regarded as low, since this interferes with current practise of quality-driven harvest operations picking only florets suitable for the fresh market. Alternative harvest methodologies similar to the kale harvest could entail the harvest of the larger part of the broccoli plant with a facility-based sorting procedure. Another alternative is a mechanised leaf harvest after the last floret harvest. This could be viable since the broccoli plants continue to grow after harvest of the florets. However, cuts from floret removal may become subject to infections and mould, which could cause problems with food safety in the downstream process. In order to determine if this can be a viable option, detailed field studies are required to investigate if the feedstock quality could be adequate with mechanical harvest and how this would affect the value of the resulting products.

#### 3.4.2. Kale

Economic feasibility of the milled biomass using kale leaves as feedstock is much more likely to be achieved compared to broccoli, since most leaves used are harvested in the same step as harvesting kale leaves for conventional marketing as a fresh vegetable. The leaves that are made available for protein extraction are derived from the quality-based sorting step in the leaf processing facility and imply no further harvesting costs, with the exception of transport costs.

For a milled biomass product (pathway A), costs and revenues are comparable when the milled biomass is marketed for only the nutritional value of the protein, indicating that a more detailed assessment is required to evaluate if there is a potential to develop this pathway commercially. Still, the simple process of drying and milling the leaves to prepare a health product seems to be an interesting option mostly for kale leaves, since the current production setup does not require costly field operations for additional harvest. A simple process adjustment can provide the feedstock with only transportation costs straining the economic balance. If health benefits from fibre and phenolic compounds can be substantiated, the economic feasibility of such a milled product could improve considerably.

White and green protein extraction (pathway B), is not an economically feasible option under the investigated conditions. Similar to broccoli, this is mainly due to the small fractions of protein that was recovered. The literature on the topic suggests the application of an ultrafiltration (UF) step or similar as one way of increasing the white protein recovery (Koschuh et al., 2004). From a cost perspective, a major part of UF cost is related to membrane replacement (Yu et al., 2020), but Bals and Dale (2011) suggested a low-cost and effective way to restore fouled membranes, which could decrease UF cost. However, the present study showed that more than 50% of the protein was still retained in the pulp after the juicing step, indicating additional fractionation early in the process (e.g. additional juicing steps or enzymatic treatments) are needed to reach feasibility for the protein fractionation. Also, mining other components, such as bioactive components and fibre would contribute positively to process economic feasibility.

**Table A1 – Overview of commercial horse feed products.**

| Product | Digestibility [g] | Fibre content [%] | Protein content [%] | Protein price [€/kg] | Source |
|---|---|---|---|---|---|
| Krafft Groov Original, 20 kg | 81.8 | 18 | 11 | 8.9 | https://www.granngarden.se/hastfoder-krafft-groov-original-20-kg/p/1235439 |
| Krafft Groov Protein, 20 kg | 85.2 | 16 | 13.5 | 8.0 | https://www.granngarden.se/hastfoder-krafft-groov-protein-20-kg/p/1235440 |
| Krafft Groov Extra Protein, 20 kg | 82.4 | 16 | 17 | 6.6 | https://borjes.se/stall-skotsel/hastfoder-stro/foder/krafft-groov-extra-protein-20kg/270 |
| Best Horse Basic Pellets | 90.0 | ? | 11 | 7.9 | https://www.foderonline.se/hastfoder/best-horse-basic-pellets.html |
| Best Horse Müsli Classic | 90.0 | ? | 11.4 | 8.0 | https://www.foderonline.se/hastfoder/best-horse-musli-classic.html |
| Best Horse Müsli Classic, havrefritt | 90.0 | ? | 11.8 | 7.9 | https://www.foderonline.se/hastfoder/best-horse-musli-classic-havrefritt.html |
| RS Mustang Protein Müsli | 85.0 | 9 | 14 | 9.3 | https://www.hooks.se/hast/hastfoder/20-kg-protein-musli-rs-mustang |
| RS Mustang Trottning | 97.0 | 10 | 11.5 | 9.3 | https://www.hooks.se/hast/hastfoder/hastfoder-2/pellets-20-kg-trotting-rs-mustang |
| RS Mustang Diet Pellets | 112.0 | 20 | 14.7 | 7.6 | https://www.hooks.se/hast/hastfoder/hastfoder-2/20-kg-diet-pellets-rs-mustang |
| RS Mustang Fibre Original Müsli | 93.0 | 12 | 11.2 | 10.4 | https://www.hooks.se/hast/hastfoder/hastfoder-2/musli-fiber-orginal-rs-mustang |
| RS Mustang Lusernpellets | 0.0 | 27 | 15 | 7.0 | https://www.hooks.se/hast/hastfoder/hastfoder-2/lusern-rs-mustang |
| RS Mustang Breed Pellets | 95.0 | 14 | 12 | 7.8 | https://www.hooks.se/hast/hastfoder/hastfoder-2/pellets-breed-rs-mustang |
| RS Mustang Active Pellets | 90.0 | 8 | 10.5 | 9.8 | https://www.hooks.se/hast/hastfoder/hastfoder-2/pellets-active-rs-mustang |
| RS Mustang Slobber Mash | 90.0 | 7.2 | 11.2 | 10.0 | https://www.hooks.se/hast/hastfoder/hastfoder-2/slobber-mash |
| RS Mustang Alround Müsli | 85.0 | 9 | 10.7 | 10.0 | https://www.hooks.se/hast/hastfoder/hastfoder-2/musli-allround-rs-mustang |
| **Minimum** | **0.0** | **7.2** | **10.5** | **6.6** | |
| **Maximum** | **112.0** | **27.0** | **17.0** | **10.4** | |
| **Average** | **84.4** | **13.9** | **12.4** | **8.6** | |

For the combined protein fraction (pathway C), marketing as a horse feed has a good potential to achieve economic feasibility but requires further investigation. The horse feed market in Sweden is relatively large with a high number of horses kept for recreational and tournament purposes. As this requires that the feed product is safe for animals as a large component of their diet, further research is needed to investigate if the product possesses an acceptable content of anti-nutritional components. However, other specific nutritional or animal health-related components are interesting to investigate in order to motivate the higher product price required to reach economic sustainability.

For all three production pathways, the focus of a more detailed assessment should be on product quality enabling a better value assessment and market placement. This should also include an assessment of the stability of dried products.

## 4. Conclusions

Both broccoli and kale cultivation result in substantial amounts of residuals, in terms of stems and leaves, with the potential to be used as a raw material for producing protein-rich or other health promoting products for humans and animals, in particular in countries with large production volumes. The leaves of the two crops behave similarly when fractionated, with dry matter, protein, phenolics and fibre content and recovery similarly divided into the different fractions. Thus, for both crops, a high protein and a significant phenolic content is obtained in all fractions, although the protein content is higher in all fractions of broccoli than in the corresponding fractions of kale. The highest protein content is obtained in the GPF and WPC for both crops making these fractions interesting for food and feed production purposes. However, the protein recovery is clearly highest in the P fraction of both crops, with around 50% of the proteins ending up in this fraction thereby calling for an improved protein fractionation from the P. All juice fractions contain high amounts of phenolics indicating these fractions to be of importance for phenolics fractionation after a more thorough evaluation of their composition and solubility. A significant content of dietary fibres is only present in the P fraction of both crops.

Protein fractionation from broccoli and kale residuals results in large differences in costs and revenues depending on the planned products. For both crops, the most economically feasible use of the crop residues, such as the leaves, is a direct milling of the leaves to produce a flour to be used as a food additive with health claim. Higher feasibility is obtained for kale than for broccoli, due to a lower feedstock production cost of kale than broccoli. For broccoli, the production cost of the biomass to feed the protein fractionation facility is a large part of the cost, due to the fact that an extra harvest of the broccoli leaves is needed. A change in this procedure so that the leaves can be harvested together with the florets and thereafter sorted (similar to the current situation for kale), or a cheaper harvest procedure used, should reduce the cost for

protein fractionation of broccoli. For kale, the cost for drying of the products produced is a significant part of the costs.

The revenues for the full fractionation of the broccoli and kale residual leafy biomass are extremely low, mainly due to the fact that the protein recovery in the WPC is very low, thereby resulting in substantially higher revenues for a limited protein fractionation with a CPF as the final product. The full fractionation resulting in a GPF and a WPC is only economically feasible if feedstock costs are significantly decreased (i.e. the leaf harvest procedure changed) and/or nitrogen recovery to the WPC significantly increased (i.e. by higher nitrogen recovery from the P fraction). Also, additional fractionation to develop an increased number of added-value products e.g. phenolics and dietary fibres, would contribute to economic feasibility for the full fractionation of broccoli and kale leafy residues.

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## Declaration of interests

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

## Declaration of Competing Interest

The authors report no declarations of interest.

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## Appendix A

## References

Andersson, I., Backlund, A., 2008. Structure and function of rubisco. Plant Physiol. Biochem. 46 (3), 275–291, http://dx.doi.org/10.1016/j.plaphy.2008.01.001.

Angiolillo, L., Spinelli, S., Conte, A., Del Nobile, M.A., 2019. Extract from broccoli byproducts to increase fresh filled pasta shelf life. Foods 8 (12), 621, http://dx.doi.org/10.3390/foods8120621.

Ascard, J., Håkansson, B., Söderlind, M., 2008. Ekonomi – Kalkyler för odling av grönsaker på friland. Swedish Board of Agriculture, Jönköping, Sweden.

Baker, C.G.J., McKenzie, K.A., 2005. Energy consumption of industrial spray dryers. Drying Technol. 23 (1–2), 365–386, http://dx.doi.org/10.1081/DRT-200047665.

Bals, B., Dale, B.E., 2011. Economic comparison of multiple techniques for recovering leaf protein in biomass processing. Biotechnol. Bioeng. 108 (3), 530–537, http://dx.doi.org/10.1002/bit.22973.

Bals, B.D., Dale, B.E., Balan, V., 2012. Recovery of leaf protein for animal feed and high-value uses. In: Bergeron, C., Carrier, D.J., Ramaswamy, S. (Eds.), Biorefinery Co-products: Phytochemicals, Primary Metabolites and Value-added Biomass Processing. Wiley, Chichester, pp. 179–198.

Berndtsson, E., 2019. Dietary Fibre and Phenolic Compounds in Broccoli (*Brassica oleracea* Italica Group) and Kale (*Brassica oleracea* Sabellica Group). A Literature Study about the Potential Uses of Side Streams. Faculty of Landscape Architecture, Horticulture and Crop Production Science, Swedish University of Agricultural Sciences, Alnarp, Sweden.

Berndtsson, E., Nynäs, A., Newson, W., Langton, M., Andersson, R., Johansson, E., Olsson, M., 2019. The Underutilised Side Streams of Broccoli and Kale–valorisation via Proteins and Phenols, Sustainable Governance and Management of Food Systems: Ethical Perspectives. Wageningen Academic Publishers, pp. 74–81, http://dx.doi.org/10.3920/978-90-8686-892-6_21.

Berndtsson, E., Andersson, R., Johansson, E., Olsson, M.E., 2020. Side streams of broccoli leaves: a climate smart and healthy food ingredient. Int. J. Environ. Res. Public Health 17 (7), 2406, http://dx.doi.org/10.3390/ijerph17072406.

Biegańska-Marecik, R., Radziejewska-Kubzdela, E., Marecik, R., 2017. Characterization of phenolics, glucosinolates and antioxidant activity of beverages based on apple juice with addition of frozen and freeze-dried curly kale leaves (Brassica oleracea L. var. acephala L.). Food Chem. 230, 271–280, http://dx.doi.org/10.1016/j.foodchem.2017.03.047.

Campas-Baypoli, O.N., Sánchez-Machado, D.I., Bueno-Solano, C., Núñez-Gastélum, J.A., Reyes-Moreno, C., López-Cervantes, J., 2009. Biochemical composition and physicochemical properties of broccoli flours. Int. J. Food Sci. Nutr. 60 (Sup 4), 163–173, http://dx.doi.org/10.1080/09637480802702015.

Colas, D., Doumeng, C., Pontalier, P.Y., Rigal, L., 2013. Green crop fractionation by twin-screw extrusion: influence of the screw profile on alfalfa (Medicago sativa) dehydration and protein extraction. Chem. Eng. Process. Process Intensif. 72, 1–9, http://dx.doi.org/10.1016/j.cep.2013.05.017.

Damborg, V.K., Jensen, S.K., Weisbjerg, M.R., Adamsen, A.P., Stødkilde, L., 2020. Screw-pressed fractions from green forages as animal feed: chemical composition and mass balances. Anim. Feed Sci. Technol. 261, 114401, http://dx.doi.org/10.1016/j.anifeedsci.2020.114401.

de Visser, C.L.M., Schreuder, R., Stoddard, F., 2014. The EU's dependency on soya bean import for the animal feed industry and potential for EU produced alternatives. Oilseed Fats Crops Lipids 21 (4), D407, http://dx.doi.org/10.1051/ocl/2014021.

Dewanto, V., Wu, X., Adom, K.K., Liu, R.H., 2002. Thermal processing enhances the nutritional value of tomatoes by increasing total antioxidant activity. J. Agric. Food Chem. 50 (10), 3010–3014, http://dx.doi.org/10.1021/jf0115589.

Dinelli, G., Segura Carretero, A., Di Silvestro, R., Marotti, I., Fu, S., Benedettelli, S., Ghiselli, L., Fernández Gutiérrez, A., 2009. Determination of phenolic compounds in modern and old varieties of durum wheat using liquid chromatography coupled with time-of-flight mass spectrometry. J. Chromatogr. 1216 (43), 7229–7240, http://dx.doi.org/10.1016/j.chroma.2009.08.041.

Dolores Megías, M., Meneses, M., Madrid, J., Hernández, F., Martínez-Teruel, A., Cano, J.A., 2014. Nutritive, fermentative and environmental characteristics of silage of two industrial broccoli (Brassica oleracea var. Italica) by-products for ruminant feed. Int. J. Agric. Biol. 16 (2), 307–313.

Dominguez-Perles, R., Moreno, D.A., Carvajal, M., Garcia-Viguera, C., 2011. Composition and antioxidant capacity of a novel beverage produced with green tea and minimally-processed byproducts of broccoli. Innovative Food Sci. Emerg. Technol. 12 (3), 361–368, http://dx.doi.org/10.1016/j.ifset.2011.04.005.

Domínguez-Perles, R., Martínez-Ballesta, M.C., Carvajal, M., García-Viguera, C., Moreno, D.A., 2010. Broccoli-derived by-products—a promising source of bioactive ingredients. J. Food Sci. 75 (4), C383–C392, http://dx.doi.org/10.1111/j.1750-3841.2010.01606.x.

Edwards, R.H., Miller, R.E., De Fremery, D., Knuckles, B.E., Bickoff, E., Kohler, G.O., 1975. Pilot plant production of an edible white

fraction leaf protein concentrate from alfalfa. J. Agric. Food Chem. 23 (4), 620–626, http://dx.doi.org/10.1021/jf60200a046.
Edwards, C.A., Havlik, J., Cong, W., Mullen, W., Preston, T., Morrison, D.J., Combet, E., 2017. Polyphenols and health: interactions between fibre, plant polyphenols and the gut microbiota. Nutr. Bull. 42 (4), 356–360, http://dx.doi.org/10.1111/nbu.12296.
Enochian, R.V., 1980. Producing Pro-Xan (Leaf Protein Concentrate) from Alfalfa: Economics of an Emerging Technology. Department of Agriculture, Economics, Statistics, and Cooperatives Service.
Fink, M., Feller, C., Scharpf, H.C., Weier, U., Maync, A., Ziegler, J., Paschold, P.J., Strohmeyer, K., 1999. Nitrogen, phosphorus, potassium and magnesium contents of field vegetables — recent data for fertiliser recommendations and nutrient balances. J. Plant Nutr. Soil Sci. 162 (1), 71–73, http://dx.doi.org/10.1002/(SICI)1522-2624(199901)162:1<71::AID-JPLN71>3.0.CO;2-0.
Foegeding, E.A., Plundrich, N., Schneider, M., Campbell, C., Lila, M.A., 2017. Protein-polyphenol particles for delivering structural and health functionality. Food Hydrocolloids 72, 163–173, http://dx.doi.org/10.1016/j.foodhyd.2017.05.024.
Gao, X., Björk, L., Trajkovski, V., Uggla, M., 2000. Evaluation of antioxidant activities of rosehip ethanol extracts in different test systems. J. Sci. Food Agric. 80 (14), 2021–2027, http://dx.doi.org/10.1002/1097-0010(200011)80:14<2021::AID-JSFA745>3.0.CO;2-2.
Ghumman, A., Singh, N., Kaur, A., 2017. Chemical, nutritional and phenolic composition of wheatgrass and pulse shoots. Int. J. Food Sci. Technol. 52 (10), 2191–2200, http://dx.doi.org/10.1111/ijfs.13498.
Goupy, P.M., Varoquaux, P.J.A., Nicolas, J.J., Macheix, J.J., 1990. Identification and localization of hydroxycinnamoyl and flavonol derivatives from endive (Cichorium endivia L. cv. Geante Maraichere) leaves. J. Agric. Food Chem. 38 (12), 2116–2121, http://dx.doi.org/10.1021/jf00102a003.
Gupta, K., Wagle, D., 1988. Nutritional and antinutritional factors of green leafy vegetables. J. Agric. Food Chem. 36 (3), 472–474, http://dx.doi.org/10.1021/jf00081a016.
Hermansen, J.E., Jørgensen, U., Lærke, P.E., Manevski, K., Boelt, B., Jensen, S.K., Weisbjerg, M.R., Dalsgaard, T.K., Danielsen, M., Asp, T., 2017. Green Biomass-protein Production Through Bio-refining. DCA-Nationalt Center for Fødevarer og Jordbrug.
Hojilla-Evangelista, M.P., Selling, G.W., Hatfield, R., Digman, M., 2017. Extraction, composition, and functional properties of dried alfalfa (Medicago sativa L.) leaf protein. J. Sci. Food Agric. 97 (3), 882–888, http://dx.doi.org/10.1002/jsfa.7810.
Ivey, K.L., Hodgson, J.M., Croft, K.D., Lewis, J.R., Prince, R.L., 2015. Flavonoid intake and all-cause mortality. Am. J. Clin. Nutr. 101 (5), 1012–1020, http://dx.doi.org/10.3945/ajcn.113.073106.
Jha, R., Fouhse, J.M., Tiwari, U.P., Li, L., Willing, B.P., 2019. Dietary fiber and intestinal health of monogastric animals. Front. Vet. Sci. 6 (48), http://dx.doi.org/10.3389/fvets.2019.00048.
Kaszás, L., Alshaal, T., El-Ramady, H., Kovács, Z., Koroknai, J., Elhawat, N., Nagy, É., Cziáky, Z., Fári, M., Domokos-Szabolcsy, É., 2020. Identification of bioactive phytochemicals in leaf protein concentrate of Jerusalem artichoke (Helianthus tuberosus L.). Plants 9 (7), 889, http://dx.doi.org/10.3390/plants9070889.
Kim, Y., Je, Y., 2016. Dietary fibre intake and mortality from cardiovascular disease and all cancers: a meta-analysis of prospective cohort studies. Arch. Cardiovasc. Dis. 109 (1), 39–54, http://dx.doi.org/10.1016/j.acvd.2015.09.005.
Kmiecik, W., SŁUpski, J., Lisiewska, Z., 2010. Comparison of amino acid content and protein quality in raw broccoli and in broccoli after technological and culinary processing. J. Food Process. Preserv. 34 (s2), 639–652, http://dx.doi.org/10.1111/j.1745-4549.2009.00422.x.
Koschuh, W., Povoden, G., Hong Thang, V., Kromus, S., Kulbe, K., Novalin, S., Krotscheck, C., 2004. Production of leaf protein concentrate from ryegrass (Lolium perenne x multiflorum) and alfalfa (Medicago sauva subsp. sativa). Comparison between heat coagulation/centrifugation and ultrafiltration. Desalination 163, 253–259, http://dx.doi.org/10.1016/S0011-9164(04)90197-X.
Kyle, J.A.M., Sharp, L., Little, J., Duthie, G.G., McNeill, G., 2010. Dietary flavonoid intake and colorectal cancer: a case–control study. Br. J. Nutr. 103 (3), 429–436, http://dx.doi.org/10.1017/S0007114509991784.
Lisiewska, Z., Kmiecik, W., Korus, A., 2008. The amino acid composition of kale (Brassica oleracea L. var. acephala), fresh and after culinary and technological processing. Food Chem. 108 (2), 642–648, http://dx.doi.org/10.1016/j.foodchem.2007.11.030.
Liu, R.H., 2003. Health benefits of fruit and vegetables are from additive and synergistic combinations of phytochemicals. Am. J. Clin. Nutr. 78 (3), 517S–520S, http://dx.doi.org/10.1093/ajcn/78.3.517S.
Liu, M., Zhang, L., Ser, S.L., Cumming, J.R., Ku, K.-M., 2018. Comparative phytonutrient analysis of broccoli by-products: the potentials for broccoli by-product utilization. Molecules 23 (4), 900, http://dx.doi.org/10.3390/molecules23040900.
Manach, C., Williamson, G., Morand, C., Scalbert, A., Rémésy, C., 2005. Bioavailability and bioefficacy of polyphenols in humans. I. Review of 97 bioavailability studies. Am. J. Clin. Nutr. 81 (1 Suppl), 230s–242s, http://dx.doi.org/10.1093/ajcn/81.1.230S.
Mariotti, F., Tomé, D., Mirand, P.P., 2008. Converting nitrogen into protein—beyond 6.25 and Jones' factors. Crit. Rev. Food Sci. Nutr. 48 (2), 177–184, http://dx.doi.org/10.1080/10408390701279749.
Martinez, K.B., Mackert, J.D., McIntosh, M.K., 2017. Chapter 18 — polyphenols and intestinal health. In: Watson, R.R. (Ed.), Nutrition and Functional Foods for Healthy Aging. Academic Press, pp. 191–210, http://dx.doi.org/10.1016/B978-0-12-805376-8.00018-6.
Melchini, A., Traka, M.H., 2010. Biological profile of erucin: a new promising anticancer agent from cruciferous vegetables. Toxins 2 (4), 593–612, http://dx.doi.org/10.3390/toxins2040593.
Merodio, C., Sabater, B., 1988. Preparation and properties of a white protein fraction in high yield from sugar beet (Beta vulgaris L) leaves. J. Sci. Food Agric. 44 (3), 237–243, http://dx.doi.org/10.1002/jsfa.2740440305.
Muneer, F., Hovmalm, H.P., Svensson, S.-E., Newson, W.R., Johansson, E., Prade, T., 2021. Economic viability of protein concentrate production from green biomass of intermediate crops: a pre-feasibility study. J. Cleaner Prod. (294), 126304, http://dx.doi.org/10.1016/j.jclepro.2021.126304.
Natesh, H., Abbey, L., Asiedu, S., 2017. An overview of nutritional and antinutritional factors in green leafy vegetables. Horticult Int J 1 (2), 00011, http://dx.doi.org/10.15406/hij.2017.01.00011.
Nynäs, A.-L., Newson, W., Johansson, E., Submitted to Foods 2021. The Green Leaf Biomass Source — Its Impact on Protein Fractionation Outcomes, and a Rationale for Its Utilization in a Food Concept.
Olagaray, K.E., Bradford, B.J., 2019. Plant flavonoids to improve productivity of ruminants — a review. Anim. Feed Sci. Technol. 251, 21–36, http://dx.doi.org/10.1016/j.anifeedsci.2019.02.004.
Olsen, H., Aaby, K., Borge, G.I.A., 2009. Characterization and quantification of flavonoids and hydroxycinnamic acids in curly kale (Brassica oleracea L. Convar. acephala var. sabellica) by HPLC-DAD-ESI-MSn. J. Agric. Food Chem. 57 (7), 2816–2825, http://dx.doi.org/10.1021/jf803693t.
Peschel, W., Sánchez-Rabaneda, F., Diekmann, W., Plescher, A., Gartzía, I., Jiménez, D., Lamuela-Raventós, R., Buxaderas, S., Codina, C., 2006. An industrial approach in the search of natural antioxidants from vegetable and fruit wastes. Food Chem. 97 (1), 137–150, http://dx.doi.org/10.1016/j.foodchem.2005.03.033.
Pojić, M., Mišan, A., Tiwari, B., 2018. Eco-innovative technologies for extraction of proteins for human consumption from renewable protein sources of plant origin. Trends Food Sci. Technol. 75, 93–104, http://dx.doi.org/10.1016/j.tifs.2018.03.010.

Rana, S., Kamboj, J.K., Gandhi, V., 2011. Living life the natural way–wheatgrass and health. Funct. Foods Health Dis. 1 (11), 444–456, http://dx.doi.org/10.31989/ffhd.v1i11.112.

Ranawana, V., Campbell, F., Bestwick, C., Nicol, P., Milne, L., Duthie, G., Raikos, V., 2016. Breads fortified with freeze-dried vegetables: quality and nutritional attributes. Part II: breads not containing oil as an ingredient. Foods 5 (3), 62, http://dx.doi.org/10.3390/foods5030062.

Röös, E., Carlsson, G., Ferawati, F., Hefni, M., Stephan, A., Tidåker, P., Witthöft, C., 2020. Less meat, more legumes: prospects and challenges in the transition toward sustainable diets in Sweden. Renew. Agric. Food Syst. 35 (2), 192–205, http://dx.doi.org/10.1017/S1742170518000443.

Rosenfeld, D.L., Burrow, A.L., 2017. Vegetarian on purpose: understanding the motivations of plant-based dieters. Appetite 116, 456–463, http://dx.doi.org/10.1016/j.appet.2017.05.039.

Santamaría-Fernández, M., Molinuevo-Salces, B., Kiel, P., Steenfeldt, S., Uellendahl, H., Lübeck, M., 2017. Lactic acid fermentation for refining proteins from green crops and obtaining a high quality feed product for monogastric animals. J. Cleaner Prod. 162, 875–881, http://dx.doi.org/10.1016/j.jclepro.2017.06.115.

Satheesh, N., Workneh Fanta, S., 2020. Kale: review on nutritional composition, bio-active compounds, anti-nutritional factors, health beneficial properties and value-added products. Cogent Food Agric. 6 (1), 1811048, http://dx.doi.org/10.1080/23311932.2020.1811048.

Saura-Calixto, F., 2011. Dietary fiber as a carrier of dietary antioxidants: an essential physiological function. J. Agric. Food Chem. 59 (1), 43–49, http://dx.doi.org/10.1021/jf1036596.

SCB, 2019. Prices on Natural Gas for Industrial Consumers 2007–2019. Statistics Sweden, Stockholm, Accessed on 25 March 2020 from https://www.scb.se/contentassets/0769fa54864a4e58adc8a7a69d61c110/tabeller_2007-2019_1halvar_sv200313.xls.

Schmidt, S., Zietz, M., Schreiner, M., Rohn, S., Kroh, L.W., Krumbein, A., 2010. Genotypic and climatic influences on the concentration and composition of flavonoids in kale (Brassica oleracea var. sabellica). Food Chem. 119 (4), 1293–1299, http://dx.doi.org/10.1016/j.foodchem.2009.09.004.

Selma, M.V., Espín, J.C., Tomás-Barberán, F.A., 2009. Interaction between phenolics and gut microbiota: role in human health. J. Agric. Food Chem. 57 (15), 6485–6501, http://dx.doi.org/10.1021/jf902107d.

Shi, M., Ying, D.-Y., Ye, J.-H., Sanguansri, L., Augustin, M.A., 2020. Broccoli byproducts for protection and co-delivery of EGCG and tuna oil. Food Chem. 326, 126963, http://dx.doi.org/10.1016/j.foodchem.2020.126963.

Sinclair, S., MacManus, R., 2009. Protein Extraction from Pasture. Cost-benefit Analysis: a Feasibility Study Evaluation of Both Investment and Economic Implications. A Report Prepared for the Sustainable Farming Fund, Ministry of Agriculture.

Singleton, V.L., Rossi, J.A., 1965. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. Am. J. Enol. Vitic. 16 (3), 144–158.

Strid, I., Eriksson, M., Andersson, S., Olsson, M., 2014. Svinn av isbergssallat i primärproduktionen och grossistledet i Sverige. Swedish Board of Agriculture, Jönköping, Sweden.

Surampudi, P., Enkhmaa, B., Anuurad, E., Berglund, L., 2016. Lipid lowering with soluble dietary fiber. Curr. Atheroscler. Rep. 18 (12), 75, http://dx.doi.org/10.1007/s11883-016-0624-z.

Tenorio, A.T., Gieteling, J., De Jong, G.A., Boom, R.M., Van Der Goot, A.J., 2016. Recovery of protein from green leaves: overview of crucial steps for utilisation. Food Chem. 203, 402–408, http://dx.doi.org/10.1016/j.foodchem.2016.02.092.

Thavarajah, D., Siva, N., Johnson, N., McGee, R., Thavarajah, P., 2019. Effect of cover crops on the yield and nutrient concentration of organic kale (Brassica oleracea L. var. acephala). Sci. Rep. 9 (1), 10374, http://dx.doi.org/10.1038/s41598-019-46847-9.

Udenigwe, C.C., Okolie, C.L., Qian, H., Ohanenye, I.C., Agyei, D., Aluko, R.E., 2017. Ribulose-1,5-bisphosphate carboxylase as a sustainable and promising plant source of bioactive peptides for food applications. Trends Food Sci. Technol. 69, 74–82, http://dx.doi.org/10.1016/j.tifs.2017.09.001.

Vosloh, C.J., 1976. Leaf Protein Concentrate (Pro-Xan) from Alfalfa: an Economic Evaluation. US Department of Agriculture, Economic Research Service, [National Economic.

Wang, J., Kinsella, J.E., 1975. Composition of alfalfa leaf protein isolates. J. Food Sci. 40 (6), 1156–1161, http://dx.doi.org/10.1111/j.1365-2621.1975.tb01041.x.

Wang, S., Melnyk, J.P., Tsao, R., Marcone, M.F., 2011. How natural dietary antioxidants in fruits, vegetables and legumes promote vascular health. Food Res. Int. 44 (1), 14–22, http://dx.doi.org/10.1016/j.foodres.2010.09.028.

Williamson, G., 2017. The role of polyphenols in modern nutrition. Nutr. Bull. 42 (3), 226–235, http://dx.doi.org/10.1111/nbu.12278.

Yi, X.W., Yang, F., Liu, J.X., Wang, J.K., 2015. Effects of replacement of concentrate mixture by broccoli byproducts on lactating performance in dairy cows. Asian-Australas J. Anim. Sci. 28 (10), 1449–1453, http://dx.doi.org/10.5713/ajas.15.0016.

Yu, H., Li, X., Chang, H., Zhou, Z., Zhang, T., Yang, Y., Li, G., Ji, H., Cai, C., Liang, H., 2020. Performance of hollow fiber ultrafiltration membrane in a full-scale drinking water treatment plant in China: a systematic evaluation during 7-year operation. J. Membr. Sci. 613, 118469, http://dx.doi.org/10.1016/j.memsci.2020.118469.

Zhang, Y., Jiang, Z., Wang, L., Xu, L., 2017. Extraction optimization, antioxidant, and hypoglycemic activities in vitro of polysaccharides from broccoli byproducts. J. Food Biochem. 41 (5), e12387, http://dx.doi.org/10.1111/jfbc.12387.

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A more sustainable food production system is needed to meet the demands of a growing population combined with challenges related to a changing climate. Green leafy biomass is a potential source of food protein, and producing leaf protein concentrates through a biorefinery approach could improve food system sustainability. In this thesis, leaf protein was successfully extracted from a large range of leafy species. The protein concentrates had good foam stabilising properties, making them useful in food applications.

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