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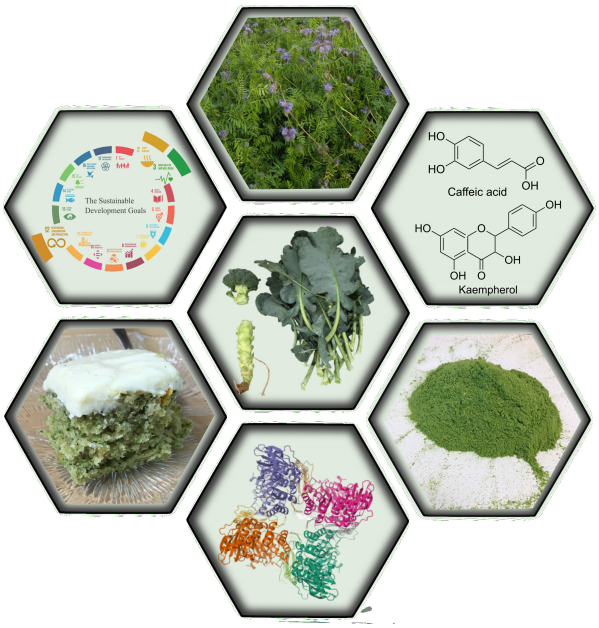
Turning over a new leaf

Extracting protein, phenolic compounds and dietary fibre from green leafy biomass

Emilia Berndtsson



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Extracting protein, phenolic compounds and dietary fibre from green leafy biomass

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Cover: Hexagon structure, containing chemical structure of phenolic compounds and RuBisCO, image of broccoli plant and a broccoli cake, green powder from broccoli leaves, the Sustainable Development Goals and phacelia leaves. Copyright Emilia Berndtsson.
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Turning over a new leaf: Extracting protein, phenolic compounds and dietary fibre from green leafy biomass

Abstract

Global food security faces mounting pressures from climate variability, geopolitical conflicts, price volatility, and diminishing arable land, resulting in chronic hunger for over 724 million people worldwide in 2022. This thesis investigates the potential for extracting valuable nutrients from currently underutilised green leaf biomass from harvested crops and intermediate crops through biorefinery processes.

Our findings reveal that broccoli and kale leaves represent significant untapped biomass resources, though economic constraints currently limit commercial harvesting, particularly for broccoli. These leaves contain substantial amounts of dietary fibre but comparatively lower protein levels than intermediate crops. Among biorefinery fractions from intermediate crops, the green protein and white protein fractions yielded protein compositions suitable for human and animal nutrition, predominantly consisting of RuBisCO-rich fractions.

Both harvest timing and fertilisation significantly influenced protein content and extractability, highlighting the importance of strategic planning when selecting biomass for protein extraction. Regarding phenolic compounds, the green juice, white juice, and brown juice fractions demonstrated the highest concentrations, primarily in the form of flavonoids. In broccoli, these interannual variations are likely attributable to differences in soil conditions, light exposure and temperature variations.

Although not presented as a comprehensive solution to global food insecurity, this research demonstrates that systematic utilisation of currently discarded leaf biomass could significantly contribute to more sustainable food systems. By improving resource efficiency, enhancing the nutritional quality of food products, and providing alternative protein sources, the environmental impact could be significantly reduced compared to conventional animal protein production.

Keywords: green leafy biomass, intermediate crops, biorefinery, phenolic compounds, dietary fibre, green protein, food, feed

Att vända blad: Extraktion av protein, fenoliska ämnen och kostfibrer från biomassa från gröna blad.

Sammanfattning

Den globala livsmedelssäkerheten står inför ökande påfrestningar från klimatvariationer, geopolitiska konflikter, volatila priser och minskande areal odlingsbar mark, vilket resulterat i kronisk hunger hos fler än 724 miljoner människor i världen under 2022. Denna avhandling undersöker potentialen i att extrahera värdefulla näringsämnen från idag underutnyttjad grön bladbiomassa från skördade grödor och mellangrödor genom bioraffinaderiprocesser.

Våra resultat visar att blad från broccoli och grönkål utgör betydande outnyttjade biomassaresurser, även om ekonomiska begränsningar för närvarande hämmar kommersiell skörd, särskilt för broccoli. Dessa blad innehåller betydande mängder kostfiber men jämförelsevis lägre proteinnivåer jämfört med mellangrödor. Bland bioraffinaderifraktionerna från mellangrödorna uppvisade fraktionerna gröna proteiner och vita proteiner proteinsammansättningar lämpliga för människo- och djurföda, huvudsakligen bestående av RuBisCO-rika fraktioner.

Både skördetidpunkt och gödsling påverkade signifikant innehållet och extraherbarheten av protein, vilket understryker vikten av strategisk planering vid val av biomassa för proteinextraktion. Beträffande fenoliska föreningar uppvisade den gröna juicen, vita juicen och bruna juicen de högsta koncentrationerna, främst i form av flavonoider. För broccoli kan dessa årliga variationer sannolikt tillskrivas skillnader i markförhållanden, ljusexponering och temperaturvariationer.

Även om det inte presenteras som en heltäckande lösning på den globala livsmedelsförsörjningens osäkerheter visar denna avhandling att systematiskt utnyttjande av idag kasserad bladbiomassa skulle kunna bidra till mer hållbara livsmedelssystem. Genom att förbättra resurseffektiviteten, öka näringskvaliteten i livsmedelsprodukter och tillhandahålla alternativa proteinkällor skulle miljö- och klimatpåverkan avsevärt kunna minska jämfört med konventionell animalisk proteinproduktion.

Nyckelord: sidoströmmar, mellangrödor, bioraffinaderi, fenoliska ämnen, kostfibrer, växtproteiner, mat, foder

Preface

The purpose of a storyteller is not to tell you how to think, but to give you questions to think upon.

- Brandon Sanderson, The Way of Kings

Problem är bara möjligheter i arbetskläder / Problems are just opportunities wearing work clothes.

- Mulle Meck

Dedication

To my family, who never stopped believing in me.
To Anton, who always encouraged me.
To Frej, who makes me want to make the world a better place.

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List of publications

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I. 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

II. 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) residual leaves — A pre-feasibility assessment and evaluation of fraction phenol and fibre content*. Food and Bioproducts Processing, 130, 229–243. https://doi.org/10.1016/j.fbp.2021.10.004

III. Nynäs, A.-L., Berndtsson, E., Newson, W.R., Persson Hovmalm, H. & Johansson, E. (2024). *Protein Fractionation of Leafy Green Biomass at the Pilot Scale: Partitioning and Type of Nitrogen in the Fractions and Their Usefulness for Food and Feed*. ACS Food Science & Technology, 4 (1), 126 138. https://doi.org/10.1021/acsfoodscitech.3c00426

IV. Berndtsson, E., Nynäs, A.-L., Newson, W.R., Persson Hovmalm, H. & Johansson, E. *Phenolic compounds in fractionated green biomass: An exploration of diversity, functions, and potential applications* (manuscript)

V. Persson Hovmalm, H, Berndtsson, E., Prade, T., Chawade, A., Svensson, S.-E., Johansson, E. *Extractability of proteins in intermediate crops – Opportunities and challenges for protein fractionation in a food and feed context* (manuscript)

All published papers are published open access

The contribution of Emilia Berndtsson to the papers included in this thesis was as follows:

I. Planned the study together with supervisors, collected samples and analysed dietary fibre and phenolic compounds with the aid from laboratory technicians. Analysed the data and performed statistical analyses with the aid of the supervisors and laboratory technicians. Wrote the manuscript with input from the co-authors.

II. Collected samples and analysed the content of phenolic compounds in broccoli and kale samples with the aid from laboratory technicians. Discussed and was part of writing the manuscript.

III. Participated in runs in biorefinery. Responsible for the compilation of data. Planned and performed the statistical analyses of the data and wrote the manuscript together with co-authors. Participated actively in the review and editing process. Made the supplementary journal cover.

IV. Participated in runs in biorefinery. Responsible for the compilation of data. Planned and performed the statistical analyses of data and wrote the first draft of the manuscript together with co-authors. Created all figures and tables for the manuscript.

V. Performed statistical analyses of data. Wrote the first draft of the manuscript together with co-authors. Created most of the figures and tables for the manuscript.

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Abbreviations

BM	Biomass
P	Pulp fraction
GJ	Green juice fraction
GP	Green protein fraction
WJ	White juice fraction
WP	White protein fraction
BJ	Brown juice fraction
SDG	Sustainable Development Goal
GHG	Greenhouse gas

1. Introduction

1.1 Challenges of food security

The global food supply chain is facing unprecedented challenges that necessitate dramatic transformations in the coming decades. According to the Food and Agriculture Organization (FAO) (2023), between 690 and 783 million people worldwide faced hunger in 2022, with projections indicating that nearly 600 million will remain chronically undernourished by 2030. With Sustainable Development Goal (SDG) 2's (Zero Hunger) target date of 2030 rapidly approaching, uncovering sustainable methods to feed the growing population has become critically urgent.

This challenge of food security is compounded by the need to minimise the stress on the environment, e.g. from greenhouse gas (GHG) emissions from the food production, through more efficient use of limited resources such as land, water, and fertilisers. The current food distribution pattern highlights a profound inequity: whilst approximately 724 million people were undernourished in 2022, concurrently about 880 million people were classified as obese (FAO 2024). Indeed, this paradox underscores the fundamental issues of food access and distribution as opposed to merely production capacity.

1.2 Impact of climate change on agriculture

Climate change has reduced food security and rendered it more difficult to meet the SDGs, e.g. SDG 2 (IPCC et al. 2023). The covid-19 pandemic, ongoing wars and armed conflicts, and increased food prices have all negatively impacted on the estimated number of people in hunger, with levels remaining above pre-covid-19 levels and far off track to achieve SDG 2 (FAO et al. 2023). All of the global sustainability goals (*United Nations Sustainable Development* 2025) can be connected to food and food production, but this thesis focuses on three of the SDGs (Figure 1):

- Zero hunger (SDG 2)
- Good health and well-being (SDG 3)
- Responsible consumption and production (SDG 12)

Figure 1: The Sustainable Development Goals primarily addressed in this thesis.

The goals highlighted are Sustainable Development Goal (SDG) 2, SDG 3 and SDG 12.

The global food system faces a significant paradox: whilst there is an urgent need to increase food production to meet the growing demand, we are simultaneously confronted with a "shrinking land challenge", wherein arable land is diminishing due to conservation efforts, urban expansion and climate change impacts (Brain et al. 2023). As a result, a smaller portion of land is responsible for feeding an expanding population. The current human population of 8 billion is projected to reach almost 10 billion in 2050 (FAO 2017). As climate change intensifies the pressure on traditional agricultural systems, developing innovative approaches to valorise agricultural by-products becomes not only beneficial but essential for building resilient and sustainable food systems for future generations. This challenging paradox directly impacts our ability to achieve SDG 2 (Zero Hunger) and emphasises

the importance of utilising agricultural side-streams. These undervalued resources can help address food security concerns whilst also supporting SDG 12 (Responsible Consumption and Production) through more efficient resource utilisation. Additionally, optimising these side-streams can contribute to SDG 3 (Good Health and Well-being) by potentially expanding access to nutritious food products.

Global warming is predicted to increase at least 1.5 °C above pre-industrial levels in the coming decades, which will negatively impact on food security (IPCC et al. 2023). For the four major crops which provides two thirds of the human caloric intake (wheat, rice, maize and soybean) each degree-Celsius increase in global mean temperature would, on average, reduce global yields of wheat by 6.0%, rice by 3.2%, maize by 7.4%, and soybean by 3.1% (Zhao et al. 2017).

1.3 Food production and climate change

To achieve the goal of reducing CO_2 emissions by 48% from the 2019 levels by 2030, as suggested by the IPCC (2023), it is necessary to alter our diets to include more plant-based protein. This dietary change is crucial because the global food production system accounts for approximately 30% of total GHG emissions (Clark et al. 2020), with animal agriculture being particularly carbon-intensive. Consequently, the food we consume and how we produce it have substantial impact for global climate.

Working more efficiently is vital to simultaneously reduce both hunger and reduce the resource waste in food production. Kummu et al. (2012) found that around one quarter of the produced food crops (cereals, fruits and vegetables, oilseeds and pulses, and root and tubers) are lost within the food supply chain, which is enough to feed 1.9 million people with 2100 kcal/day. The production of lost and wasted food crops accounts for 24% of total freshwater resources used in food crop production, 23% of total global cropland area, and 23% of total global fertiliser use (Kummu et al. 2012). Emissions from food loss and food waste in the supply chain and waste management were 9.3 G tonnes of CO_2 equivalents in 2017, whilst the global food production emitted 18.4 G tonnes of CO_2 equivalents throughout the same year (Zhu et al. 2023). Of this, the meat and animal products represent most of the emissions (> 60%), with significant regional variation in emission patterns, and targeted solutions are needed at regional level.

Reducing food loss and waste provides three major benefits: it helps counter climate change, improves food security, and creates more sustainable food production (Rigillo 2022). This work must be prioritised, especially now considering that hunger is increasing worldwide and food prices are soaring (Rigillo 2022).

1.4 The protein shift: more green than red

The Nordic Council (Blomhoff et al. 2023) advice limiting red meat consumption to no more than 350 g per week. On a more global scale, the Eat-Lancet Commission's Planetary Health Diet recommends a maximum of 28 g/day of red meat (196 g per week), whilst maintaining total protein intake (both animal and plant-based) at approximately 56 g/day or 392 g/week (Willett et al. 2019). Accordingly, half of the protein intake should derive from legumes or other plant-based origins, supporting SDG 2 (Zero Hunger) and SDG 3 (Good Health and Well-being) (Figure 1).

However, an increased plant-based protein consumption presents a significant agricultural challenge. The current global protein production capacity is insufficient to meet the growing demand for protein (Merlo et al. 2024). To address this shortfall, we must either substantially increase yields of existing protein crops or develop new protein extraction methods from previously underutilised green biomass sources such as agricultural by-products (Jørgensen et al. 2021). As a novel option to meet these dietary recommendations, protein from agricultural side streams, which have already received inputs of fertilisers, water and energy, could be a promising and sustainable alternative. This approach would simultaneously support SDG 12 (Responsible Consumption and Production) by reducing waste and making more efficient use of resources already invested in food production systems. The question is, do these side streams contain enough of interesting nutrients of sufficient quality?

2. Background

To feed a growing population, it is crucial to have a more effective use of the produced biomass, whilst sustainably using the limited resources such as water, nutrients, land area and energy. It goes without saying that the world also needs a more equitable distribution of the produced food.

2.1 Side streams, food loss, and food waste

There are parts of cultivated plants that are not intended for human consumption and are thus considered as side streams. These side streams can consist of e.g. leaves, stems, peels, and hulls. Food loss and food waste are often mentioned together, but these terms relate to different issues in the food supply system. When attempting to analyse the material in the food supply chain, there are different phrases currently used for the side streams. The two most common used are:

- Food loss, which occurs during the production stages, from primary production including post-harvest losses, up to (not including) retailers. Items included here are all edible parts from crop and livestock that are discarded, incinerated, or otherwise thereby leaving the production or supply chain (United Nations Environment Programme 2024).
- Food waste, on the other hand, occurs at the retail and consumer levels. The items included here are any parts or substances intended for human consumption and the associated inedible parts, that are removed from the human food supply chain to be composted, discarded, or sent to a landfill or similar (United Nations Environment Programme 2024). Hence, food waste includes both edible and inedible parts (e.g. rinds, bones, pits/stones).

Field waste and side streams are more general phrases, with the former containing the plant material left in the field during production and harvest, and the latter spanning over the same area as both food loss and field waste (Figure 2).

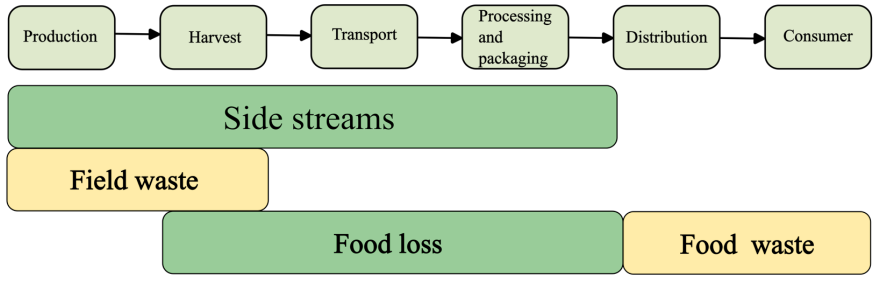

Figure 2: Expressions at different stages in the food supply chain for non-eaten produce

In many cases, there is a gap between "edible" and "inedible" parts, such as parts that could be consumed (e.g. leaves and stems) but are not due to cultural reasons or traditions. The available research, with a focus on content of nutrients and antinutrients and on the available biomass, is almost exclusively focused on the parts that are already eaten, and not on the parts that could possibly be used as food. Due to the inconsistency with the terms used, side streams, field waste, food waste and food loss, it is difficult to obtain an overview of the research field and how much potential useful material there is. However, it is indisputable that not using side streams is a loss of nutritious green biomass, and of limited resources such as water, fertiliser, farmland, and energy, all of which contribute to GHG emissions (Röös et al. 2020).

In 2023, it was estimated that within the food supply chain 8 % of the food loss occurs at the farm site, 14 % is lost between the farm and retailer, and 17 % from retailer to consumer (Brian 2023), resulting in a total loss of 39 % of produced food throughout the entire food supply chain. The Swedish Board of Agriculture (Jordbruksverket) has initiated several projects to measure the amounts of side streams in primary production. For example of projects to measure side streams, strawberries comprised 40-55 % of berries remaining on the fields after harvest (Persson Hovmalm & Nordmark 2023). Moreover, potatoes made up 7.4 % remaining on the field after harvest (Strid et al. 2023). However, it is important to note that these calculations only include parts *intended* for food uses, meaning that e.g. broccoli leaves were not included. There are unmeasured amounts of biomaterial that could possibly be used as human food in the food supply chain. In this thesis, the main focus is on green leafy side streams and intermediate crops.

2.2 The underutilised green leafy material

Crop side streams and intermediate crops, notably green leafy biomass, present an opportunity to establish local supply chains for biorefinery operations, from which compounds of interest may be extracted (Di Donato et al. 2018; Møller et al. 2021). Among these valuable compounds are phenolic compounds, dietary fibre, and plant proteins, all of which can contribute to more sustainable food and ingredient production. The main benefit of using vegetable parts that are not currently used as food today for the extraction of e.g. proteins, antioxidants, and dietary fibre is that the whole plant can be utilised (e.g. food crops such as broccoli and sugar beet). This approach directly supports SDG 12 (Responsible Consumption and Production) by reducing waste and making our food system more efficient.

In food crops grown today, different parts serve different purposes; some are primarily used as food, others could potentially be repurposed for consumption in various forms, and some parts are unsuitable for food but alternative applications could be used. To effectively utilise these parts, it is important to first quantify the availably material and analyse its composition. As Lord Kelvin aptly stated, "when you can measure what you are speaking about, and express it in numbers, you know something about it; but when you cannot measure it, when you cannot express it in numbers, your knowledge is of a meagre and unsatisfactory kind" (Thomson 1889).

Each year, approximately 5 billion tonnes of crop side streams are generated globally from cereals, legumes, oil crops, sugar crops, and tuber crops (Shinde et al. 2022). For vegetables, these side streams represent a substantial untapped resource. For example, in a pilot study investigating the production of iceberg lettuce, 65 % of the lettuce became waste (Strid et al. 2014). Of that waste, the unharvested and discarded outer leaves comprised 45 %, never harvested fields 15 % (due to overproduction or failure to meet the high quality criteria), and lastly 5 % was wasted from producer to retailer (Strid et al. 2014). Another source of underutilised leafy material for food purposes is intermediate crops or catch crops. Catch crops are specific crops sown to catch nitrate between two main crops, and intermediate crops are crops sown between two main crops to obtain soil cover, e.g. during winter period, and catch nutrients such as nitrate (Hill 2023). These catch crops and intermediate crops are ploughed down as green manure before the next main crop is sown (Poeplau & Don 2015). This biomass, both from crop side

streams and intermediate crops, could instead be used as a source material in a biorefinery.

2.2.1 Biorefinery

In a biorefinery concept, biomass is converted into value-added products and energy through various techniques (Cherubini 2010). A transition towards the circular bioeconomy has been identified as a crucial step towards increased sustainability because this concept offers a holistic method to minimise waste and maximise resource efficiency. Central to this concept is the utilisation of renewable biological resources as the basis for various products and processes (European Commission 2012). Agricultural residues, including side streams from protein fractionation, represent a substantial and underutilised feedstock with an inherent value for the production of chemicals, bioactive compounds, such as phenolic compounds, and bio-based materials (Santana-Méridas et al. 2012; Nayak & Bhushan 2019; Ortega et al. 2022; Chauhan et al. 2023).

Green leaves not only constitute the major supply of green biomass, they are also a rich source of nutritional and bioactive compounds such as phenolic compounds (Gunathilake & Ranaweera 2016; Iqbal et al. 2022; Yeasmen & Orsat 2023). These compounds offer potential benefits for human health and could contribute to sustainable food systems. Indeed, recovering multiple compounds, such as protein and phenolic compounds, from the same fractionation process of leafy green biomass has the potential to increase the feasibility of the process and to contribute to increased sustainability (Johansson et al. 2015). This integrated approach supports circular economy principles and addresses key challenges in sustainable resource management.

The protein fractions obtained from the biorefinery process represent a promising alternative protein-rich feedstock for monogastric animals, particularly swine and poultry (Stødkilde et al. 2018; Santamaría-Fernández & Lübeck 2020). These protein concentrates derived from green leafy biomass could substantially reduce dependence on imported protein sources such as soybean meal, thereby enhancing agricultural sustainability and resource circularity (Cong & Termansen 2016; Njakou Djomo et al. 2020; Karlsson et al. 2021). Furthermore, preliminary feeding trials have demonstrated favourable digestibility coefficients and amino acid profiles

comparable to conventional protein sources, suggesting minimal need for supplementation (Stødkilde et al. 2019).

2.2.2 Phenolic compounds: Why should we eat more of them?

More than 8000 different phenolic compounds have been identified in plants, and these can be classified into distinct categories including phenolic acids, flavonoids, tannins, stilbenes and lignans depending on their chemical characteristics (Zhang et al. 2022; Pop et al. 2023). Phenolic compounds serve crucial functions in plants, such as contributing to pigmentation and defence mechanisms that enhance their resilience against environmental stressors such as pathogens and UV radiation (Kumar et al. 2020). Beyond their significance in plant physiology, phenolic compounds have received increasing attention for their beneficial effects on human health. Studies have demonstrated their antioxidant properties, which help to mitigate oxidative stress and potentially reducing the risk of chronic diseases including cardiovascular disorders, cancer, and diabetes (Cosme et al. 2020; Rashmi & Negi 2020; Zhang et al. 2022). Furthermore, these compounds exhibit anti-inflammatory and antimicrobial activities (Zhang et al. 2022).

Two of the largest groups of phenolic compounds are flavonoids and phenolic acids. Certain health benefits that are attributed to flavonoids are antioxidative, anti-inflammatory, anti-diabetic, anti-cancer, anti-obesity, and cardioprotective mechanisms, either by enhancing/inhibiting enzymes that have an effect on ROS levels, or by influencing gene expressions and productions related to inflammation (Ballard & Maróstica 2019). Phenolic acids, on the other hand, have been attributed to health benefits such as antioxidative, anti-inflammatory, immunoregulatory, anti-allergenic, anti-microbial, cardioprotective, and anti-cancer activities and antidiabetic properties (Rashmi & Negi 2020). Most flavonoids and phenolic acids are found in the form of glycosides, meaning that they are bound to sugars, fatty acids, or proteins (Acosta-Estrada et al. 2014).

2.2.3 Dietary fibre: How are they affecting our health?

This group of compounds have been the subject of scientific discussion since the term was coined in the 1950s (Hipsley 1953). The current definition from Codex Alimentarius (2017) describes dietary fibre as "carbohydrate polymers, or associated compounds, with a degree of polymerisation not lower than 3. Moreover, they are not digested nor absorbed in the small intestine, and they decrease the intestinal

transit time, increase stool bulk, are fermentable by gut microbiota and can reduce cholesterol levels in the blood". The chemical structure of dietary fibre is complex, with variations in the branching, crosslinking with other dietary fibre, and various degrees of methylation, acetylation, and sulfation (Carlsen & Pajari 2023). These structural differences influence the physicochemical properties, including solubility, fermentability and production of short chain fatty acids (SCFA), all of which impact the host.

An adequate intake of dietary fibre provides numerous health benefits, including positive effects on gut microbiota (Yang et al. 2013), and reduced mortality in relation to cancer, coronary heart disease, and cardiovascular disease (Kim & Je 2016). Additionally, dietary fibre helps lower blood cholesterol levels (Mandimika et al. 2012), influences gastric emptying rates (Mackie et al. 2016), and promotes peristaltic movement in the intestines (Wrick et al. 1983). Despite these benefits, most modern diets contain insufficient amounts of dietary fibre, potentially compromising people's health. In many Western countries, the average daily intake of dietary fibre is 15-25 g/day, depending on country, which falls short of the recommended daily intake of 20-38 g/day (Stephen et al. 2017). The recently updated Nordic Nutrition Recommendations (Blomhoff et al. 2023) suggests an intake of 25 g/day for women and 36 g/day for men. These recommendations emphasise that whole grain cereals, whole fruits, berries, vegetables, legumes/pulses, and nuts should be the major sources of dietary fibre.

2.2.4 Plant proteins: What impact can they have on climate?

Protein is a vital macronutrient required by both humans and animals for maintaining essential bodily functions, supporting immune system health, and enabling tissue growth and repair (Wu 2016; Yang et al. 2020). Traditional protein sources, predominately derived from animal husbandry, are associated with various environmental concerns, including GHG emissions, land degradation, and water depletion (Aleksandrowicz et al. 2016; Burke et al. 2025). The production of animal-based food and livestock feed accounts for 57 % of the global GHG emissions from food production (Xu et al. 2021), generating an urgent need for alternative protein sources with reduced environmental footprints. Green biomass, comprised of various plant-based materials such as leaves, grasses and aquatic plants, holds immense potential as a renewable reservoir of proteins (Møller et al. 2021;

Pérez-Vila et al. 2022). An increased use of this abundant resource offers several advantages in addressing both climate and food security challenges:

- **RuBisCO-rich composition**: Approximately 50% of the proteins in green biomass are RuBisCO (Feller et al. 2008), which is considered the most abundant protein in the worldwide (Bar-on & Milo 2019). Protein concentrates rich in RuBisCO have a high nutritional value and significant functional properties, which substantially enhances their potential as a valuable food ingredient (Nieuwland et al. 2021; Nynäs et al. 2023).
- **Reduced food-feed competition**: The utilisation of protein from green biomass as a food and feed source offers distinct benefits, as it currently does not negatively contribute to the food-feed competition (Santamaría-Fernández & Lübeck 2020).
- **Reduced climate impact**: When incorporated into the livestock feeding system, proteins from green biomass can reduce the environmental footprint of animal products. For instance, the climate impact of pork production is decreased by 17% when pigs were fed grass-clover protein compared to solely cereal based feed (Zira et al. 2023).

Extracting protein from green biomass presents a promising and potentially sustainable solution to meet the global protein demand (Domokos-Szabolcsy et al. 2023). This approach represents an effective use of agricultural side streams and cover crops, creating added value for the farmer by transforming what may otherwise be considered waste materials into valuable protein products. The integration of protein extraction from green biomass into existing agricultural systems could support multiple SDGs simultaneously, particularly SDG 2 (Zero Hunger) and SDG 12 (Responsible Consumption and Production) by promoting circular economy principles within food production systems.

2.2.5 Are dietary fibre, protein and phenolic compounds interacting in the biomass?

An aspect both wonderful and problematic is that the plant biomass is a matrix of many different compounds, which interact and chemically bind to each other. Although the combination of phenolic compounds and dietary fibre may significantly contribute to the overall health, the compounds are usually analysed separately due to substantial differences in their chemical

structure and biological properties (Saura-Calixto 2011; Edwards et al. 2017).

Dietary fibre is proposed to bind phenolics (Quirós-Sauceda et al. 2014; Phan et al. 2015; Gonzalez-Aguilar et al. 2017), enabling the phenolic compounds to escape digestive enzymes in the upper gastrointestinal tract and instead reach the colon intact (Perez-Jimenez et al. 2009; Palafox-Carlos et al. 2011). There, the gut microbiota can ferment both the dietary fibre and the phenolics to more easily absorbable compounds such as short-chain fatty acids (SCFA). To highlight the interactions between dietary fibre and phenolic compounds, the phrase "antioxidant dietary fibre" was suggested by Saura-Calixto (1998). Recent studies have indicated that there may be a similar situation for the interaction between phenolic compounds and proteins (Czubinski & Dwiecki 2017; Nemli et al. 2024), which further complicates the question of extracting and analysing the individual compounds separately or not.

Understanding how bioactive compounds interact within the plant matrix and which health impacts they impose remains a significant challenge. In an illuminating study involving mice that were fed diets containing either whole lingonberries, lingonberry dietary fibre fraction or lingonberry flavonoid fraction, researchers observed distinct health benefits associated with each dietary intervention (Liu et al. 2022). Whole lingonberries contributed to reduced atherosclerotic plaque formation in blood vessels, whilst the fibre fraction significantly elevated SCFA levels. Additionally, the flavonoid fraction notably enhanced populations of the beneficial gut bacterium *Akkermansia* (Liu et al. 2022).

Therefore, the interactions between proteins, phenolic compounds, and dietary fibre within plant matrices present significant analytical challenges. This complexity also raises important considerations for extraction processes and bioavailability of these components. A holistic approach is needed to preserve beneficial interactions while developing sustainable methods to utilise green biomass as valuable nutrition sources.

3. Aim and objectives

Considering the various challenges climate change poses to food production, it is essential to explore all possible crops usages, including the parts that are currently not used as food, to ensure utmost efficient utilisation. This comprehensive approach is necessary because climate change threatens to reduce agricultural yields whilst global food demand grows. Maximising the utility of each harvested crop builds resilience in our food systems, reduces waste and improves resource efficiency. Moreover, embracing more plant-based products is crucial for adopting a more environmentally sustainable diet, as plant-based food systems typically require fewer resources and produce fewer emissions.

Hence, the aim of this thesis was twofold*:*

1. Analyse the potential to utilise green leafy biomass to ensure efficient use of resources allocated to grow the crops
2. Evaluate the potential for innovative applications of green leafy biomass and intermediate crops

These aims can be further divided in objectives*:*

- To measure dietary fibre, phenolic compounds, and proteins in green leafy side streams from horticultural production and intermediate crops
- To extract phenolic compounds and protein from these side streams and intermediate crops on a larger scale
- Evaluate the potential of the extracted compounds and process fractions as food, feed, or other applications

4. Methods

The methods used in this thesis are described here briefly. Each method, whether isolating proteins or unlocking phenolic compounds, added a new dimension to the understanding of plant biomass. For more detailed descriptions, please refer to the Material and Methods in each paper.

4.1 Collection of biomass (Papers I–V)

Side stream leaves from broccoli and kale, intended for an analysis of dietary fibre, phenolic compounds, and protein content, were collected from the fields within 24 hours after the final harvest in both 2017 and 2018 (Papers I and II). Each fraction was weighed, with the numbers telling a story of abundance and waste, hope and opportunity. The collected material was transported to the laboratory in plastic bags prior to analysis.

Material for measurement of the amount of side streams of broccoli were collected simultaneously (Papers I and II), whilst kale side stream leaves were collected during the post-harvest sorting process to assess three distinct fractions: (i) leaves destined for market, (ii) leaves deemed unsuitable for sale, and (iii) resilient stems left behind (Paper II).

For Papers III and IV, fresh green biomass from red clover, lucerne, beet root tops, sugar beet tops, immature oat, white clover, hemp tops, and pea residuals, were collected once per crop from operating farms in Scania between June and October in 2020. The biomass was processed within 3 h to minimise the risk of degradation of compounds and loss of water.

For Paper V, biomass from four different intermediate crops: buckwheat, hemp, phacelia, and oilseed radish was harvested at different time points—August, September, October, and November—in 2017, with and without the application of fertiliser in the field.

4.2 Measurement of dietary fibre (Papers I and II)

Each fragment of fibre holds potential health benefits, culinary uses, and opportunities for valorisation. The content of dietary fibre in broccoli and kale leaves was determined using a standardised method (Theander et al. 1995), adapted as per Andersson et al. (1999), to enable separate quantification of soluble and insoluble dietary fibre fractions (Papers I and

II). The broccoli, freeze-dried and milled into a fine powder, was subjected to an enzymatic treatment that eased apart its structures of dietary fibre. Soluble fibres and insoluble fibres were all coaxed into yielding their constituents: sugar residues, uronic acids, and Klason lignin. These were subsequently analysed using gas chromatography, colourimetry, and gravimetry, respectively.

4.3 Plant Protein Factory process (Papers III and IV)

The Plant Protein Factory is a facility with the goal to develop and scale up techniques and evaluate the economic potential of green proteins and other compounds of interest from underutilised biomasses (*SLU Holding* 2024). The facility is located in Alnarp, Sweden, owned by the Swedish University of Agricultural Sciences. The factory process (Figure 3) was aimed at optimising the protein yield from the biomass.

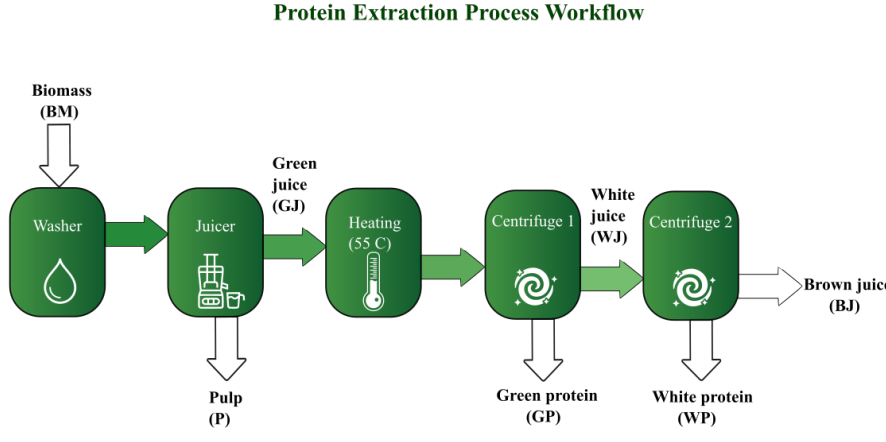

Figure 3: Process for protein extraction in the Plant Protein Factory

The fresh green biomass (BM) was washed to remove dirt and other particles, then processed in a juicer to produce a pulp fraction (P) and a green juice fraction (GJ). The GJ was heat-treated to coagulate the proteins and subsequently centrifuged to produce a green protein fraction (GP) and a white juice fraction (WJ). The WJ received an addition of citric acid to lower the pH, which caused the remaining protein to precipitate, and to obtain a

white protein fraction (WP) and a remaining brown juice fraction (BJ) after centrifugation.

4.4 Measurement of protein content (Papers II, III and V)

The next step in the journey involved quantifying the protein content within these fractions. Proteins are more than just their overall content — they are mosaics of different amino acids. The protein content in the samples was measured with Dumas method, calculating the content of nitrogen (N) and multiplying it with a factor of 6.25 according to FAO (2019) (Papers III and V) or 5.6 (Paper II). When comparing the results between the different papers in this thesis, a factor of 5.6 was used (Mariotti et al. 2008). To analyse RuBisCO specifically, the major protein behind photosynthesis (Erb & Zarzycki 2018), a size exclusion (SE)-HPLC was used (Paper III). With this analysis, the content of RuBisCO could be specifically analysed according to Desai et al. (2014). To evaluate the content of amino acids, samples were analysed according to a standard method (ISO 13903:2005) (2005) in a certified laboratory (Eurofin, LU).

4.5 Extraction and measurement of phenolic compounds (Papers I and IV)

The bioactive phenolic compounds, known for their antioxidant properties, are bound to the cell walls and other structures of the plant material. The fractions from the protein factory were also analysed for their content of phenolic compounds. The analysis of phenolic compounds commenced with an ethanol extraction to liberate the free phenolic compounds into the alcohol and make them ready for analysis. After centrifugation, a pellet remained, which contained the bound phenolic compounds and dietary fibre. The remaining pellet was initially treated with alkali hydrolysis by adding NaOH to break the ester bonds linking phenolic compounds to polysaccharides, e.g. in the cell wall. With the bonds severed, the bound phenolic compounds were released. However, even then certain compounds remained locked away (Figure 4). Following the alkali hydrolysis, the remaining pellets were treated with acid hydrolysis by adding HCl to unleash the remaining phenolics bound to sugars, fatty acids, or proteins, whilst leaving ester bonds intact.

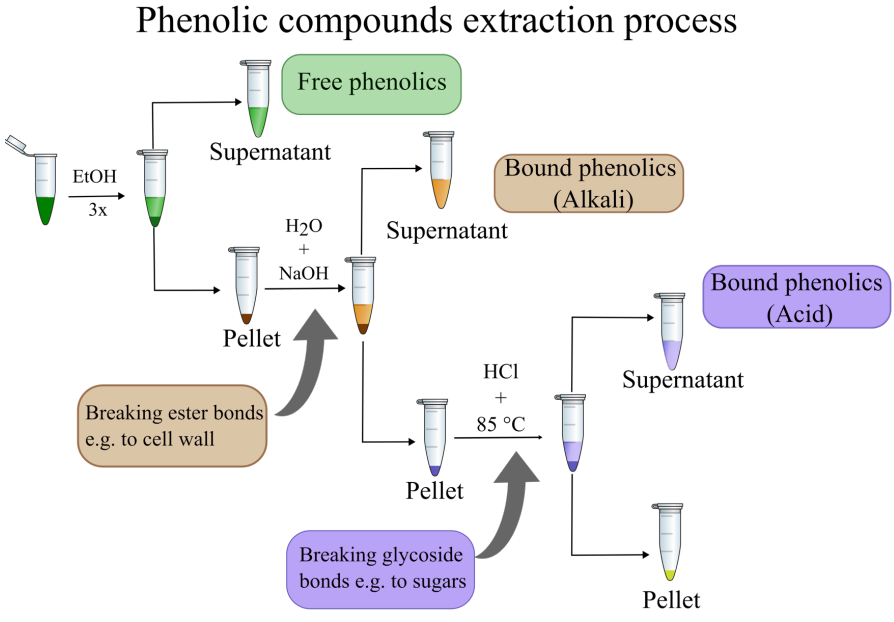

Figure 4: Extraction process for free and bound phenolic compounds.

The extracted phenolic compounds were analysed via high-performance liquid chromatography-mass spectrometry (HPLC-MS-DAD), which enabled both quantification of their abundance and tentative identification of these structurally diverse compounds. The Folin-Ciocalteu assay and FRAP method were employed to illuminate the antioxidative tapestry of the samples, thus providing glimpses into their potential as guardians against oxidative stress.

5. Aim 1: Analyse the potential to utilise green leafy biomass to ensure efficient use of resources allocated to grow the crops

5.1 Amount left after harvest: Just a few leaves, right? (Papers II and V)

Broccoli and kale function as model plants in this thesis to demonstrate the significant biomass volume currently classified as side streams in horticultural production. For broccoli, only a small part of the above-ground biomass is considered edible, which is the broccoli head (Dominguez-Perles et al. 2010). Kale leaves in particular have recently garnered attention for their high content of health beneficial compounds (Becerra-Moreno *et al.,* 2014; Šamec *et al.,* 2018).

Measurements of the broccoli plant revealed that the leaves constituted 43–78 % of the fresh weight of the whole broccoli plant and amounted to 64–84 % of the crop residues after the broccoli head was removed (Paper II). The marketable broccoli head only comprised approximately 20 % of the total wet weight of the plant (Figure 5). Even when using the lowest estimates, this translates to 3.8 t dry matter (DM) (25 t fresh weight (FW)) of residual biomass per hectare.

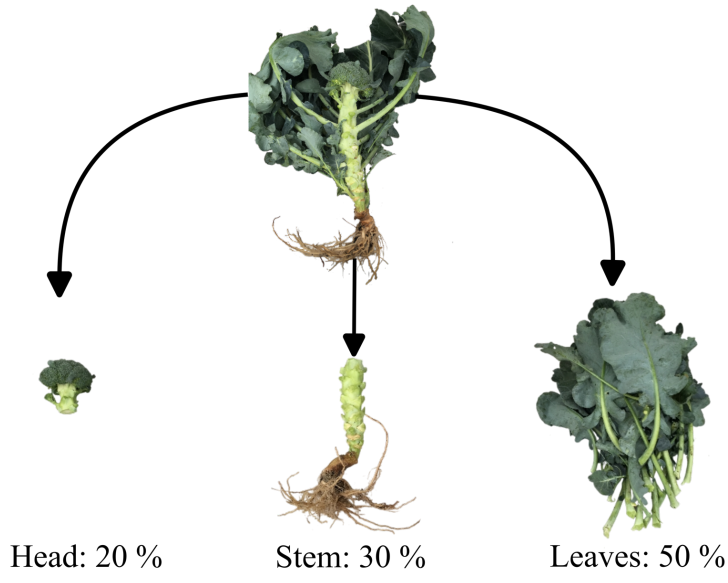

Figure 5: An example of division of a broccoli plant.

Here divided into head, stem + root, and leaves, and the mean percentage that each part composes.

In Sweden in 2023, the cultivated areal of broccoli was 447 hectare (ha) (Karlsson 2024) indicating that at least 1,699 t DM (11,175 t FW) of broccoli leaves remained on the field after harvest. In terms of FW, this volume is comparable to the total food waste (from the plate, serving and from the kitchens) from all school lunch catering services in Sweden in 2022; 11,500 tonnes (Figure 6) (Fritz & Jonsson 2023). Most of the broccoli side streams in the field today are ploughed into the field as a green fertiliser (Liu et al. 2018).

Regarding kale, the rejected leaves account for approximately 16 % of the plant's wet weight, whilst the marketable leaves correspond to 50 % and the stems constitute the remaining 34 % of the plant's wet weight (Paper II). In 2023, 175 ha was used for kale cultivation in Sweden (Karlsson 2024), resulting in an amount of rejected kale leaves amounting to 52.5 tonnes DM (1,347 t FW) of residual biomass that could be used for fractionation of protein, phenolic compounds, and fibre, amongst other valuable compounds.

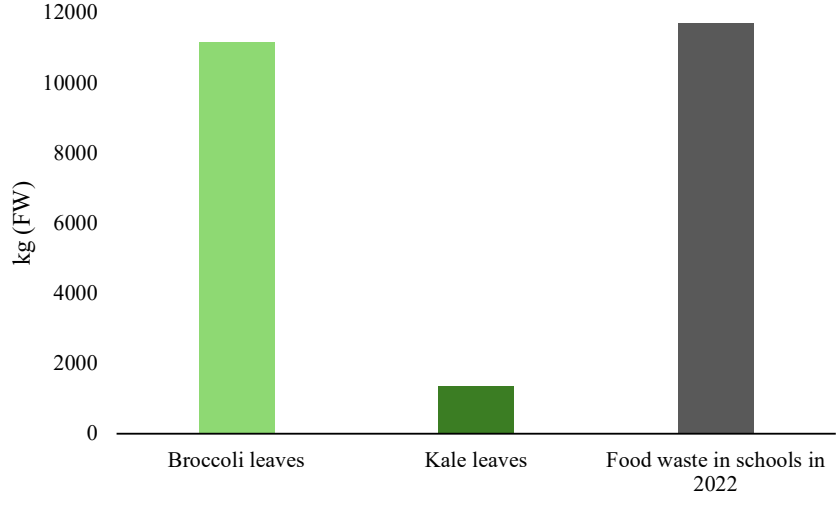

Figure 6: Estimate of side stream in broccoli and kale production.

Compared to the amount of all wasted food in Swedish schools in 2022.

Another readily available source of green leafy biomass is intermediate crops. When analysing this type of biomass, a preliminary approach to estimate cultivation area is by analysing data from farmers' economic support applications, which provide documented records of land use and crop selection. In 2023, the area was approximately 86,000 ha for catch crops (crops specific ally sown to catch nitrate between two main crops) and 77,000 ha for intermediate crops (crops sown between two main crops to obtain soil cover and catch nutrients such as nitrate) (Hill 2023), demonstrating a significant potential biomass source for extracting high-value compounds.

Hence, there is a substantial amount of green leaf material that could be valorised into new products or be used as feed stock into a biorefinery to extract compounds of interest.

5.2 Fibre content in broccoli and kale leaves (Papers I–II)

Both broccoli and kale leaves exhibited a high content of total dietary fibre compared to the other vegetables (Table 1). For broccoli leaves (Paper I) the total fibre content remained stable between the years, despite considerable

variations in growing conditions, particularly regarding temperature and rainfall. This consistency suggests a reliable nutritional profile that could be valuable for commercial applications.

Noteworthily, the total dietary fibre content of broccoli and kale leaves exceeded that of certain products marketed specifically as ”fibre-rich” such as oat bran (Table 1). The analysis focused on total dietary fibre content rather than distinguishing between soluble (SDF) and insoluble dietary fibre (IDF). However, the findings from Paper I revealed that for the broccoli leaves 90% of the total dietary fibre consisted of insoluble dietary fibre, which may have influenced the extractability on other compounds and may also influence the texture of food when incorporated. The high fibre content discovered in these typically discarded materials presents a significant opportunity for resource optimisation in crop production. Furthermore, these results highlight the potential for developing innovative food and feed applications that capitalise on these fibre-rich materials, potentially creating new value-added products from what is currently considered as waste.

Table 1: Levels of total dietary fibre in some vegetables and vegetable parts.

Sample	Mean [g/100 g DW]	Reference
Onion	47.2	(Kalala et al. 2018)
Cabbage outer leaves	40.9	(Tanongkankit et al. 2012)
Kale leaves	40.7	Paper II
Brussels sprouts	39.2	(Nowak et al. 2025)
Broccoli florets	38.7	(Nowak et al. 2025)
Broccoli florets	36.0	(Kalala et al. 2018)
Broccoli leaves	35.2	Paper II
Cauliflower (curd)	29.7	(Kalala et al. 2018)
Broccoli leaves	28.2	Paper I
Carrot	24.1	(Theander et al. 1995)
Oat bran	18.4	(Theander et al. 1995)
Apple	17.9	(Theander et al. 1995)
Green peas	16.7	(Theander et al. 1995)

In the fractionation process, most of the total dietary fibre from the biomass concentrates in the P fraction, with progressively smaller quantities distributed across the juice and protein fractions (Figure 7). This distribution pattern represents a viable opportunity to maximise resource efficiency from horticultural side streams. It is reasonable to hypothesise that the later fractions (GP, WJ, WP, and BJ) may contain a higher proportion of soluble dietary fibre, whilst the predominant fibre content in the P fraction likely consists of insoluble fibre components. This differentiation opens innovative pathways for targeted applications across food, feed and other potential markets, since insoluble dietary fibre increases the volume of the stool and reduces transit time (Carlsen & Pajari 2023). Whereas soluble dietary fibre instead as functionalities such as lowered the serum cholesterol levels, improves glycaemic control, and delays gastric emptying (McRorie & McKeown 2017; Carlsen & Pajari 2023). Soluble dietary fibre can also be used for improving texture and nutritional content in bread (Renzetti et al. 2025).

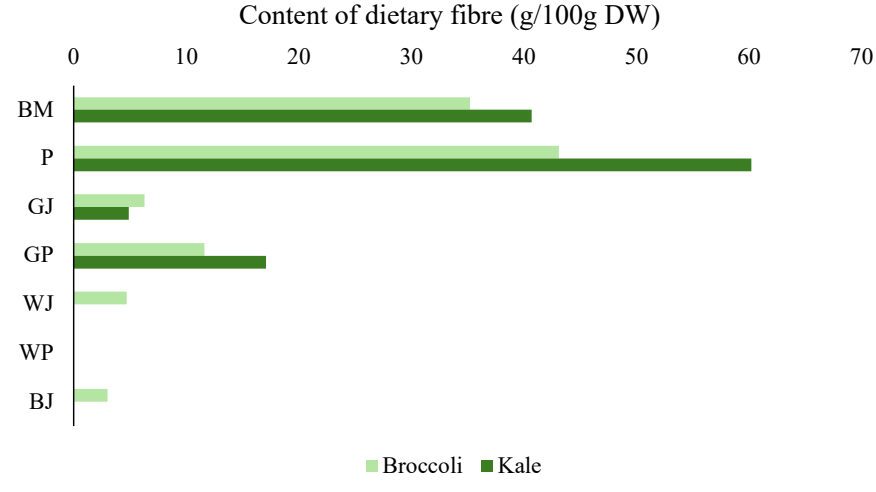

Figure 7: Content of dietary fibre in different fractions from broccoli and kale leaves.

The process fractions are biomass (BM), pulp (P), green juice (GJ), green protein (GP), white juice (WJ), white protein (WP), and brown juice (BJ).

The concentrated insoluble fibre in the P fraction could be developed into high-fibre food ingredients or sustainable packaging materials, whilst the

soluble fibre components in the juice and protein fractions could serve as functional food ingredients with potential prebiotic properties. This strategic separation generates multiple value streams from what would otherwise be considered waste material, thereby ensuring holistic utilisation of all nutrients present in the original horticultural side streams.

When attempting to expand our understanding of dietary fibres' complex health impacts, it is essential to implement advanced analytical methodologies for comprehensive characterisation of structural composition and functional bioactivity profiles. Categorising fibre into soluble and insoluble components, or alternatively into fermentable or not fermentable (Williams et al. 2019), would provide greater insights into their potential applications. Different dietary fibre types exert distinct physiological effects, each contributing uniquely to human and animal health outcomes. This detailed characterisation would enable more efficient utilisation of horticultural side streams through a precise identification of valuable fibre components.

5.3 Protein content (Papers II, III and V)

5.3.1 Extractable proteins (Papers II, III and V)

In the laboratory scale process for broccoli and kale (Paper II), the highest protein contents were found in the GP and WP fractions (Figure 8). Both broccoli and kale leaves contained lower total protein compared to the intermediate crops (as shown when comparing Figures 8 and 9). The differences between these leafy biomasses may be attributed to species-specific characteristics or processing differences during the dry or wet fractionation. However, both the laboratory-scale protein fractionation and pilot-scale fractionation were conducted with limited replication, which highlights the need for further studies to properly evaluate the influence of e.g. crop species and harvest date on the protein content and extractability.

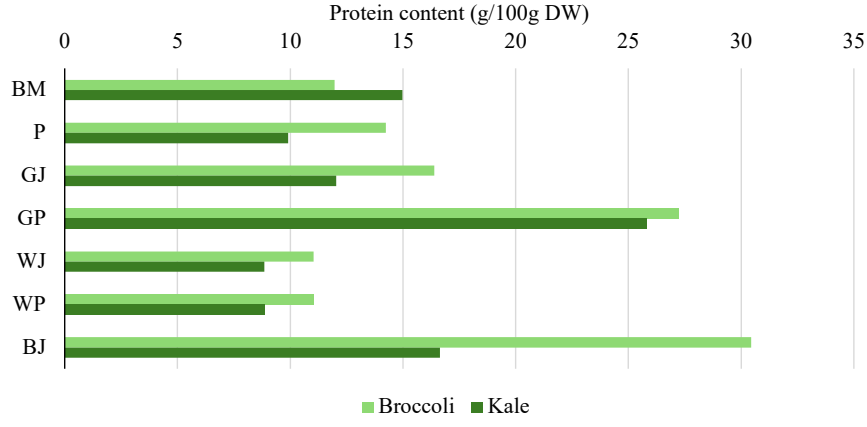

Figure 8: Protein content from lab scale protein wet extraction for broccoli and kale leaves.

The pilot-scale wet fractionation process (Paper III) of cover crop and green leafy biomass demonstrated notable variations in protein content. Immature oat WP exhibited the highest total protein content of 34.2 g/100g DW (Figure 9). Most proteins were concentrated in the protein fractions (GP and WP) for lucerne, pea, sugar beet, white clover and oat, although some water-soluble proteins migrated through the entire process to the BJ fraction. For red beet, red clover, and hemp, most of the proteins were found in GP and with GJ or WP. Amino acids constituted the largest proportion of nitrogen in all intermediate crops fractions (Paper III) whilst 30-49% of the nitrogenous compounds—primarily water-soluble components—remained unidentified.

Identifying the pure RuBisCO peaks on the chromatograms (Paper III) was challenging, therefore we instead looked at the RuBisCO rich peak regions of different biomass sources, where the content ranged from 70.2 to 207.5 mg/g when analysed by (SE)-HPLC. Further, the unidentified proteins (C, E) were less than 1% of the total (Table 2). The highest levels of total possible RuBisCO were found in the WJ and BJ fractions, which was expected since native RuBisCO is water soluble.

A consistent pattern can be seen through the fractionation process (Paper III), where all protein types and amino acids exhibit similar migration behaviour, resulting in an insignificant separation of RuBisCO from other proteins. This pattern extends to essential versus nonessential amino acids, which likewise showed minimal differentiation. Nevertheless, earlier studies have demonstrated that despite the fact that the protein fractions from green biomass contains a heterogenous mixture of proteins, there seems to be no negative impact on the functional properties of these fractions (Nynäs et al. 2023). Similar findings regarding protein functionality in fractions have been reported in studies of leaf protein concentrates from various plant sources (Tamayo Tenorio et al. 2016; Nynäs et al. 2021).

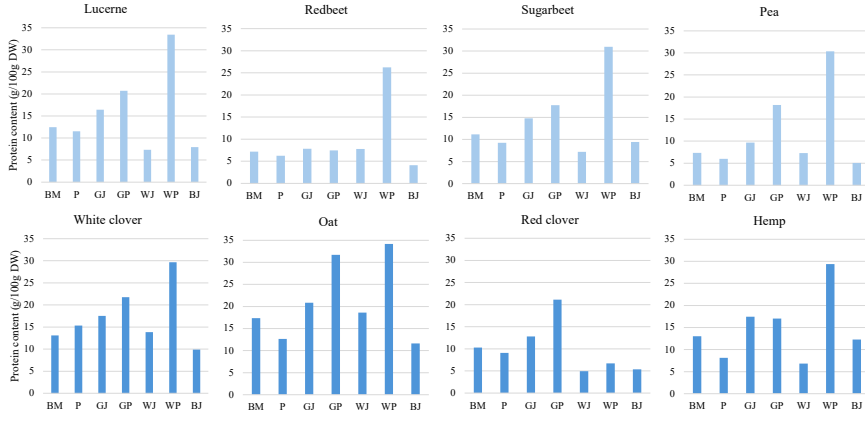

Figure 9: Protein content in the fractions from protein extraction process for the individual intermediate crops and green leafy biomasses.

Table 2: Mean values and standard deviation of protein components (mg/g dry weight) in different fractions as analysed with SE-HPLC. Peak regions can be found in Paper III.

Source	Soluble Peak A, B, D	Soluble Peak C, E	Insoluble Peak A, B, D	Insoluble Peak C, E	Total protein
Fraction					
BM	123 ± 42.2 ^{C}	0.58 ± 0.23 ^{C}	18.8 ± 8.25 ^{B}	0.06 ± 0.05 ^{B}	142 ± 46.5 ^{C}
P	56.0 ± 15.0 ^{D}	0.32 ± 0.13 ^{D}	13.8 ± 3.56 ^{C}	0.06 ± 0.04 ^{BC}	70.3 ± 16.5 ^{D}
GJ	166 ± 60.9 ^{B}	1.00 ± 0.55 ^{B}	18.4 ± 12.1 ^{B}	0.06 ± 0.04 ^{BC}	186 ± 67.6 ^{B}
GP	78.5 ± 94.0 ^{D}	0.52 ± 0.59 ^{CD}	11.6 ± 4.39 ^{CD}	0.06 ± 0.04 ^{BC}	90.7 ± 95.0 ^{D}
WJ	214 ± 59.1 ^{A}	1.14± 0.38 ^{AB}	8.87 ± 5.75 ^{D}	0.03 ± 0.03 ^{BC}	224 ± 60.9 ^{A}
WP	122 ± 64.2 ^{C}	0.66 ± 0.41 ^{C}	24.9 ± 14.1 ^{A}	0.13 ± 0.14 ^{A}	148 ± 69.8 ^{C}
BJ	219 ± 53.4 ^{A}	1.34 ± 0.74 ^{A}	3.32 ± 2.87 ^{E}	0.02 ± 0.02 ^{C}	223 ± 54.3 ^{A}

Total protein is calculated as the sum of Soluble Peaks A, B, D, C and E and Insoluble Peaks A, B, D, C, and E. Values in columns followed by the same letter does not significantly differ ($p<0.05$) using the Duncan post hoc test.

Significant variations in nitrogenous compound quantities were observed across biomass sources. Nitrogen content is influenced by multiple factors, including crop species, harvest date, and biomass disruption during processing (Muneer et al. 2021; Nynäs et al. 2021; Stødkilde et al. 2021). A substantial portion of the proteins (in nitrogen form) from intermediate crops, broccoli, and kale accumulated in the solid P fraction, possibly either because proteins are bound to dietary fibre components (Damborg et al. 2020) or insufficient particle milling that prevented complete protein release (Nynäs et al. 2021). A subsequent study exploring various coagulation temperatures (50°C, 55°C, 60°C and 65°C) and centrifugation techniques to enhance white protein yield from alfalfa/lucernce, discovered that an optimal coagulation temperature of 55°C improved white protein recovery. However, challenges persisted regarding a reduction in yield during green protein separation from white juice and the subsequent white protein extraction (dos Passos & Ambye-Jensen 2024). These findings underscore the need for further process optimisation to maximise protein extraction efficiency.

Different extraction methods have an impact on the extracted proteins and their functionality in various ways. A wet fractionation of broccoli leaves, followed by either a microwave radiation or lacto-fermentation of the GJ (Domokos-Szabolcsy et al. 2022), increased the crude protein content with microwave radiation to 34.3 %, and with lacto-fermentation to 39.2 %, from protein content of 27.4 % in the GJ. A dry fractionation, as was used in Paper V, is more energy efficient and can produce enriched protein fractions with retained functionality, as compared to wet fractionation (Schutyser & van der Goot 2011). Accordingly, dry fractionation of legume seeds gave a higher protein yield per unit of energy used in the process (55.8 g protein/MJ), compared to wet fractionation (14.6 g protein/MJ), but the dry fractionation protein had lower purity (Schutyser et al. 2015).

An alternative method to improve the protein yield is to combine dry fractionation and wet fractionation, which resulted in a yield of 29.1 g protein/MJ, which is almost double compared to only wet fractionation (Schutyser et al. 2015). In faba beans, a combined dry and wet fractionation recovered 87 % of the total seed protein (as compared to 55 % in a dry fractionation) and at the same time simultaneously consumed 5.5 times less water per kg extracted proteins compared to a one step wet fractionation (Dumoulin et al. 2021). When comparing the effect of the process on the protein, the fractionation process, wet or dry, impacted the functional

properties (Hopf et al. 2024). Dry fractionated pulse proteins had higher solubility and emulsifying capacity and were lighter in colour compared to wet fractionated pulse proteins. Dry fractionated proteins had, however, lower water and oil binding capacity (Hopf et al. 2024). Thus, different extraction methods have different impacts on the characteristics of the obtained proteins, which is worth to considering in future studies.

These findings demonstrate how valuable proteins can be efficiently extracted from horticultural side streams and other underutilised green leafy biomasses, thereby ensuring more a complete utilisation of crop resources. They also identify promising fractions for innovative food and feed applications, particularly the protein-rich GP and WP fractions which could help address the growing demand for sustainable protein sources.

5.3.2 The impact of harvest time and fertilisation (Paper V)

The protein concentration within biomass fluctuates based on several key factors, including crop species, harvest date, and fertilisation protocols. In Paper V, the protein yield (kg protein per hectare) was calculated across four distinct crops under varying harvest periods and fertilisation regimes (Figure 10) to identify which horticultural side streams offer the most promising protein resources. Not all crops proved suitable or economically viable for protein extraction processes. Buckwheat, for instance, poses challenges due to its limited protein-rich biomass production, which is decreasing with later harvest dates (Figure 10). Hemp, by contrast, exhibits elevated protein levels and substantial biomass volume when both properly fertilised and harvested later in the season, thus establishing itself as a superior candidate for protein fractionation endeavours.

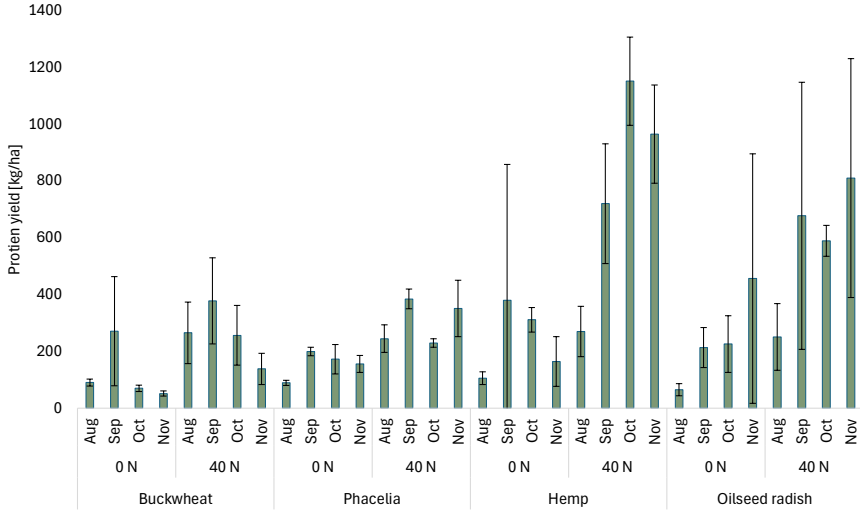

Figure 10: Protein yield for buckwheat, phacelia, hemp and oilseed radish.

The protein yield is measured during different harvest dates, with (40 N) or without (0 N) fertilisation, from Paper V. Bars show the mean values and error bars show the standard deviation.

These insights offer valuable guidance for the strategic selection of crops when developing protein extraction systems from green leafy biomass. By prioritising high-protein, high-biomass crops such as hemp, it is possible to maximise resource efficiency and enhance the economic viability of the fractionation process. This allows for more productive innovative applications of the extracted protein fractions, whether for human food ingredients, animal feed supplements, or other potential markets requiring sustainable protein sources. An important next step will be to conduct comprehensive economic assessments to evaluate the feasibility of using intermediate crops for protein extraction, for both the growers and industry. Additionally, ecological sustainability analyses will be crucial to ensure that any developed processes align with broader environmental objectives. These evaluations will help determine whether the biological potential of certain crops translates into practical, sustainable solutions for resource-efficient food systems. By optimising harvest timing and fertilisation protocols for specific high-performing crops such as hemp, both the quantity and quality of extractable proteins from green leafy biomass can be improved, creating new value from what would otherwise be underutilised resources.

5.4 Phenolic compounds (Papers I, II, and IV)

5.4.1 Broccoli and kale (Papers I and II)

In the quest for phenolic compounds in green leafy biomass from broccoli and kale, the highest amounts of total phenolic compounds were found for WP (Figure 11), followed by the juice fraction (GJ, WJ, and BJ) (Paper II). Of the total phenolic compounds, between 55–95 % were free phenolic compounds (extractable with ethanol as a solvent), with a lower share in the P and GP fractions (Table 3).

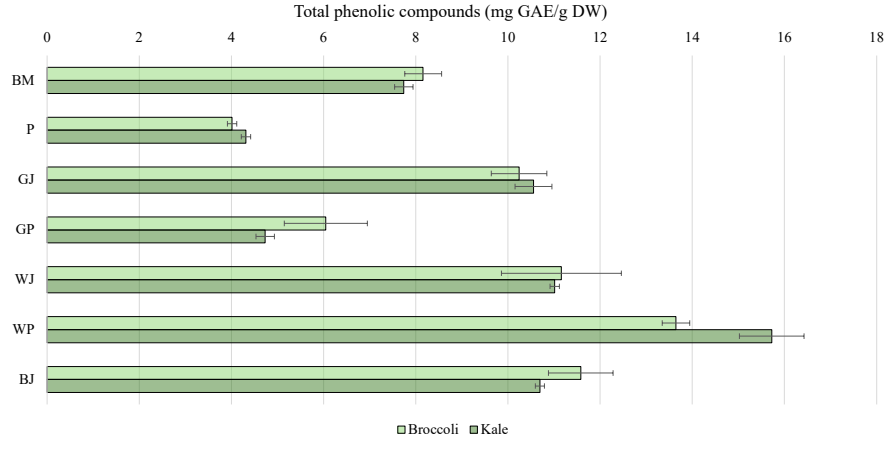

Figure 11: Total amount of phenolic compounds in process fractions from broccoli and kale leaves (Paper II)

In another study on the wet fractionation process (Domokos-Szabolcsy et al. 2022), the most abundant flavonoids in the GJ were quercetin and kaempferol, and the content of quercetin and kaempferol were increased 10-fold with microwave radiation and at least one order of magnitude with lactic-fermentation in the resulting protein rich fraction. In our study, the total phenolic compound content increased from the GJ to the WP, and it would be interesting in future studies to analyse if the content of kaempferol and quercetin could be similarly increased to the levels from Domokos-Szabolcsy et al (2023) with an alternative method.

Table 3: Content of free and bound phenolic compounds, measured by Folin Ciocaltreu assessement, measured in mg GAE/mg DW). (Paper II)

Crop	Component	BM	P	GJ	GP	WJ	WP	BJ
Broccoli leaves	Free phenolic compounds	6.7 ± 0.4	2.4 ± 0.2	9.2 ± 0.6	4.5 ± 0.8	10.7 ± 1.3	13.1 ± 0.3	11.0 ± 0.7
	Total phenolic compounds	8.2 ± 0.4	4.0 ± 0.1	10.2 ± 0.6	6.0 ± 0.9	11.2 ± 1.3	13.6 ± 0.3	11.6 ± 0.6
Kale leaves	Free phenolic compounds	6.2 ± 0.2	2. 4 ± 0.1	9.0 ± 0.4	2.8 ± 0.1	10.5 ± 0.2	13.5 ± 0.6	10.3 ± 0.1
	Total phenolic compounds	7.7 ± 0.2	4.3 ± 0.1	10.6 ± 0.4	4.7 ± 0.2	11.0 ± 0.1	15.7 ± 0.7	10.7 ± 0.1

It is important to remember that the content of phenolic compounds may differ between years (Paper I), as was found in Brassicas due to differences in light intensity, soil conditions, and insect attacks (Cartea et al. 2011). The levels of phenolic compounds in kale have been shown to increase when the temperature decreases due to an accumulation of secondary metabolites (Neugart et al. 2012, 2018). This trend can be observed when comparing the levels of phenolic compounds in 2017 and 2018 for broccoli (Table 4), as the summer in 2018 was exceptionally dry and warm, resulting in a lower number of phenolic compounds compared to 2017 (Table 4 and Paper I). It is reasonable to assume that a similar trend can be found for kale, and other green leafy vegetables, as well.

Table 4: Comparing the content of phenolic compounds by methanol extraction.

Sample	mg/g DW	Reference
Broccoli leaves, 2017	10.8–15.2	Paper I
Kale leaves	10.6	(Olsen et al. 2009)
Broccoli leaves	8.2	Paper II
Kale leaves	7.7	Paper II
Broccoli leaves, 2018	6.3–7.5	Paper I
Broccoli florets	1.7–2.2	(Torres-Contreras et al. 2017)

5.4.2 Intermediate crops (Papers III-IV)

In the eight biomass sources, most of the phenolic compounds were found in free form, and of these flavonoids and phenolic acids made up over 90 % of the present phenolic compounds present (Paper IV). When focusing on the process fraction instead of the individual crops, it becomes clear that most of the free phenolic compounds concentrate in the juice fractions (Table 5). Regarding the free flavonoids, most were dissolved into the GJ at juicing or into the BJ in the end of the process. For the free phenolic acids, in addition to high concentrations in GJ and BJ, there were also high amounts in WJ. It is possible that the current method alters some of the phenolics due to oxidation and hence the recovery rate is lower compared to during optimal conditions, as has been shown by Shi et al (2022) (Paper IV).

Table 5: Content of total free phenolic compounds, divided into flavonoids and phenolic acids, in the fractions, all crops combined, from the fractionation process (Paper IV).

	Free flavonoids (µg/ml)	Free phenolic acid (µg/ml)
BM	745.9 ± 525.2 ^{ABC}	58.9 ± 49.3 ^{B}
P	430.9 ± 371.7 ^{A}	17.9 ± 24.3 ^{A}
GJ	1097.0 ± 1118.0 ^{C}	111.1 ± 83.8 ^{C}
GP	641.5 ± 563.8 ^{AB}	35.4 ± 38.8 ^{AB}
WJ	1044.4 ± 1324.7 ^{BC}	105.1 ± 67.6 ^{C}
WP	642.7 ± 579.9 ^{AB}	45.3 ± 41.2 ^{AB}
BJ	1074.8 ± 1238.6 ^{C}	87.8 ± 68.4 ^{C}

5.5 The green trinity: Is there a problem with protein, fibre and phenolic compounds occurring together?

The presence of dietary fibre may adversely affect protein extractability in biorefinery processes. Previous research indicates that soluble dietary fibre suppresses protein digestibility in the digestive tract (Dégen et al. 2007; Stødkilde et al. 2018), which is attributed to the gel-forming properties of these fibres or the presence of tannins (Sarkar et al. 2022). Considering that combining the extraction of proteins and phenolic compounds is proposed to enhance the economic viability of biorefinery processes, understanding the interactions between these compounds and others present, such as dietary fibre, is essential.

A principal component analysis (PCA) indicates that there is no significant correlation between amino acids (Ess amino and Noness Amino) and bound phenolic compounds (PC, alk and PC, acid), as the angle between the vectors is almost 90° (Figure 12). Instead, there was a significant correlation between the amino acids and between the bound phenolic compounds, which is confirmed with a correlation analysis (Table 6).

The amino acids and the free phenolic compounds, on the other hand, show a relationship (Figure 12), as the angle between the vectors is relatively small. The correlation between amino acids and free phenolic compounds were, however, not significant according to correlation analysis (Table 6), which suggests that the relationship might only be present within certain fractions. These results seem reasonable as amino acids are predominantly found in protein fractions (GP and WP) and in GJ, whilst bound phenolic

compounds are mainly present in juice fractions (GJ, WJ, and BJ) and in BM and P. Free phenolic compounds are primarily located in juice fractions (GJ, WJ, and BJ). This indicates that different fractions might be of interest when seeking high protein extraction yield or high phenolic compound yield from the biorefinery process.

Table 6: Spearman rank correlation analysis of the phenolic compounds and amino acids from intermediate crops (Paper III and IV)

	PC, Alk	PC, Acid	PC, Free	Ess AA	Noness AA
PC, Alk	1.00				
PC, Acid	0.74 ***	1.00			
PC, Free	-0.02	-0.02	1.00		
Ess AA	-0.04	0.03	0.02	1.00	
Noness AA	0.00	0.04	0.03	0.89 ***	1.00

PC, alkali = phenolic compounds extracted with alkali hydrolysis. PC, acid = phenolic compounds extracted with acid hydrolysation. PC, Free= phenolic compounds, free. Ess AA = essential amino acids. Noness AA = nonessential amino acids.

*** $p < 0.005$. Only values marked with *** were significant

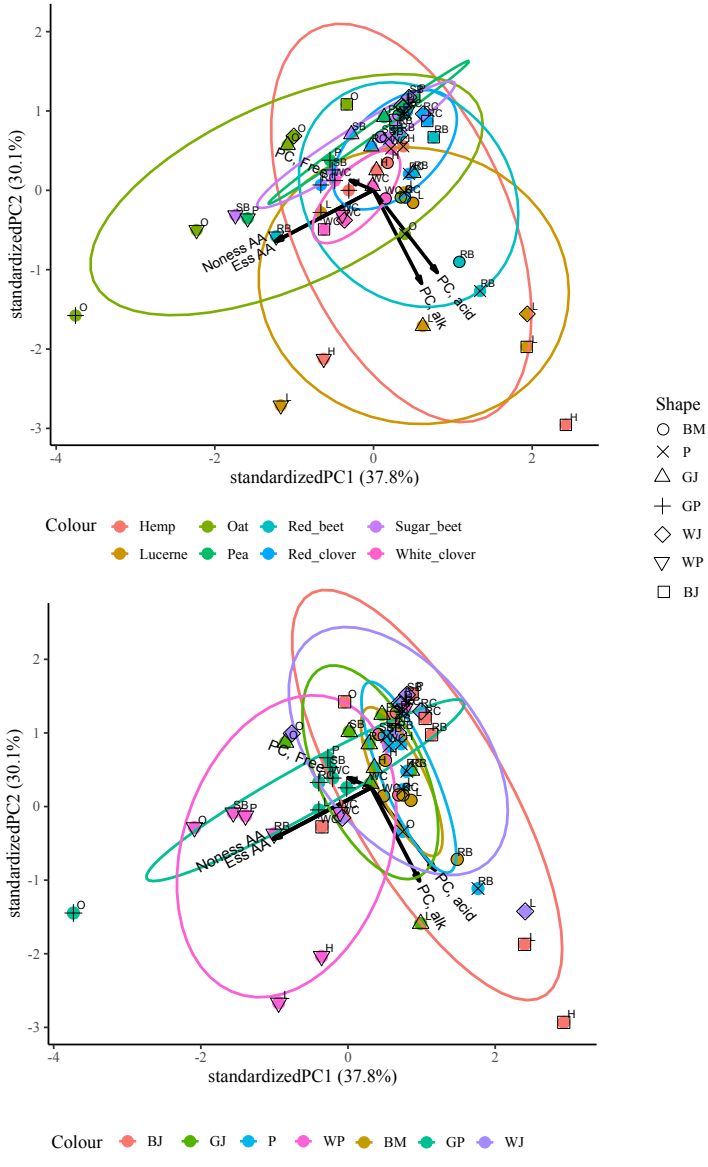

Figure 12: Principal component analysis (PCA) with combined nitrogen and phenolic compounds for intermediate crops and green leafy biomass.

Upper figure shows crops as groups, whilst the lower figure shows process fractions as groups. PC, alkali = phenolic compounds extracted with alkali hydrolysis. PC, acid = phenolic compounds extracted with acid hydrolysation. PC, Free= phenolic compounds, free. Ess AA = essential amino acids. Noness AA = nonessential amino acids. (Papers III and IV).

Since phenolic compounds and dietary fibre might interact and bind to each other, a crucial next step in optimising the biorefinery process is to analyse both soluble and insoluble dietary fibre content in the biomass and fractions. In broccoli leaves, a negative correlation was indicated between free phenolic compounds (green) and soluble dietary fibre constituents (blue mand Sol), visible with they being on opposite sides of 0 on Principal component 1-axis. At the same time, a positive correlation was indicated between bound phenolic acids (orange) and certain soluble dietary fibre constituents, due to both their positive values on Principal component 1-axis and close position to each other (Figure 13). Insoluble fibre constituents (blue and Insol) indicated a negative correlation with certain phenolic acids. The Pearson correlation analysis can be found in Paper I. This suggests that a higher insoluble dietary fibre content reduces extractable phenolic compounds, possibly due to interactions between insoluble fibre and phenolic compounds. Conversely, a higher soluble dietary fibre content enhances the extractability of bound phenolic acids. Therefore, analysing the correlations between phenolic compounds, proteins and dietary fibre in the same samples is vital to understand the possible interactions and how they could affect structure, bioavailability and functionality. This knowledge would aid in finding innovative applications of side streams and green leafy biomass and also evaluating the potential of extracted compounds as food, feed or other products through optimised extraction processes.

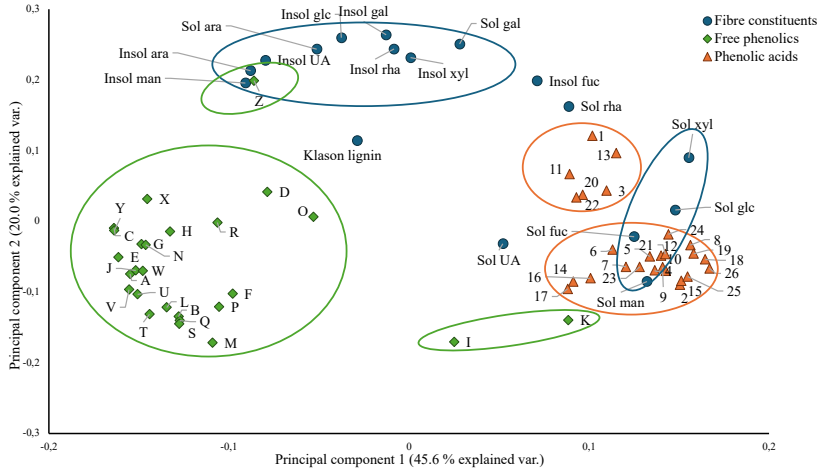

Figure 13: Loading plot for the principal component analysis (PCA) for dietary fibre constituents, free phenolics and phenolic acids (after hydrolysis) from broccoli leaves.

From Paper I. The dietary fibre consistent are Klason lignin and sugar residues. Insol = insoluble fibre constituent. Sol= soluble fibre constituent.

6. Aim 2: Evaluate the potential for innovative applications of green leafy biomass and intermediate crops

The innovative utilisation of horticultural side streams and other underutilised green leafy biomass sources represents a remarkable opportunity to transform what was once considered waste or underutilised biomasses into valuable resources. By thoroughly analysing the fibre, protein, and phenolic compound profiles within these raw materials, it is possible to strategically direct each component toward optimal applications, creating multiple streams of beneficial products with significant economic and environmental advantages.

6.1 Food

Horticultural side streams offer tremendous potential as food ingredients. Adding 10% vegetable powder from sources such as carrot, tomato, broccoli florets, and beetroot can significantly enhance the nutritional and functional attributes of oil-free bread (Ranawana et al. 2016). Broccoli leaf powder is particularly promising, as it has been successfully incorporated into gluten-free sponge cake, improving not only its mineral content, antioxidant capacity (Drabińska et al. 2018), and protein levels but also enhancing technological and sensory qualities (Krupa-Kozak et al. 2019). If red or processed meat is subsidised with legumes, vegetables, cereals, and fruits, there is a significantly reduced risk for type 2 diabetes in adult males (Maukonen et al. 2023). Thereby, there is an incentive to include more vegetables into our diets.

6.1.1 Fibre

The dietary fibre extracted from green leafy biomass presents excellent opportunities for food enrichment. In broccoli and kale, the highest amounts of total dietary fibre were found in BM, P, and GP (Figure 8). However, to maximise both the functional and nutritional benefits, deeper knowledge of the individual dietary fibre composition is essential. The significant levels of dietary fibre and phenolic compounds found in broccoli leaves makes this

side stream particularly valuable as a food supplement, since it is capable of substantially increasing nutritional value whilst contributing to socio-economic and environmental sustainability.

6.1.2 Phenolic compounds

A regular intake of flavonoids and other phenolic compounds has been linked to positive health outcomes in numerous studies (e.g. the extensive reviews by Wang et al. (2020), Rocchetti et al. (2022) and Matsumura et al. (2023)). Extracting these valuable compounds from green leafy biomass for use as food ingredients or additives could deliver significant health benefits, and here the GJ and WJ fractions contain the highest amounts of phenolic compounds. As shown in a study by Lafarga et al. (2019), incorporating broccoli leaves and stems at just a 2% concentration in bread can increase phenolic content and antioxidant capacity whilst maintaining overall product acceptability.

6.1.3 Protein

The biorefinery fractions from green leafy biomass contain varying levels of essential amino acids. When assessed using amino acid scoring, i.e. comparing available amounts against human and livestock requirements, most fractions demonstrated sufficient quantities (score of 1 or above) (Paper III). Products developed from fractions with a lower amino acid content could be effectively supplemented with amino acid-rich ingredients from sources such as cereals. The WP, and possibly the GP fractions, are particularly promising for human consumption as they contain sufficient profiles of all essential amino acids (Table 1, Paper III). Broccoli powder derived from dried florets, leaves, or stalks serves as an excellent natural food supplement, offering high levels of both amino acids and fatty acids alongside beneficial physicochemical properties (Campas-Baypoli et al. 2009; Liu et al. 2018).

When developing novel food products from these sources, allergen considerations remain important, especially regarding potential cross-contamination with known allergens such as soy, pea, birch, and grass. Pure RuBisCO has a benefit towards other plant proteins such as soy protein due to it being considered a non-allergenic protein (Grácio et al. 2023). In the EU, there is a rigorous legislation framework (European Parliament & Council of the European Union 2015) from which the European Food and

Safety Authority (EFSA) will assess and approve novel food products, a process that takes approximately one year.

6.2 Feed

Many biorefinery fractions show excellent potential as animal feed ingredients. Fibre-rich fractions such as P are particularly suitable for ruminants, whilst GP may better serve swine or poultry nutrition needs (Paper III). Some fractions, e.g. GP, would benefit from supplementation with cereals to address limiting amino acids such as methionine and cysteine. Moreover, certain amino acids are limiting in food and feed for both human and animals due to their dietary requirements (Paper III). Additionally, these materials show promise as feed additives with high phenol content, potentially reducing intestinal parasites and supporting immune function in livestock (Williams et al. 2017). However, even if fractions from crops such as hemp have interesting levels of proteins (Paper II) and protein yield later in the season (Paper V), there is still the issue of antinutrients such as nitrate which make the fractions unsuitable to be used as either food or feed.

6.3 Other applications

Fractions less suitable for direct food or feed applications—due to either antinutrient content or a high water percentage—can still offer valuable opportunities within a circular bioeconomy. The P fraction, which is rich in cellulose and associated compounds (Møller et al. 2021; Pérez-Vila et al. 2022), could serve as a substrate for pyrolysis (Valizadeh et al. 2022), anaerobic digestion for biofuel production, or raw material for lignocellulose extraction (Gundupalli et al. 2022). It also displays potential for removing pollutants from wastewater (Karić et al. 2022) or as a substrate for biogas production (Møller et al. 2021).

Juice fractions, which contain the highest concentrations of phenolic compounds, present exciting possibilities for health applications, including potential treatments for diabetes (Nunes et al. 2021). However, previous research has demonstrated additional applications: alfalfa brown juice as a bio-stimulant for French marigold (Kisvarga et al. 2020; Barna et al. 2021), lucerne extract as a green preservative in cosmetics (Rodrigues et al. 2013), and phenolic extracts as natural preservatives in meat products (Kim et al.

2013; Burri et al. 2019). Alfalfa GJ could be used for biofuel production (Mechmech et al. 2015), and fractions from sugar beet, lucerne, kale, phacelia, red clover, oilseed radish, and red beet have potential as a source of active compounds for skin care products (Prawitz 2020). BJ can be used to produce dietary fibre for feed (Njakou Djomo et al. 2020), as a sugar source for PHA production (Colombo et al. 2017), or biofuel (Santamaría-Fernández et al. 2018; Feng et al. 2021).

To achieve economic viability in processing green leafy biomass, a simultaneous extraction of multiple compounds—such as both proteins and phenolic compounds—appears necessary. Indeed, pre-feasibility assessments of phenol and fibre extraction from broccoli and kale (Paper II) confirm the economic necessity of extracting more than one compound group to achieve process viability.

By developing these innovative applications, underutilised, green leafy biomass can be transformed into valuable resources, thereby supporting a more sustainable and circular bioeconomy whilst capturing nutritional and functional benefits that would be otherwise lost (Figure 14).

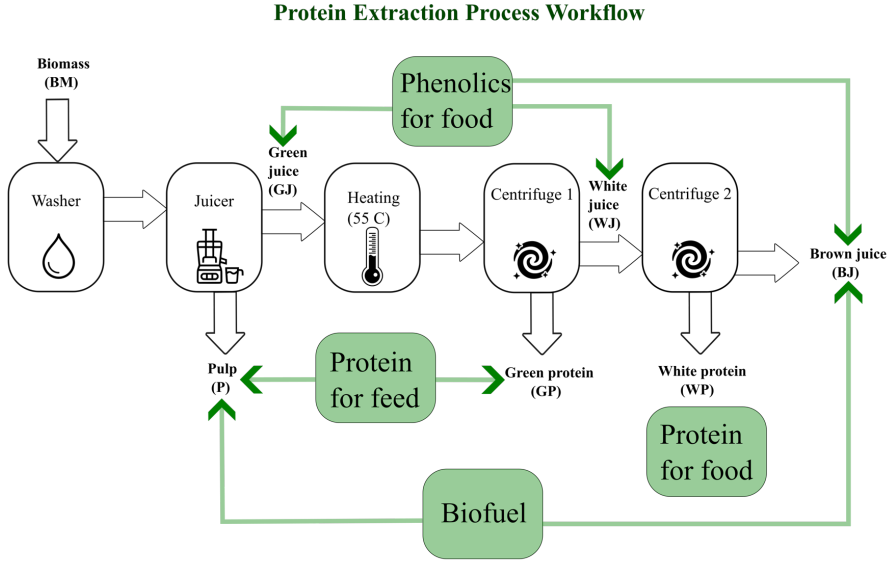

Figure 14: Some suggested uses for the different biorefinery fractions

7. Conclusions

The main findings in this thesis demonstrate the considerable potential for sustainable green leafy biomass utilisation:

- Discarded broccoli and kale leaves show substantial promise as a source of protein, dietary fibre and phenolic compounds, with superior amounts of dietary fibre compared to total fibre-rich products such as oat bran. Economically viable extraction methods, however, await development.
- Leaves from intermediate crops represent an untapped resource for the extraction of phenolic compounds, such as flavonoids and phenolic acids, and proteins, with composition varying depending on the crop species, plant maturity, and cultivation conditions.
- Significant chemical interactions exist between soluble dietary fibre and phenolic acid and flavonoids, whilst results suggest that amino acids and phenolic compounds may also interact, thus influencing extraction efficiency and bioavailability.
- The protein extraction process from green leafy biomass yields multiple valuable fractions with distinct applications: pulp and green protein fractions are well-suited for ruminant, swine, and poultry feed; white protein fractions show particular promise for human food applications; whereas brown juice can serve as either biogas feedstock or as a rich source of phenolic compounds, which persist throughout the processing chain.
- The economic viability can be substantially enhanced through multi-compound extraction processes that target multiple high-end components simultaneously. However, further research is necessary to develop optimised extraction methods that can efficiently recover different compounds without compromising the yield or quality of other target compounds.

These findings provide meaningful insights into our understanding of sustainable biomass utilisation, and present promising strategies toward a more comprehensive use of agricultural by-products. They also show

that it is potentially possible to reduce low-value side streams whilst increasing the value derived from existing production systems.

8. Future perspectives

8.1 Contribution of this thesis

This thesis has provided key measurements of side streams in broccoli and kale production, whilst also demonstrating the feasibility of extracting phenolic compounds and dietary fibre through a process that simultaneously targets protein extraction from a variety of green leafy side biomasses. These different types of raw material represent valuable sources of phenolic compounds, proteins and dietary fibre, which can be utilised in food, animal feed or various other applications. This includes extracting health-beneficial compounds such as dietary fibre and phenolic compounds, creating protein-rich ingredients for food and feed, or providing a source material for e.g. the production of biogas. The implementation of these methods could enhance farmers' economic viability by improving the utilisation of limited resources involved in green biomass production. Additionally, the domestic production of these compounds could reduce the need for import of e.g. soy protein, potentially decreasing global transportation.

8.2 Future recommendations.

Based on the findings in this thesis, the following recommendations are proposed for future research and development.

1. **Development of a comprehensive biorefinery process:** A fully integrated biorefinery process should aim to extract protein, fibre, and phenolic compounds simultaneously. Additionally, it is imperative to assess potential anti-nutritional factors, such as nitrates. For instance, this thesis identified that hemp contains toxic levels of nitrate in specific juice fractions. Strategies should therefore be developed to either remove these undesirable compounds or repurpose them effectively. Whilst the biorefinery concept has been applied to intermediate crops in this thesis, its potential should be explored for a broader range of crops and

agricultural by-products to minimise waste and losses within the food production chain.

2. **Examination of fibre content:** A detailed analysis of fibre content, examining the proportions of soluble and insoluble fibre across different crops, is essential. Certain types of fibre may offer distinct health benefits, influence the retention of other compounds such as proteins and phenolic compounds, and contribute to advancements in food technology.
3. **Evaluation of phenolic compounds:** Further investigation into the phenolic composition of green leafy biomasses, such as broccoli and kale leaves, is warranted. It is particularly important to examine the presence of flavonoids (such as kaempferol and quercetin) and phenolic acids (including caffeic acid), considering their potential health implications.
4. **Enhancing protein recovery in biorefinery processes:** The current inefficiencies in protein fraction recovery within the biorefinery process present a barrier to economic viability. Enzymatic treatment may be required to improve the recovery of both proteins and phenolic compounds, thereby minimising losses.
5. **Ensuring food safety and prototype development:** Food and feed safety must be prioritised. Certain plant-derived materials may contain harmful compounds that could become concentrated during the extraction process from green biomass, necessitating rigorous safety assessments. To facilitate the commercialisation of biorefinery-derived products, prototype development should be undertaken alongside consumer testing to assess acceptability and market potential.
6. **Evaluate the impact on soil organic matter:** The potential depletion of soil organic matter due to increased crop utilisation requires careful evaluation. Root systems play a crucial role in soil organic matter accumulation. The development of robust or deep root systems is fundamental for the formation of soil organic matter in arable land, as demonstrated by Kaštovská et al. (2024). The use of intermediate crops that are incorporated as green fertiliser may mitigate the adverse effects of removing larger portions of certain crops, especially the roots of the intermediate crops (Aronsson et

al. 2023). Hence, there is a balance to be struck between the extraction of compounds from green leafy biomass and using the green leafy biomass as green fertiliser.

In conclusion, these recommendations aim to improve the overall efficiency, safety and sustainability of biorefinery processes, ultimately contributing to meaningful advancements in sustainable food production and resource management. Whilst challenges remain, the path towards more circular agricultural systems appears increasingly promising and attainable.

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Popular science summary

The green leaves we throw away – how we can make use of the whole plant in a hungry world

The production of food that we place on our plates is becoming increasingly uncertain. Weather vagaries, global conflicts, soaring prices and shrinking farmland have led to more than 724 million people worldwide living with hunger as a constant companion. Every bite therefore needs to count – we must begin using our crops more wisely and efficiently. Simultaneously, those of us in the Western world need to rethink our diet. Less meat, more plant-based protein, more fibres and antioxidants would benefit both our health and the planet's wellbeing. Instead of relying solely on traditional protein crops such as beans and lentils, there is an untapped resource right before our eyes: the green leaves left behind in the fields.

While climate change makes cultivation increasingly difficult, enormous amounts of biomass are wasted. Broccoli and kale leaves are discarded. Intermediate crops grown between main seasons are usually ploughed under, turned into basic animal feed or used for biogas. These green treasures have received the same resources – water, nutrients, land area and labour – as the parts we harvest for food. Why not make use of everything?

The results in this thesis show that leaves from broccoli, kale, sugar beet, hemp and similar crops can be used to extract valuable nutrients through biorefining. Through this process, high-quality protein and antioxidants can be extracted from what would otherwise become green manure. Both harvest time and fertilisation affected the amount of protein that could be extracted, with higher levels later in the season if fertilisation was used. Hemp and oil radish in particular proved to contain sufficient biomass to make protein extraction economically viable.

The biorefinery process yields several useful fractions. The protein fractions extracted can be used as food or as locally produced feed for pigs, poultry and cattle. Some fractions, particularly from hemp, contained nitrate levels too high for use as feed, but can instead be utilised in other ways such as raw material for biogas. The juice fractions are rich in antioxidants that can serve as natural preservatives, while the fibres, both soluble and insoluble, can enrich everyday foods such as bread and pasta. By using more of the cultivated biomass, we could produce more high-quality protein on the

same amount of arable land whilst reducing our dependence on imported soya protein for animal feed and food.

The question is whether we can save the world by using more of our crops? Perhaps not as the sole solution, but it would definitely be an important step towards more secure food provision and a more sustainable future.

Populärvetenskaplig sammanfattning

De gröna bladen vi slänger – hur vi kan använda hela växten i en hungrig värld

Produktionen av maten som vi lägger på våra tallrikar blir alltmer osäker. Vädrets nycker, konflikter i världen, skenande priser och krympande odlingsmarker har lett till att över 724 miljoner människor världen över lever med hungern som ständig följeslagare. Varje tugga behöver därför räknas – vi måste börja använda våra grödor på ett klokare och mer effektivt sätt. I västvärlden behöver vi samtidigt tänka om kring vår kost. Mindre kött, mer växtbaserat protein, mer fibrer och antioxidanter skulle gynna både vår hälsa och planetens välmående. Istället för att enbart förlita oss på traditionella proteingrödor som bönor och linser, finns en outnyttjad resurs mitt framför våra ögon: de gröna bladen som lämnas kvar på åkrarna.

Samtidigt som klimatförändringarna gör odling allt svårare så slösas enorma mängder biomassa bort. Broccoli- och grönkålsblad kasseras. Mellangrödor som odlas mellan huvudsäsongerna plöjs oftast ner, blir till enkelt djurfoder eller går till biogas. Dessa gröna skatter har fått samma resurser – vatten, näring, markyta och arbete – som de delar vi skördar till bland annat mat. Varför inte ta vara på allt?

Resultaten i denna avhandling visar att blad från broccoli, grönkål, sockerbetor, hampa och liknande grödor kan användas för att utvinna värdefulla näringsämnen genom bioraffinering. Genom denna process kan högkvalitativt protein och antioxidanter utvinnas från det som annars skulle bli till gröngödsling. Både skördetid och gödsling påverkade mängden protein som kunde utvinnas, med högre halter senare på säsongen om gödsling användes. Särskilt hampa och oljerättika visade sig innehålla tillräckligt mycket biomassa för att kunna göra proteinutvinningen ekonomiskt lönsam.

Bioraffinaderiprocessen ger flera användbara beståndsdelar eller fraktioner. De proteinfraktioner som utvinns kan användas som mat eller som lokalt producerat foder till svin, höns och kor. Vissa fraktioner, särskilt från hampa, innehöll för höga halter av nitrat för att användas som foder, men kan istället nyttjas på andra sätt såsom råvara till biogas. Juicefraktionerna är rika på antioxidanter som kan fungera som naturliga konserveringsmedel, medan fibrerna, både lösliga och olösliga, kan berika vardagliga livsmedel som bröd

och pasta. Genom att använda mer av den odlade biomassan skulle vi kunna producera mer högkvalitativt protein på samma mängd odlingsbar mark och samtidigt minska vårt beroende av importerat sojaprotein för exempelvis djurfoder och mat.

Frågan är om vi kan rädda världen genom att använda mer av våra grödor? Kanske inte som enda lösning, men det skulle definitivt vara ett viktigt steg mot en säkrare matförsörjning och en mer hållbar framtid.

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I

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Article

Side Streams of Broccoli Leaves: A Climate Smart and Healthy Food Ingredient

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Abstract: Human consumption of fruits and vegetables are generally below recommended levels. Waste from the production, e.g., of un-used parts such as broccoli leaves and stem when producing broccoli florets for food, is a sustainability issue. In this study, broccoli leaves were analyzed for the content of various dietary fibre and phenolics, applying the Uppsala method and HPLC analyses, respectively. The results showed that broccoli leaves had comparable levels of dietary fibre (26%–32% of dry weight (DW)) and phenolic compounds (6.3–15.2 mg/g DW) to many other food and vegetables considered valuable in the human diet from a health perspective. A significant positive correlation was found among soluble dietary fibre and phenolic acids indicating possible bindings between these components. Seasonal variations affected mainly the content of conjugated phenolics, and the content of insoluble dietary fibre. This study verified the importance of the use of broccoli production side streams (leaves) as they may contribute with health promoting components to the human diet and also socio-economic and environmental benefits to the bioeconomic development in the society.

Keywords: broccoli; dietary fiber; gut microbiota; health; leaves; phenolic compounds; side steams

1. Introduction

Human health benefits from diets being rich in fruits and vegetables have been verified in a range of studies, and is partly due to an association with a reduction in cardiovascular disease and cancer mortality [1]. Both fruit and vegetables, as well as other plant based foods, are rich in compounds that are suggested to have health beneficial properties [2]. Of these compounds, in particular dietary fiber and bioactive compounds such as phenolics are reported as beneficial when sufficiently consumed [3–5].

Dietary fiber is a term used for naturally occurring carbohydrate polymers that are not digested nor absorbed in the small intestine, and that have health beneficial properties [6]. Dietary fiber can be divided into two fractions, soluble (SDF) and insoluble (IDF) dietary fiber, due to the solubility in water. Most plant foods contain a combination of SDF and IDF [7]. Dietary fiber has been shown to promote health benefits, such as lowering cholesterol in the blood [8], have an impact on the rate of gastric emptying [9], and promote peristaltic movement in the intestines [10]. In addition, dietary fiber is important as energy source for the gut microbiota, which will use the dietary fiber to produce short chained fatty acids (SCFA) [11]. These SCFA can be absorbed and can help in regulating the metabolism and immune system of the host [11]. A diet that contains several types of dietary fiber has been suggested to lead to a gut microbiota with an increased diversity, which in turn could have health beneficial effects [11–13]. Fruit and vegetables have been shown to be good sources of dietary fiber [14]. The edible parts of vegetables in the Brassica family usually contain dietary fiber in moderate to high

amounts [15–17]. Given the recent interest in increasing the resource efficiency by using side streams of different produce, broccoli leaves could be an attractive new source of dietary fiber.

In earlier studies, dietary fiber and phenolic compounds have been analyzed separately due to substantial differences in their chemical structure and biological properties, even though the phenolic compounds that are associated with the dietary fiber might have a significant contribution to the overall health [18,19]. Dietary fiber is proposed to bind phenolics [20–22], enabling these compounds to escape digestive enzymes in the upper gastrointestinal tract and instead reach the colon intact [23,24]. There, the gut microbiota can ferment both the dietary fiber and the phenolics to more easily absorbable compounds.

Phenolic compounds are defined as substances possessing an aromatic ring bearing one or more hydroxyl group including their functional derivatives [25]. In plants, the phenolic compounds have various functions, such acting as anti-feedant, anti-pathogenic, and protective agents (e.g., for UV light) [25]. They also provide pigmentation of plants, are attractants for pollinators, make the cell walls impermeable for gas and water, and contribute to physical stability of the plant [25]. Phenolic compounds are often complex molecules, that are transformed into molecules of smaller size by the gut microbiota before absorption, which increases the bioavailability of these compounds [26]. Most phenolic compounds have antioxidative properties, hence protecting the cells from, e.g., free radicals [27]. Furthermore, the phenolic compounds have been implicated as involved in improving the vascular health [28], lower the risk for developing certain types of cancer [29] and lower the risk of chronic inflammations [3,30]. Phenolic compound may also have an impact on the diversity of the gut microbiota, if they can reach the colon intact [26]. Leafy green vegetables usually contain high levels of phenolic compounds [31]. In Brassica vegetables, including broccoli, a large number of phenolic compounds have been identified [32–34], mainly from the parts already used as food, such as the broccoli florets and kale leaves. This indicates that broccoli leaves should contain phenolic compounds in comparable amounts.

The florets in broccoli (*Brassica oleracea* Italica group) have been shown to contain health beneficial compounds, such as vitamin K and C, minerals, dietary fiber, phenolic compounds, glucosinolates and folic acid [35–37]. The broccoli leaves, on the other hand, are not as well studied as the florets, but have been shown to have higher levels of phenolic compounds as compared to the florets [38,39]. The stem in broccoli contains large amount of insoluble fiber and low amounts of soluble fiber [40].

From the currently applied greenhouse production systems of broccoli, it has been estimated that only 10% of the above ground biomass ends up as broccoli florets for consumption. The rest (90%) of the above ground broccoli plants (which includes stems, leaves, and inflorescences of insufficient size) becomes waste [41]. Previous experiments have shown that 70% of the total weight of the broccoli plants is wasted in the field, while 45–50% of the harvested edible broccoli florets are wasted during processing and transportation [42]. Such parts of the broccoli plant, today cultivated and edible but not used as food, are interesting sources for use as novel food products. These side streams have a potential to be used as functional ingredients to improve the nutritional values of different food products.

The aim of this study was to evaluate the content and composition of dietary fiber and phenolic compounds in broccoli leaves, and to investigate potential relationships between the content and composition of these groups of compounds. A second aim was to discuss possible impact on health from consumption of broccoli leaves, based on the evaluated content and composition of these compounds. Furthermore, the study aimed to describe possible food applications of broccoli leaves as a side stream from commercial broccoli (florets) production.

2. Materials and Methods

2.1. Plant Material

Broccoli leaves were collected on the fields at a commercial production site located in the southern part of Sweden, in the vicinity of 56°24′38.5″N 12°39'34.5"E. The grower used the same broccoli

cultivar ´Beneforte´, known for its nutritional high value [43], throughout the whole production site. The broccoli florets to be commercialized were harvested in October during the two years of sampling, 2017 and 2018. The leaves for this study were collected within 24 h after the final harvest of the broccoli florets. Leaves were collected from a total of four fields; two fields in 2017 and two fields in 2018 (denominated Field 1 (2017), Field 2 (2017), Field 3 (2018) and Field 4 (2018)). In each field, three squares (1.5 × 1.5 m) were randomly positioned (excluding edges of the fields) and ten plants were selected from each square. The plants were cut approximately 2 cm above ground, excluding the roots and most woody lower section. The plants were then transported to the lab in plastic bags, washed under flowing water to rinse away visible dirt, air dried and the whole leaves (including midvein and petiole) were thereafter placed pairwise in bags and stored at –80 °C to minimize the degradation of phenolic compounds.

2.2. Water Content Determination and Milling

Water content for analysis was determined by weighing the frozen samples before and after freeze-drying for 48 h. The freeze-dried samples were milled using an Ultra Centrifugal Mill ZM 200 (Retsch GmbH, Haan, Germany) equipped with a sieve with pore size <0.5 mm. The powder was stored in +4°C in dark plastic containers until analysis.

2.3. Analysis of Dietary Fibere

The components of dietary fiber were analyzed according to the Uppsala method [44], with modification according to Andersson et al. [45] for separate analysis of soluble and insoluble dietary fiber components (sugar residues); Klason lignin, uronic acid (UA), rhamnose (rha), fucose (fuc), arabinose (ara), xylose (xyl), mannose (man), galactose (gal) and glucose (glc). Following previous experiences and method descriptions [44,45], analysis were performed in duplicates. The analytical results are reported on a dry matter basis (DW). Dry matter was determined by drying the milled samples at 105 °C for 16 h.

2.4. Analysis of Phenolic Compounds

All samples were analyzed in triplicates, and measurements of phenolic compounds were according to Lin et al. [46], with some modifications as described below. Similar as in our previous study [47], a methanol extraction was applied as described below, following common practice for phenolic compounds [48–50].

2.4.1. Methanol Extraction

For each sample, 2 mL 60% MeOH were added to 100 mg freeze-dried leaf sample in an Eppendorf tube and vortexed (Combi-spin FVL-2400, Biosan, Latvia) for 5 seconds until mixed. The tubes were put in ultrasonic bath (Sonorex Digitec DT 100 H, Bandelin, Germany) at 35 °C, for 60 min in order to extract the phenolic compounds from the tissues, and thereafter chilled shortly in cold water. The tubes were centrifuged at 4 °C and 21,000× *g* in a Centrifuge 5427 R (Eppendorf, Hamburg, Germany), for 10 min to separate sufficient supernatant from pellet. An aliquot of the supernatant was saved as methanol extract for analysis with HPLC, while one other aliquot was analysed further with alkaline hydrolysis. Compounds analysed from the methanol extraction are denominated as *conjugated phenolics*, since the phenolic compounds in Brassica are commonly found as conjugated to sugars and organic acids [51]. The conjugated phenolics normally include naturally occurring flavonoid glycosides and phenolic acids glycosides [51]. The conjugated phenolics were therefore further subdivided into two groups (called *Flavonoids* and *Phenolic Acids Derivatives*, respectively), based on their retention time in the chromatogram (Figure S1).

2.4.2. Hydrolysis

After the methanol extraction, alkaline hydrolysis was used on the supernatant from samples in order to liberate the phenolic acids from their glycoside.

For the alkaline hydrolysis, 200 µL 2 M NaOH was added to 500 µL supernatant from the methanol extraction for each sample and the tube was shortly vortexed to mix. Then, the tube was put on a shaking bed at 2 °C for hydrolysis during 18 h. Thereafter, 280 µL 6 M HCI was added and the tube was again vortexed. A liquid-liquid extraction was performed by adding 2 × 500 µL ethylacetate to extract the released phenolic compounds. The top phase was collected and the ethylacetate was evaporated under N_2 until dryness. The residue was dissolved in 100 µL 100% MeOH and the tube was placed in ultrasonic bath, at 25 °C, for 5 min to dissolve the sample. An amount of 100 µL of the solution was transferred to a HPLC vial for analysis with HPLC. Compounds analyzed from the alkaline hydrolysis are denominated *phenolic acids*. The phenolic acids (after hydrolysis) were further subdivided into two groups (called Group 1 and Group 2) based on their retention time in the chromatogram (Figure S1).

2.4.3. HPLC Analysis

In order to identify the phenolic compounds, the samples were analyzed by HPLC-MS. The phenolic compounds were identified by their particular spectra, their UV-maxima, molecular weight and retention time, and were compared with previous literature [32–34]. For the methanol extract, kaempferol-3-O-rutinoside (Extrasynthèse, France) was used as an external standard and for alkaline hydrolysis caffeic acid (Sigma, Germany) was used.

The individual phenolic compounds were analyzed in a HPLC–DAD–ESI(-)–MS system, Agilent 1260 (Agilent Technologies, Waldbronn, Germany). The system consisted of binary pump (0.700 mL/min), thermostated column compartment (35 °C), with a Triart C_{18} ExRS column (YMS, 150 mm × 3 mm, particle size 3µm and pore size 8 nm), an autosampler, a diode array detector (DAD) (350 nm for methanol extract and 280 nm for alkaline hydrolysis), a mass spectrometer (Agilent 6120, ionization mode API-ES negative polarity, gas temperature of 350 °C, drying gas 12.0 L/min, m/z 130–800). Data acquisition was made with Chemstation software (B04.03-SP1 [version 87], Agilent Technologies, Waldbronn, Germany). Injection volume was 3.00 µL per sample. The mobile phase consisted of a binary solvent system using water acidified with 0.5% formic acid (solvent A) and 0.1% formic acid in acetonitrile (solvent B). The gradient increased linearly from 0–3% B (v/v) at 0–7 min, to 3–12% B at 7–13 min, to 12–14% B at 13–17 min, to 14–35% B at 17–26 min, held at 35% B at 26–28 min, decreased to 3% B at 28–32.5 min and held at 3% B at 32.5–35 min.

2.5. Statistical Analysis

Data were expressed as mean ± standard deviation (SD). Statistical analysis was made in RStudio Team (2016, US), version 1.1.456 [52], with the packages ggplot2, ggbiplot, dplyr, emmeans, lme4 and lmerTest.

Variation in content of different compounds in plants is related to variation among genotypes and environment of cultivation, know to play an equal role and being related to selection of cultivars and environments used [53,54]. It is known from a broad range of studies that environmental variation is due to multitude of factors including year, site and field variation, originating from variation in soil, temperature, precipitation etc. [55,56]. Comparisons of environmental effects on compounds evaluated in the study were carried out applying a general linear model analysis of variance (ANOVA) comparing effects of years and fields. When significant differences ($p < 0.05$) were found, the differences between the means were evaluated by the use of Tukey post-hoc test (build in the command compact letter display (CLD)). A principal component analysis (PCA) was made to investigate the relationship between content of dietary fiber and phenolic compounds. Each data point was the average from two (dietary fiber) or three (phenolic compounds) sample replications. Content of dietary fiber and phenolic compounds in the analyzed broccoli leaves were compared with content in other comparable

food items with data collected from literature. Due to different levels of digits presented in various publications concerning this data, all numbers were rounded to one decimal level.

3. Results

3.1. Dietary Fiber and Water Content in Broccoli Leaves

The majority of the dietary fiber in broccoli leaves consisted of IDF, comprising 23.8%–30.6% of the DW as compared to the SDF constituting 2% of the DW (Table 1). Cultivation location impacted the concentration of IDF in the leaves (23.8–30.6% of DW), and significant differences were found between Field 1 and 4 (Table 1). No significant differences were found for SDF and total dietary fiber (TDF) among fields, and neither among years for the total content of IDF, SDF, or TDF in the leaves (data not shown).

Table 1. Total content of dietary fiber in broccoli leaves, divided into insoluble (IDF), soluble (SDF) and total (TDF) dietary fiber. TDF was calculated as the sum of IDF and SDF.

	IDF [% of DW]	SDF [% of DW]	TDF [% of DW]
Field 1 (2017)	30.6 ^{b} ± 4.2	1.9 ^{a} ± 0.4	32.6 ^{a} ± 4.4
Field 2 (2017)	25.0 ^{ab} ± 0.7	1.8 ^{a} ± 0.3	26.8 ^{a} ± 0.5
Field 3 (2018)	25.3 ^{ab} ± 1.5	2.0 ^{a} ± 0.1	27.3 ^{a} ± 1.6
Field 4 (2018)	23.8 ^{a} ± 1.2	2.3 ^{a} ± 0.2	26.0 ^{a} ± 1.3

Data is expressed as mean ± SD (n = 6). Values followed by the same letters do not differ significantly ($p < 0.05$) by using the Tukey post hoc test.

Significant differences were found for the content of dietary fiber constituents (Klason lignin and sugar residues) in samples originating from different fields and years. Similarly, as for the total content of dietary fiber, the content of the individual soluble fiber constituents was generally low in comparison with the content of insoluble fiber constituents. Among the analyzed dietary fiber (Table 2), the most abundant constituents were Insol glc, Insol UA, and Insol xyl. Significant differences were found among samples from different fields in content of individual constituents for Insol UA, Insol ara, Sol ara, Sol xyl, Sol man, and Sol glc (Table 2).

Table 2. Content of dietary fiber constituents (Klason lignin and sugar residues) in broccoli leaves from four fields.

	Klason lignin	Insol UA	Insol rha	Insol fuc	Insol ara	Insol xyl	Insol man	Insol gal	Insol glc
Field 1 (2017)	1.8 ^{a} ± 0.6	8.1 ^{b} ± 0.6	0.7 ^{a} ± 0.0	0.2 ^{a} ± 0.0	2.6 ^{b} ± 0.6	2.6 ^{a} ± 0.7	1.0 ^{a} ± 0.0	1.5 ^{a} ± 0.2	12.2 ^{a} ± 1.9
Field 2 (2017)	1.7 ^{a} ± 0.5	7.5 ^{ab} ± 0.4	0.7 ^{a} ± 0.1	0.1 ^{a} ± 0.0	1.6 ^{ab} ± 0.1	1.7 ^{a} ± 0.2	0.9 ^{a} ± 0.0	1.2 ^{a} ± 0.1	9.6 ^{a} ± 0.2
Field 3 (2018)	1.8 ^{a} ± 0.4	7.3 ^{ab} ± 0.4	0.7 ^{a} ± 0.0	0.2 ^{a} ± 0.0	1.1 ^{a} ± 0.1	2.1 ^{a} ± 0.5	0.9 ^{a} ± 0.1	1.3 ^{a} ± 0.1	1.0 ^{a} ± 0.8
Field 4 (2018)	1.6 ^{a} ± 0.4	6.9 ^{a} ± 0.4	0.7 ^{a} ± 0.0	0.2 ^{a} ± 0.0	1.4 ^{a} ± 0.2	1.7 ^{a} ± 0.1	0.9 ^{a} ± 0.1	1.3 ^{a} ± 0.1	9.3 ^{a} ± 0.7
Fields, 2017	1.8 ^{a} ± 0.5	7.8 ^{a} ± 0.6	0.7 ^{a} ± 0.1	0.2 ^{a} ± 0.0	2.1 ^{a} ± 0.7	2.1 ^{a} ± 0.7	1.0 ^{a} ± 0.1	1.4 ^{a} ± 0.2	10.9 ^{a} ± 1.9
Fields, 2018	1.7 ^{a} ± 0.4	7.1 ^{b} ± 0.4	0.7 ^{a} ± 0.0	0.2 ^{a} ± 0.0	1.3 ^{b} ± 0.2	1.9 ^{a} ± 0.4	0.9 ^{b} ± 0.1	1.3 ^{a} ± 0.1	9.6 ^{a} ± 0.8
		Sol UA	Sol rha	Sol fuc	Sol ara	Sol xyl	Sol man	Sol gal	Sol glc
			[10^{-2}]	[10^{-2}]	[10^{-1}]	[10^{-2}]	[10^{-1}]	[10^{-1}]	[10^{-1}]
Field 1 (2017)		1.0 ^{a} ± 0.4	5.6 ^{a} ± 0.9	1.3 ^{a} ± 0.6	3.3 ^{b} ± 0.5	2.3 ^{ab} ± 0.1	1.1 ^{ab} ± 0.2	3.1 ^{a} ± 0.4	0.8 ^{ab} ± 0.1
Field 2 (2017)		1.1 ^{a} ± 0.2	4.1 ^{a} ± 0.7	1.1 ^{a} ± 0.6	2.3 ^{a} ± 0.4	1.6 ^{a} ± 0.1	1.0 ^{a} ± 0.2	2.5 ^{a} ± 0.5	0.7 ^{a} ± 0.1
Field 3 (2018)		1.1 ^{a} ± 0.1	5.3 ^{a} ± 1.2	3.1 ^{a} ± 0.8	2.2 ^{a} ± 0.3	3.2 ^{b} ± 0.4	1.4 ^{ab} ± 0.1	2.9 ^{a} ± 0.3	1.1 ^{b} ± 0.1
Field 4 (2018)		1.4 ^{a} ± 0.2	5.9 ^{a} ± 0.7	2.1 ^{a} ± 0.3	2.4 ^{ab} ± 0.2	3.2 ^{b} ± 0.4	1.4 ^{b} ± 0.1	2.8 ^{a} ± 0.1	1.1 ^{b} ± 0.3
Fields, 2017		1.1 ^{a} ± 0.3	4.8 ^{a} ± 0.1	1.2 ^{a} ±0.1	2.8 ^{a} ± 0.5	1.9 ^{a} ± 0.9	1.0 ^{a} ± 0.2	2.8 ^{a} ± 0.4	0.8 ^{a} ± 0.1
Fields, 2018		1.2 ^{a} ± 0.2	4.7 ^{a} ± 1.0	2.1 ^{b} ± 0.1	2.2 ^{a} ± 0.3	2.4 ^{b} ± 0.7	1.2 ^{b} ± 0.2	2.7 ^{a} ± 0.4	0.9 ^{b} ± 0.1

Values are mean [% of DW] ± SD. Values followed by the same letters do not differ significantly at $p < 0.05$ by using the Tukey post hoc test. From each field, three plants were analysed in duplicates. Leaves, 2017 and Leaves, 2018 are the total amount of the constituent from the two fields from each year respectively. Insol: insoluble. Sol: Soluble. The sugar residues are annotated UA: uronic acid. rha: rhamnose. fuc: fucose. ara: arabinose. xyl: xylose. man: mannose. gal: galactose. glc: glucose.

Higher content was found for leaves from 2017 as compared to those from 2018 of the insoluble fiber constituents Insol UA, Insol ara, and Insol man (Table 2). The content was instead lower in leaves from 2017 as compared to those from 2018 of some soluble fiber constituents Sol fuc, Sol xyl, Sol man, and Sol glc (Table 2).

The water content in broccoli leaves, measured before and after freeze-drying of the samples, was approximately 80%, with 84.8 ± 1.5% in 2017, and 80.9 ± 2.9% in 2018.

3.2. Phenolic Compounds in Broccoli Leaves

Year of cultivation impacted significantly the amount and composition of phenolic compounds in broccoli leaves. Thus, a significantly higher content of conjugated phenolics (compounds analyzed in methanol extract, mainly phenolic compounds conjugated to sugars and phenolic acids [51]) was found in leaves harvested in 2017 (10.8–15.2 mg/g DW) as compared to 2018 (6.3–7.5 mg/g DW), while the content of phenolic acids (compounds analyzed in methanol extract after alkaline hydrolysis) did not differ significantly in leaves harvested in different years (Table 3).

Table 3. Content of phenolic compounds in broccoli leaves.

	Conjugated Phenolics [mg/g DW]	Phenolic Acids (after Hydrolysis) [mg/g DW]
Field 1, 2017	$10.8^{ab} \pm 1.8$	$4.4^{a} \pm 1.7$
Field 2, 2017	$15.2^{b} \pm 4.8$	$3.6^{a} \pm 1.0$
Field 3, 2018	$6.3^{a} \pm 1.1$	$5.3^{a} \pm 1.2$
Field 4, 2018	$7.5^{a} \pm 0.6$	$5.7^{a} \pm 1.1$

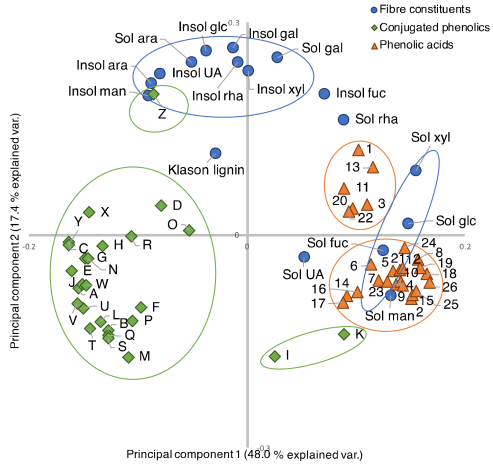
Values shown are the mean of three replicates [% of DW] ± SD. Values followed by the same letters do not differ significantly at $p < 0.05$ by using the Tukey post hoc test. For Field 4, one replicate out of nine were removed due to experimental error.

Thereby, similar amounts of conjugated phenolics and phenolic acids were found in leaves harvested in 2018, while 2.5–5 times higher levels of conjugated phenolics as compared to phenolic acids (after hydrolysis) were noted in leaves harvested in 2017. In addition, a second group of compounds was detected in the phenolic acids chromatogram in leaves from 2018, which were not found in those from 2017 (Figure S1). No significant difference was found neither in the content of conjugated phenolics, nor in content of phenolic acids in the broccoli leaves from the different fields.

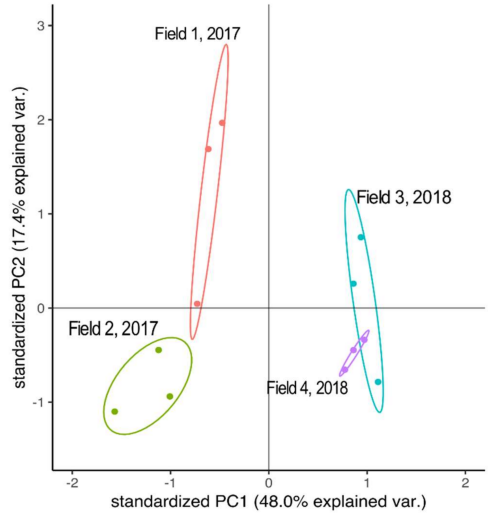
3.3. Relationship among Dietary Fiber and Phenolic Compounds in Broccoli Leaves

Principal component analysis visualized a close relationship among some of the soluble dietary fiber (Sol fuc, Sol xyl, Sol man, Sol glc) and the phenolic acids and also two of the conjugated phenolics (I and K), as could be seen from their positive values on PC1 from the loading plot (Figure 1b). In addition, a significant and positive Pearson correlation ($p < 0.05$) was found for the Group 1 of phenolic acids (Peaks 1–6 in chromatogram) and two of the dietary fiber constituents; Sol xyl and Sol glc, while Sol man was significant at $p < 0.06$ (Table 4). Furthermore, for Group 2 of the phenolic acids (Peaks 7–26 in chromatogram), significant positive Pearson correlations ($p < 0.05$) were found with Sol fuc, Sol xyl, Sol man and Sol glc, and negative with Insol UA, Insol ara, and Insol man, respectively (Table 4).

Most of the conjugated phenolics showed negative values for PC1 in the loading plot, thereby indicating a negative relationship with the above mentioned soluble dietary fibers (Sol fuc, Sol xyl, Sol man, Sol glc) (Figure 1a), which was also verified by a negative Pearson correlation between these dietary fiber and both the group Flavonoids (peak B–O in chromatogram) and the group Phenolic acids Derivatives (peak P–Z in chromatogram (Table 4). Furthermore, the Flavonoids also showed significant Pearson correlations with Insol fuc ($p < 0.05$) and with Sol rha, Sol fuc and Sol man at $p < 0.06$.



(**a**)



(**b**)

Figure 1. Loading plot (a) and score plot (b) for the principal component analysis for dietary fiber constituents (Klason lignin and sugar residues), conjugated phenolics and phenolic acids (after hydrolysis) from broccoli leaves. Each data point is the mean from three replications, n = 3. Insol: insoluble. Sol: soluble. UA: uronic acid. Rha: rhamnose. Fuc: fucose. Ara: arabinose. Xyl: Xylose. Man: mannose. Gal: galactose. Glc: glucose. For a tentative identification of the peaks in conjugated phenolics see Table 5. For the phenolic acids, Peaks 2, 4, 5, 8, 9, 10, 16 and 19 have a tentative identification (Table S1), and HPLC and MS spectra can be found in Figure S7 and Figure S8.

Table 4. Pearson correlation coefficients among dietary fiber constituents and groups of phenolic compounds.

	Group 1	Group 2	Phenolic Acid Derivatives	Flavonoids	Colour Legend
Klason lignin	−0.41	−0.05	0.04	0.02	
Insol UA	−0.47	−0.59	0.36	0.11	
Insol rha	−0.18	−0.11	0.01	−0.18	
Insol fuc	0.14	0.25	−0.43	−0.61	*p*−value
Insol ara	−0.42	−0,65	0.44	0.16	> 0.05
Insol xyl	0.08	−0.13	−0.04	−0.21	0.05–0.01
Insol man	−0.36	−0.59	0.48	0.27	0.01–0.001
Insol gal	−0.12	−0.22	0.10	−0.23	<0.001
Insol glc	−0.18	−0.33	0.16	−0.06	
Sol UA	0.54	0.33	0.09	−0.14	
Sol rha	0.47	0.42	−0.44	−0,57	
Sol fuc	0.39	0.72	−0.76	−0.56	
Sol ara	−0.29	−0.46	0.23	0.00	
Sol xyl	0.69	0.83	−0.72	−0.88	
Sol man	0.56	0.71	−0.73	−0.56	
Sol gal	−0.02	0.02	−0.21	−0.35	
Sol glc	0.74	0.84	−0.72	−0.66	

Flavonoids: Peaks B-O from the chromatogram of methanol extract (conjugated phenolics). Phenolic acid derivatives: Peaks P-Z from the chromatogram of methanol extract (conjugated phenolics) (Figure S1). Group 1: Peaks 1–6 in chromatogram after alkaline hydrolysis (phenolic acids). Group 2: Peaks 7–26 in the chromatogram after the alkaline hydrolysis (phenolic acids). The scatter plots with significant *p*-values can be found in Figures S2–S6 in Supplementary Materials.

Table 5. Tentative identification of phenolic compounds in methanol extract of broccoli leaves

Peak ID	Ret.time [min]	DAD [nm]	MS Scan(−)	MS Sim	Suggested Identification
A	10.37	326, 299	353, 1138.8	353	caffeoylquinic acid (chlorogenic acid)
B	13.06	341, 318	1157, 609		K-3-O-(sinapoyl)-sophoroside-7-O-diglucoside
C	13.29	346	771, 1159		K-3-O-(sinapoyl)-sophoroside-7-O-glucoside
D	13.66	333	1538		isorhamnetin-3-O-(disinapoyl)-sophorotrioside-7-O-diglucoside
E	14.03	327	963, 1125	963.4	K-3-O-(methoxycaffeoyl)-sophoroside-7-O-diglucoside
F	14.267	333	269	933	Unidentified phenolic compound
G	14.35	334	933, 1097	933.4	K-3-O-caffeoyl-sophoroside-7-O-diglucoside
H	14.56	340	993		Q-3-O-(sinapoyl)-sophoroside-7-O-glucoside
I	14.74	333	963	963.4	K-3-O-hydroxyferuloyl-sophoroside-7-O-glucoside
J	14.88	330	1139, 1175	933.4	K-3-O-caffeoyl-sophoroside-7-O-glucoside
K	15.05	332	1139		K-3-O-sinapoyl-sophorotrioside-7glucoside
L	15.23	336	977	977.5	K-3-O(sinapoyl)-sophoroside-7glucoside
M	15.35	339	1109	1109.5	K-3-O(feruloyl)sophoroside-7-O-diglucoside
N	15.60	332	947	947.5	K-3-O(feruloyl)sophoroside-7-O-glucoside
O	15.70	269, 341	428.2, 195, 425		Unidentified phenolic compound
P	20.62	330	731, 975, 1123, 1367		Unidentified phenolic compound
Q	20.97	332	771, 1507		K-3-O(disinapoyl)sophorotrioside-7-O-diglucoside
R	20.26				Unidentified phenolic compound
S	21.33	331	1538		isorhamnetin-3-O-(disinapoyl)-sophorotrioside-7-O-diglucoside
T	21.62	329	1316		Q-3-O(disinapoyl)sophorotrioside7-O-diglucoside
U	22.71	330	753	1402	disinapoyl-diglucoside
V	23.07	327	723		sinapoyl-feruloyl- diglucoside
W	23.33	326	693		diferuloyl-diglucoside
X	24.16	324	959		trisinapoyl-diglucoside
Y	24.48	325	617, 653, 1236		phenolic acid derivate

K stands for kaempferol, Q stands for quercetin. All peaks were not detectable in all samples. For MS Scan (−), the main fragments are reported.

The rest of the fiber constituents (Insol ara, Sol ara, and Sol gal) showed no relationship to any of the individual phenolics, indicated by their relatively close to zero PC1 values and relatively high positive values on PC2 (Figure 1), which was also verified by the Pearson correlations coefficients (Table 4).

The PCA also clearly depicted the higher content of phenolic acids and soluble fiber constituents (Sol fuc, Sol xyl, Sol man, Sol glc) in leaves from 2018 (Field 3 and Field 4), as compared to leaves from 2017, the latter instead having a higher content of conjugated phenolics (compare Figure 1a

with Figure 1b). Leaves from Field 2 showed low levels of insoluble fiber, indicated by their negative PC2 values, while insoluble fiber showed positive PC2 values. Field 1 leaves showed generally large variation of insoluble fiber content. Based on all the dietary fiber constituents and phenolic compounds detected with HPLC, the first two principal components accounted for 48.0 and 17.4% of the variation respectively, adding up about 65% of the variation.

4. Discussion

The present study clearly showed that broccoli leaves, today commonly not used as food, have high content of both dietary fiber and phenolic compounds and also that the content of some of the dietary fiber constituents and phenolic compounds co-varied. Broccoli leaves turned out as having high content of compounds regarded as healthy, which make them of interest as potential component for the food industry. Environmental and climate change concern has increased the interest in using edible side-streams of food production for new food products, which also would increase the amount of food available globally. Furthermore, a high content of dietary fiber and phenolic compounds combined is of interest from a health perspective. The co-variation of these compounds might be of specific relevance as a major factor affecting the uptake mechanism in the human intestine.

Here, we have for the first time, to our knowledge, shown a co-variation in broccoli leaves among content of certain phenolic and dietary fiber, i.e., some of the phenolic acids showed a positive correlation with some of the SDF (Sol fuc, Sol xyl, Sol man and Sol glc; (Table 4)). Three of the mentioned dietary fiber constituents (Sol fuc, Sol xyl and Sol glc) are known as being the main parts of the complex soluble dietary fiber xyloglucan [57]. Previous studies have suggested a possibility that phenolic compounds are bound to the complex dietary fiber xyloglucan [58].

Previous results have indicated that phenolic compounds can be strongly bound to dietary fiber, thereby they should be considered as one collective group, denominated as antioxidant dietary fiber [19,23,59]. However, previous studies have also pointed out that phenolics are a large and diverse group of compounds localized in several parts of the plant cell; in the vacuole, in the chloroplast, in the nuclei, and also in the cell wall [60]. In a study of chicory leaves, the fractions of foliar parenchyma cells were found to have higher concentration of phenolics as compared to vein fractions [61], indicating that cells in the veins with thicker cell walls, constituting of dietary fiber, had lower concentrations of phenolics. The results from the present study showed corresponding results, i.e., in this investigation the dietary fiber constituents of the broccoli leaves present in highest concentration in this investigation (Insol UA, Insol xyl, and Insol glc) showed in general no significant correlation with the analyzed phenolics, and some of both IDF and SDF constituents showed negative correlation with different phenolic groups. Hence, the major part of the phenolics found in this investigation should not be bound to cell walls, i.e., the dietary fiber, but rather be present in other parts of the cells or in cells with thinner cell wall. However, the phenolic compounds are possibly not easily extracted from the fiber matrix with only organic solvent. As described in the materials and method section, we have used methanol extraction following similar procedure as recommended and used in other publications and also by us on other brassica species [48–50]. However, the results from the present study indicate that additional phenolic compounds might be present in broccoli leaves not able to be extracted with methods generally adopted and commonly used for phenolics extraction in plants. To be able to evaluate content of all phenolic compounds, and including all cell wall bound phenolic compounds, alternative extraction procedures with a more efficient disruption of the cell wall can be considered, including enzymatic [62], ultrasonic [63] and ultrasonic assisted enzymatic extraction [64,65].

Broccoli leaves, with their mean content of TDF at 26%–32% of the DW, have an intermediate content of TDF, as compared to other types of food and vegetables (Table 6). Thus, the content of dietary fiber in broccoli leaves is higher than that in oat brans, carrots and apples, but lower content as compared to onions, cabbage outer leaves, kale leaves and the broccoli florets. This makes broccoli leaves an interesting raw material for food from a health perspective.

Table 6. Comparing levels of total dietary fiber in food.

Sample	Mean [% of DW]	Reference
Potex	80.4	[44]
Onion	47.2	[16]
Kale leaves	42.7	[15]
Cabbage outer leaves	40.9	[17]
Broccoli florets	36.0	[16]
Broccoli leaves	26–32	[present study]
Cauliflower (curd)	29.7	[16]
Carrot	24.1	[44]
Oat bran	18.4	[44]
Apple	17.9	[44]
Green peas	16.7	[44]
Rye bread	10.3	[44]
White bread	4.6	[44]

Despite, as discussed above, that content of phenolics might possibly be higher in broccoli leaves than possible to measure with the applied methodology, the levels were found similar as previously reported for kale, and higher as compared to the broccoli florets (Table 7). Thus, from perspective of phenolic content, the broccoli leaves are an interesting component for the food industry. The content of conjugated phenolics in the present study varied between the years, with 10.8–15.2 mg/g DW for 2017 as compared to 6.3–7.5 mg/g DW for 2018. At the same time, the content of phenolic acids did not vary significantly between years, but were approximately 3.6–5.7 mg/g DW.

Table 7. Comparing content of phenolic compounds by methanol extraction.

Sample	mg/g DW	Reference
Broccoli leaves, 2017	10.8–15.2	[present study]
Kale leaves	10.6	[33]
Broccoli leaves, 2018	6.3–7.5	[present study]
Broccoli florets	1.7–2.2	[66]

Both the content of dietary fiber and phenolics varied between the two years of this study, though the former to somewhat lower degree. This might be due to the different weather conditions during these years, with an exceptionally warm and dry summer in Sweden 2018 (maximum and mean temperature in 2018 were 28.6 °C and 16.4 °C respectively, compared to 20.8 °C and 14.3 °C respectively in 2017, according to Swedish Meteorological and Hydrological Institute (SMHI)). The levels of phenolic compounds in kale, another member of the Brassica family, have been shown to increase when the temperature decreases due to an accumulation of secondary metabolites [67,68]. The amount of phenolic compounds in Brassica also depend on genetic variation (both within and among species) and on environmental factors as well as biotic and abiotic stresses (e.g., insect attacks, light, temperature, nutrients, water, growing conditions, and UV radiation) [51]. Furthermore, in this investigation the broccoli leaves were collected at commercial farms applying crop rotation, i.e., the same fields were not used for broccoli production during the two years. Instead plant materials were collected from fields in the same area both years, resulting in that variation between the two years might also be due to differences between fields. Lastly, water content in the broccoli leaves differed significantly between

the years, which also indicate differences in environmental factors which might impact variation in phenolic and dietary fiber between the years. Similar water content have been reported earlier [37,42].

In this investigation we have used the common categorization of the dietary fiber in soluble and insoluble fiber. However, recently it has been questioned if these two categories are sufficient when describing the functionality of the specific type of fiber, and the perceived health effects [4,69]. At present, there is insufficient knowledge of how the individual components of both the dietary fiber and the phenolics influence the various health effects, and also possible interactions between these groups. In addition, the structural diversity of the different fiber, both within a plant, but also depending of the plant species, is likely to influence the digestion of the fiber, and thereby possibly the health effects.

Health beneficial effects from phenolic compound have been suggested to be a result of some phenolics having the opportunity to travel along the intestines to reach the colon, and the gut microbiota, intact [3,26]. Phenolics are suggested to be strongly bound to dietary fiber, and to not be released from the food matrix by mastication, acid pH or human digestive enzymes [70]. The phenolic compounds that travels inside the gastrointestinal tract for a long time together with the dietary fiber might also have the effect that they lower the amounts of reactive oxygen species (e.g., free radicals) in the gastrointestinal tract, which would also be beneficial [19]. Dietary fiber from kale has been shown to bind bile acid and simultaneously release phenolic compounds from the matrix, thus bile acids can increase the bioaccessibility of the phenolic compounds [71], and has also been shown to have a beneficial impact on the cholesterol levels in the blood [72]. In connection to this, the gut microbiota has been shown to be altered by consumption of dietary fiber rich cruciferous vegetable, such as broccoli, cauliflower and cabbage, which could ultimately influence gut metabolism of bioactive food components and host exposure to these beneficial compounds [73]. Phenolic compounds in themselves have been shown to be beneficial for health, e.g., by increasing weight loss in obese mice and humans [74], and also to lower the mortality of some chronic diseases, mainly cardiovascular diseases and cancer [75].

The average daily intake of dietary fiber in most Western countries (15–25 g dietary fiber/day) is low compared with the recommended daily intake of dietary fiber in Europe (20–38 g/day for adults) [4]. The content of dietary fiber found in this investigation in broccoli leaves, 26%–32% of the DW, are in line with earlier studies which showed that the levels of TDF in a mixture of broccoli leaves and stems were approximately 36% [76] with lower amount of fiber in the leaves compared to the stems [42]. Hence, if broccoli side streams are used in every day food products, this will contribute to an increase in total dietary intake of fiber towards the recommended levels, while at the same time lessen the amount of the broccoli plant not used as food. Dietary fiber ingredients can also be used to improve functional properties in, e.g., meat, dairy, and wheat flour-based products [77].

Production of food requires resources such as water, fertilizers, farmland, and energy. Currently, the generated amount of food waste correspond to production on 0.9 million hectares of farmland, release of 3.49 GT carbon dioxide equivalents (CO_2e), and use of 306 km^3 of drinkable water [78]. In these calculations, the biomass not harvested but that could be eaten was not included. A more complete use of the agriculturally produced biomass would contribute to an increased productivity with less field waste, which would have a beneficial impact on the global climate. Furthermore, the different side steams from fruit and vegetable production are a readily available resource, and can be used as new food products, but also as a raw material for extraction of valuable compounds [79].

In the case of broccoli, the florets only make up 15% of the total biomass of the broccoli plant, while the leaves make up a total of 47%, and stems and roots make up the remaining 38% [37]. Broccoli powder from dried florets, leaves or stalks can be used as a natural food supplement since these powders contain high levels of amino acids and fatty acids and also good physicochemical properties [37,42]. An addition of 10% vegetable powder (carrot, tomato, broccoli florets, and beetroot), has been shown to increase the nutritional and functional attributes in oil-free bread [80]. Broccoli florets increased levels of protein, fat, vitamin E and also antioxidant capacity in these breads. Broccoli

leaves and stems have been shown to increase the phenolic content and antioxidant capacity in bread when added in a concentration of 2% (w/w), while still have an overall acceptability [81]. In addition, broccoli leaf powder has been proposed for use in gluten free sponge cake to increase the content of minerals, antioxidant capacity and protein [82], and also to increase the technological and sensory quality of gluten free sponge cake [83]. Hence, with the levels of dietary fiber and phenolic compounds found in broccoli leaves in this study, future food uses of this side stream would be of interest as a food supplement to increase nutritional values. Furthermore, added-value use of the side streams of broccoli leaves contributes to socio-economic and environmental sustainability to the bioeconomy of our modern society.

5. Conclusions

Broccoli leaves, a side stream in the broccoli production, contain high levels of dietary fiber and phenolics, comparable with other vegetables currently used as food. Covariation of some SDF of the dietary fiber xyloglucan and phenolic acids may indicate interactions between these components that most likely influence the bioavailability of the phenolics in the human intestine. To further elucidate the relationship between dietary fiber and phenolic acid interactions and effort on bioavailability is of relevance and would require further research combining biology/agronomy and medical expertise. Yearly variation in weather conditions affected the content of conjugated phenolics in the broccoli leaves. Lower levels were recorded during a season with hot and dry weather conditions than in a season with cooler and rainier weather. As highly nutritious and readily available, broccoli leaves is an interesting source to be used as a functional ingredient to increase the nutritional content in different types of food, with resulting potential health benefits.

Supplementary Materials: The following are available online at http://www.mdpi.com/1660-4601/17/7/2406/s1, Figure S1: examples of chromatograms, Figure S2: correlation between the group phenolic acids (methanol extraction) and dietary fiber constituents, Figure S3: correlation between the group flavonoids (methanol extraction) and dietary fiber constituents, Figure S4: correlation between Group 1 (Peaks 1–6 in the chromatogram for alkaline hydrolysis) and the constituents of dietary fiber, Figure S5: correlation between phenolic Group 2 (Peaks 7–26 in the chromatogram for the alkaline hydrolysis) and the insoluble dietary fiber, Figure S6: correlation between phenolic Group 2 (Peaks 7–26 in chromatogram for the alkaline hydrolysis) and the soluble constituents of dietary fiber. Table S1: suggested identification for the peaks in alkaline hydrolysis of broccoli leaves. Figure S7: examples of HPLC and MS spectra for phenolic compounds in methanol extract. Figure S8: examples of HPLC and MS spectra for phenolic compounds after alkaline hydrolysis

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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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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-

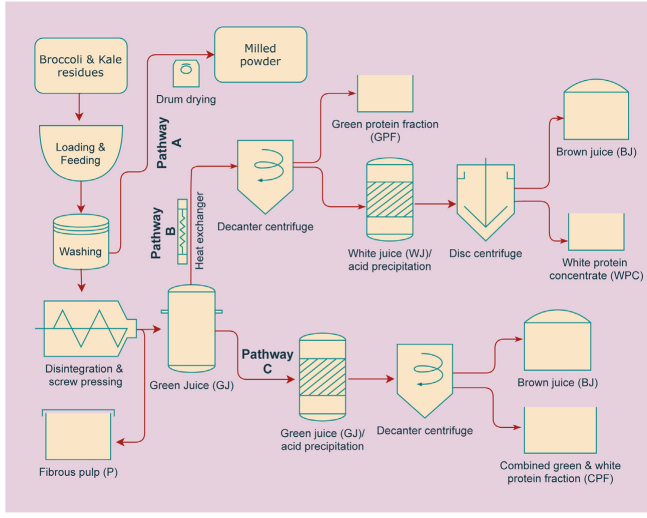

Fig. 1 – Overview of proposed use of broccoli and kale residual leaves as dried and milled biomass (pathway A), and material flow in protein extraction pathways (B and C), with different fractions and side products.

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-

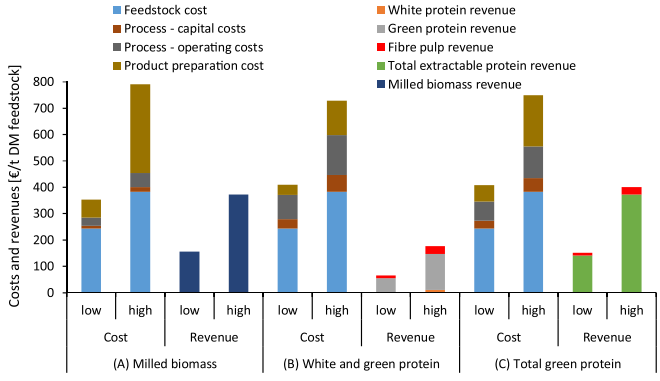

Fig. 2 – Cost and revenues [€/t DM of feedstock] of broccoli leaf-derived products in the three production pathways given as low–high range. 'Process' refers to extraction of proteins and production formulation refers to drying or ensiling for the different product fractions. Revenue for milled biomass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

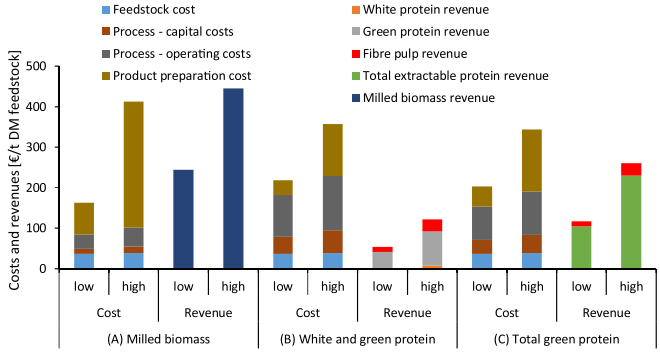

Fig. 3 – Cost and revenues [€/t DM of feedstock] of kale leaf-derived products in the three production pathways given as low–high range. 'Process' refers to extraction of proteins and 'production formulation' refers to drying or ensiling for the different product fractions. Revenue for milled biomass 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

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Article

Protein Fractionation of Leafy Green Biomass at the Pilot Scale: Partitioning and Type of Nitrogen in the Fractions and Their Usefulness for Food and Feed

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ABSTRACT: Fractionation of green biomass often results in fractions with insufficient protein content or quality for food or feed. To understand ways forward, we evaluated the fate of nitrogen (N) and the food or feed suitability of six pilot-scale fractions. The *N* was present mainly as amino acids (AA) in all fractions (<87%), however, the protein was partly degraded or insoluble in the majority of samples. All protein types and AAs traveled similarly through the fractionation process, giving insignificant separation of RuBisCO versus other proteins, and essential versus nonessential AAs. Water-soluble *N* compounds were enriched in juice fractions (90−95%), while the protein fractions contained the highest insoluble protein content (13−17%). AA composition in pulp and green juice verified their suitability as feed for ruminants and pigs, respectively. Fractionation of green biomass for food and feed is indeed important, although for sustainable industrial applications, further evaluations are required regarding process feasibility, antinutritional components, and brown juice uses.

KEYWORDS: *biorefinery, plant protein, sustainable food production, protein shift, local protein feed*

1. INTRODUCTION

Vegetable protein sources that can contribute food to the human population and feed domesticated animals have been increasingly investigated during the past decades.[1] This growing interest is a response to two of the largest challenges that humankind has ever faced: an increasing global population, predicted to reach 9.7 billion in 2050,[2] and accelerating climate change.[3] Meat consumption at its current level, and with current production systems, is unsustainable as the requirements of resources, such as land and energy, for each protein unit are too high.[4,5] Additionally, the system in Western countries to feed ruminants, horses, pigs, and chickens with soy mainly produced in South America contributes negatively to the sustainability of the food system.[6] Therefore, finding alternative sources of high-quality protein to feed both humans and domesticated animals is of utmost importance, and of equal importance is that these alternatives offer mitigations of the negative impacts, or at least cause minimal environmental burden.[1,7]

Several promising alternative protein sources for food and feed are suggested in the literature, e.g., insects,[8] algae,[9] and green leafy biomass, the latter is globally available in large quantities in the form of plant leaves.[10−12] This feedstock contains the protein ribulose-1,5-bisphospate-carboxylase/oxygenase (RuBisCO), which catalyzes carbon fixation in the photosynthetic cycle.[13] Approximately 50% of the proteins in green biomass is RuBisCO[14] and the protein is also the most abundant in the world.[15] Protein concentrates rich in RuBisCO have a high nutritional value and significant functional properties, which strongly enhances their attractiveness as a food ingredient.[16,17] The use of protein from green biomass as a feed source is beneficial as currently, it does not contribute negatively to the food-feed competition, which is in place for some plant protein sources. Also, the negative impact of meat production is reduced if protein from green biomass is used as feed, e.g., the climate impact of pork is decreased by 17% when fed grass-clover protein.[7] The concept of using green leafy biomass as a protein source is not new (the history is comprehensively reviewed by Domokos-Szabolcsy et al.),[10] but the development of novel technologies and processes, together with an ever more urgent need for alternative protein sources, has resulted in a renewed interest in this protein source.[18]

Conversion of green biomass to valuable protein for food and feed, while using the side-streams as feed, biofertilizer, and/or bioenergy, is perceived as a feasible, sustainable, and circular system to produce future products,[21] especially if a large diversity of green biomass can be utilized to cover availability across years, seasons and site.[19] The commonly used process (Figure 1) for protein fractionation of green leafy biomass for producing food and feed consists of three steps: (1) pressing of the leaves to separate the protein-rich green juice (GJ) and the fibrous pulp (P), (2) precipitation of the green protein (GP) fraction in the GJ through heating, leaving

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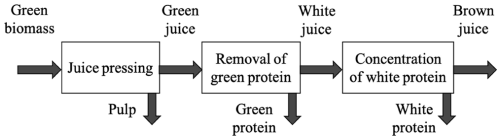

Figure 1. Overview of the protein fractionation process.

a "white" juice (WJ) for further fractionation, and (3) precipitation of a "white" protein (WP) fraction from WJ through acid treatment, leaving a brown juice (BJ) as a residual.[19] Here, the GP, and also the *P* fraction, could be of potential interest as a feed protein source, while the WP fraction has potential as a human food protein source.[20] However, the protein recovery from this fractionation method (Figure 1), is generally low, i.e., around 50% of the nitrogen (N) in the original green biomass remains in the *P* fraction and never reaches the protein fractions.[19,21] This low protein recovery results in reduced economic feasibility for the process and reduces process sustainability.[20,21] Optimized extraction processes are also crucial for reaching environmental sustainability.[22] Furthermore, the harvest occasion (year, season, and site) and biomass type largely influence the protein yield of the fractions.[19,21,23,24] Additionally, the path of *N* along the fractionation process, and the form of *N* in the final fractions have to date only been studied in the laboratory scale.[19] To secure the development of a feasible and sustainable industrial process for protein fractionation from green leafy biomass, additional knowledge is required as to (i) the fate of *N* in the different fractions, (ii) the variability of outcome from various sources, and (iii) impact from scaling-up of the process. Furthermore, the differentiation of amino acid (AA) composition in the fractions and limitations for their uses in food and feed purposes have until now not been evaluated and would need further elaboration to reach a better assessment of the product value.

Therefore, the aim of the present study was to evaluate how *N* in its various forms travels along a pilot protein fractionation pathway from leafy green biomass to different fractions. To understand the ratio of variation in nitrogenous compounds in the different fractions, a broad array of green biomass sources was used for the evaluation. An additional aim of this study was to understand the AA composition of the different fractions and what impact this has on the potential for utilization in food and feed products. The study was carried out on a pilot scale to enable an understanding of the differences in outcomes in industrial settings with those from the laboratory scale procedures. Furthermore, the impact of the fractionation methodology used on an industrial scale and possible target products are discussed.

2. MATERIAL AND METHODS

2.1. Biomass. Eight types of leafy agricultural biomass were collected in 2020 from operating farms in the Scania region of Sweden. Red clover, lucerne, beetroot tops, sugar beet tops, immature oat (hereafter referred to as oat), and white clover were collected in week 25, 34, 35, 40, 41, and 42, respectively, using a Haldrup Harvester (Haldrup, DE) with an approximately 5 cm cut height. Hemp tops were collected in week 26 using a Haldrup Harvester cutting approximately 30 cm from the top of the plants. Pea residuals were collected in week 36 as field residuals from the commercial green pea harvesting. In all cases, cut material was transferred immediately to processing with a maximum travel time of approximately 3 h.

2.2. Protein Extraction/Biomass Processing. Processing of the biomass (Figure 2) occurred in a pilot-scale facility at the Swedish

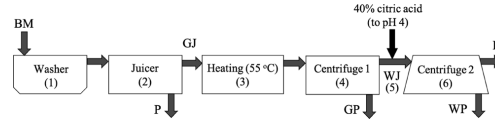

Figure 2. Green biomass fractionation process schematic (BM − unfractionated biomass, *P* − pulp, GJ − green juice, GP − green protein, WJ − white juice, WP − white protein, and BJ − brown juice).

University of Agricultural Sciences in Alnarp, Sweden. The abbreviation BM refers to the unfractionated biomass, i.e., the first fraction in the process, while "biomass" is used as a more general term in this paper. The process consisted of the following steps:

1. Washing of the biomass (BM) in a commercial salad washer (Adria, Turatti, IT) to remove soil particles and other contaminants.
2. Pressing of BM (juicer CP-10, Vincent, USA) at a process speed of approximately 250−300 kg BM/h, resulting in a dewatered pulp fraction (P) and a green juice fraction (GJ). The *P* exited the process.
3. Heating of the GJ by pumping it through two, 12 m long, silicone-lined tube-in-tube heat exchangers (Grainfather Counterflow Wort Chiller, Grainfather, NZ, approximately 65 °C heating water). The GJ reached a regulated exit temperature of 55 °C, which was sufficient for coagulating the green protein (GP). The heated GJ entered a holding tank with a residence time of 15−30 min to accommodate process variations.
4. Separation of the coagulated protein and other solids by pumping the heated GJ to a decanter centrifuge operating at approximately 4000 RCF (CA-220, Westfalia Separator AG, DE). This produced a liquid white juice (WJ) fraction and a green protein fraction (GP). The GP exited the process.
5. Acidification of the WJ with 40% w/v food-grade citric acid solution (Brenntag, DE) to reduce the pH to 4 by using an automatic pH controller (BL-7916, Hanna, USA) causing protein precipitation in a surge/holding tank with a residence time of approximately 15−60 min.
6. Separation of the precipitated white protein (WP) from the brown juice (BJ) in a self-unloading disk centrifuge at approximately 8000 RCF (SB-14, Westfalia Separator AG, DE).

All fractions (BM, *P*, GJ, GP, WJ, WP, BJ) were frozen at −80 °C, lyophilized in darkness, and stored at −20 °C pending analysis.

2.3. Chemical Composition. The total content of nitrogen (N_{total}) was measured in duplicate according to the Dumas method (Flash 2000 NC Analyzer, Thermo Scientific, USA). Crude protein values to be used for AA scores (see Section 2.4) were calculated using the *N* content (Dumas) × 6.25 according to FAO (2013).[25] Nitrate and nitrite measurements were conducted according to the standard NMKL 100[26] on single samples, and the AA content was measured according to ISO 13903:2005[27] on single samples (Eurofins, LU).

Protein (including RuBisCO) content and composition were measured in triplicate by size exclusion-high performance liquid chromatography (SE-HPLC) according to Desai et al.,[28] with modifications. This method allowed the differentiation of the peaks of the different subunits of RuBisCO, using a standard (Figure 3, red curve). Furthermore, the method allowed differentiation of all proteins present in the fractions evaluated in the present study. The presence of RuBisCO was obvious in the fractions, although other proteins were also present, both of similar sizes to the RuBisCO subunits (thereby overlapping the RuBisCO peaks) and of lower

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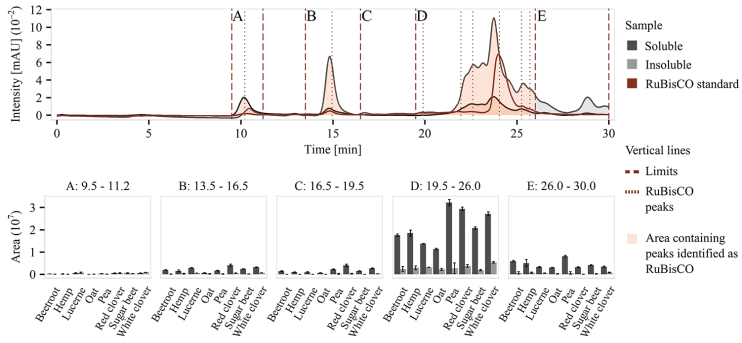

Figure 3. SE-HPLC analysis of protein (absorption at 280 nm) in green biomass from different sources. Top: Chromatogram of the RuBisCO and of "soluble" and "insoluble" protein from unfractionated biomass of oat (as a representative green biomass). The dotted vertical lines represent the approximate elution times for the RuBisCO standard. The dashed vertical lines are the integration limits used to quantify RuBisCO rich regions, and the corresponding areas are red. Bottom: Peak areas (mean of triplicate analyses) were for the different biomass sources.

molecular masses (Figure 3). Therefore, the chromatograms obtained here were divided into RuBisCO-rich peak regions (A, B, D) and regions with unidentified proteins (C, E), the latter containing less than 1% of the total protein.

For the protein analyses carried out here, 25 mg of lyophilized sample was added to 1.2 mL of 0.05 M NaH_2PO_4, pH 6.9, followed by shaking at 2000 rpm for 5 min (IKA Vibrax VXR B, IKA Werke, DE) and centrifugation at 5000 RCF for 3 min, and the supernatant (the "soluble protein") was decanted for analysis. The residual pellet was resuspended in 1.2 mL of the same extraction solution, followed by sonication for 45 s (Soniprep 150, MSE, UK) and centrifugation as above with the supernatant (the "insoluble protein") decanted for analysis. The extracts were analyzed using a Waters e2695 HPLC with a Waters 2998 PDA detector (Waters, USA). The extracts were sampled at 25 °C and the column, BioSep SEC-s3000 (Phenomenex, USA) maintained at 19 °C. An injection volume of 20 μL was used. A mobile phase of 0.05 M NaH_2PO_4, pH adjusted to 6.9, was applied at 0.5 mL/min. Absorption spectra (3D) were collected at 190 to 520 nm over 37 min, and for further analysis, spectra at 280 nm were separated. Intervals for protein integration were determined with a RuBisCO standard from spinach at a concentration of 0.565 mg/mL (Fitzgerald Industries International, USA), and chromatograms were divided into five intervals, A: 9.5−11.2, B:13.5−16.5, C: 16.5−19.5, D: 19.5−26.0 and E: 26.0−30.0 min (see Figure 3 for representative chromatograms). The RuBisCO standard was used to calculate the amount of proteins in different intervals. The total protein content was calculated as the sum of the soluble and insoluble RuBisCO-rich peak regions (A-D) and soluble and insoluble unidentified proteins (C and E).

2.4. Relative Content of Needed Amino Acids in Fractions. The AA score was calculated by comparison of the measured AA content of the intended product, in this case, a specific fraction, with the reference profile for the considered consumer (eq 1).

$$\text{AA score} = \frac{\text{AA content of the considered product (mg per gram total AA)}}{\text{Reference need for that specific AA (mg per g AA)}} \quad (1)$$

The AA score used for the calculations described below is based on chemical analysis and was not adjusted for digestibility.

To calculate the relative content of the required AAs for humans and some major domesticated animals, i.e., pigs, poultry, and cattle, in the various fractions, calculations were carried out following the literature. Thus, to calculate limiting essential AAs for humans, the content of each AA in a specific fraction was divided with the crude protein (AA/crude protein) and compared to reference values for individuals over 3 years old according to FAO.[29] For pigs, the amount of AA per unit mass of the considered fraction was used for the calculation of each pig's essential AA according to Peet-Schwering and Bikker.[30] Reference values were calculated based on 80−120 kg unbred females using a standardized ileal digestible (SID) lysine of 6.74 g/kg feed and recommended amounts of each essential AA per SID lysine.[30] For chicken, the amount of AA per unit mass of the considered fraction was used for the calculation of each chicken essential AA (which is similar to human requirements, with the addition of arginine and glycine).[31] Reference values for broiler chickens at 6−8 weeks and white egg layers were used.[31] As ruminants produce essential AA in their rumen, the required AA content in their feed has a complex relationship with their nutritional needs. Therefore, the AA score was not calculated, instead, a ratio of Lys to Met of 3:1 was used as a suitable measure which is considered desirable in lactating cattle feed.[32]

2.5. Calculation of Nitrogen Content in Nitrogenous Compounds. The ratio of nitrogen (N) in the nitrogenous compounds (nitrate, nitrite, AAs) to the total N ($N_{AA,nitrate,nitrite}/N_{total}$) was calculated based on the N content (g/mol) of each compound.

2.6. Statistical Analysis. Protein extraction of eight BM sources, used as replicates of green biomass fractionation, was carried out on a pilot scale. The content of AAs, nitrate, and nitrite was analyzed in each sample (fraction of each BM source) with single technical replicates, as the commercial testing laboratory stated their method to be robust enough. The measurement uncertainty of the analytical methods provided by the commercial testing laboratory was 10−20% for the AAs. For three of the samples with a high content of nitrate, a duplicate sample was sent to the commercial testing laboratory to check the repeatability, and similar values (not included here) were obtained.

All data analyses were performed using R Statistical Software.[33] Principal component analysis (PCA) was performed with package *ggbiplot* (v0.55). Correlation analyses were made using the package *GGally* (v 2.1.2). Anova and the following Duncan test were made using *lmerTest* (v3.1−3), *lme4* (v1.1−28), *emmeans* (v1.7.2), *multcomp* (v1.4−18), and *DescTools* (v.0.99.48). The error bars denote one standard deviation. All graphs were generated using the package *ggplot2* (v3.3.6).

3. RESULTS AND DISCUSSION

3.1. Fate of Nitrogen in Fractions along a Pilot Protein Fractionation Pathway. The significantly highest content of total N, essential and nonessential AA, and total AA was found in the GP and WP fractions (Table 1). The BJ

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Table 1. Mean Values and Standard Deviations of Amino Acids (AA), Nitrite, Nitrate, and Nitrogen (N), on dry weight basis, of the Fractions and Biomass Sources; Essential AA Are Those for Humans[a]

source	N_{total} [g/100 g]	nitrite [10^{-3} mg/g]	nitrate [mg/g]	essential AA [g/100 g]	nonessential AA [g/100 g]	total AA [g/100 g]
fraction						
BM	3.08 ± 0.90^{C}	1.59 ± 1.06^{A}	4.51 ± 7.38^{AB}	5.73 ± 1.75^{C}	9.12 ± 2.74^{B}	14.9 ± 3.60^{B}
P	2.45 ± 6.59^{CD}	4.56 ± 9.49^{A}	0.85 ± 1.59^{B}	5.83 ± 1.68^{C}	7.51 ± 1.93^{B}	13.3 ± 3.61^{B}
GJ	3.93 ± 1.19^{C}	5.79 ± 9.96^{A}	6.57 ± 11.2^{AB}	7.77 ± 2.65^{C}	11.9 ± 4.02^{B}	19.6 ± 6.25^{B}
GP	5.22 ± 1.82^{B}	138 ± 372^{A}	0.90 ± 1.25^{B}	12.9 ± 5.71^{B}	16.0 ± 6.41^{A}	28.5 ± 12.2^{A}
WJ	2.47 ± 1.22^{CD}	18.9 ± 42.8^{A}	8.46 ± 15.8^{AB}	5.08 ± 5.29^{C}	7.36 ± 4.28^{B}	12.4 ± 9.36^{B}
WP	7.40 ± 2.37^{A}	1.13 ± 1.08^{A}	3.05 ± 6.72^{AB}	18.1 ± 8.80^{A}	24.5 ± 7.92^{A}	42.6 ± 15.7^{A}
BJ	2.20 ± 0.85^{D}	4.03 ± 6.59^{A}	10.1 ± 18.8^{A}	5.04 ± 6.90^{C}	5.85 ± 2.28^{B}	10.9 ± 8.66^{B}
biomass source						
beetroot	2.55 ± 2.01^{D}	23.8 ± 44.0^{A}	0.48 ± 0.44^{B}	5.85 ± 6.22^{B}	7.66 ± 7.10^{B}	13.5 ± 13.3^{B}
hemp	2.66 ± 1.35^{D}	166 ± 394^{A}	25.0 ± 19.4^{A}	8.01 ± 6.09^{AB}	11.5 ± 6.84^{B}	19.5 ± 12.9^{B}
lucerne	4.20 ± 2.43^{B}	0.97 ± 0.91^{A}	0.32 ± 0.29^{B}	9.96 ± 8.49^{AB}	13.4 ± 8.80^{B}	23.4 ± 17.3^{B}
oat	5.63 ± 2.36^{A}	1.43 ± 1.21^{A}	7.64 ± 7.56^{B}	13.1 ± 8.83^{A}	17.6 ± 8.67^{A}	30.6 ± 17.3^{A}
pea	3.21 ± 24.8^{CD}	1.26 ± 1.00^{A}	1.17 ± 0.84^{B}	7.37 ± 7.11^{AB}	9.51 ± 8.14^{B}	16.9 ± 15.2^{B}
red clover	2.70 ± 1.60^{D}	4.31 ± 9.13^{A}	0.31 ± 0.22 ^{B}	5.55 ± 4.50^{B}	8.69 ± 4.40^{B}	14.2 ± 8.87^{B}
sugar beet	3.84 ± 2.20^{BC}	0.83 ± 0.25^{A}	3.49 ± 7.29^{B}	8.65 ± 6.92^{AB}	11.2 ± 7.84^{B}	19.9 ± 14.7^{B}
white clover	4.44 ± 2.06^{B}	0.62 ± 0.71^{A}	1.02 ± 0.71^{B}	10.6 ± 5.88^{AB}	14.9 ± 6.50^{B}	25.5 ± 6.63^{B}

[a]Total AA is the calculated sum of all AAs. Values followed by the same letter do not differ significantly at $p < 0.05$ using the Duncan post hoc test.

Table 2. Average (and range) of *N* Explained by Amino Acids (AA), Nitrate, and Nitrite for All Biomass Types and for Each Fraction

	BM	*P*	GJ	GP	WJ	WP	BJ
AA (%)	69.8 (63.1−82.0)	74.7 (71.0−81.0)	69.1 (56.5−76.3)	77.0 (63.1−84.3)	57.0 (35.8−72.6)	80.4 (60.7−87.4)	50.7 (33.0−60.8)
nitrate (%)	2.7 (0.1−10.3)	0.9 (0.1−4.9)	3.2 (0.2−15.2)	0.5 (0.0−1.9)	8.8 (0.0−56.5)	0.9 (0−5.6)	8.0 (0.6−36.8)
nitrite (‰)	0.0 (0.0−0.0)	0.1 (0.0−0.4)	0.0 (0.0−0.2)	0.9 (0.0−7.1)	0.3 (0.0−1.8)	0.0 (0.0−0.0)	0.1 (0.0−0.4)

fraction showed the significantly highest content of nitrate, while no significant differences in nitrite contents were found among the fractions (Table 1).

The AAs were found to contribute the largest share of *N* in all fractions (51−80%; Table 2). In principle, high values were found for the protein fractions (GP and WP), while the content in the juice fractions was lower (Table 2). The considerable contribution by AAs to the total *N* was also verified by a strong positive Pearson correlation ($P < 0.001$) between total *N* and both essential and nonessential AAs. The high contribution of AAs to the *N* content in biomass has previously been reported for cassava leaves, with a N_{AA}/N_{total} of 80−90%,[34] although contributions in fractions from protein fractionation have been scarcely evaluated in previous studies. However, this study indicates that AA most likely always contributes the highest share of *N* in the fractions from protein fractionation.

Essential and nonessential AAs were equally well correlated to the total *N* in the different fractions, indicating their equal fractionation along the pathway (Table 1). Only a small fraction of *N* was present as nitrite (0.002−0.26%) and nitrate (0.2−4.5%; Table 2). The contents of nitrite and nitrate in the different fractions did not correlate significantly with the *N* content nor with the content of AAs, and at least the nitrate was found to clearly travel with the juices along the fractionation pathway (Table 1). Also, the PCA (with sugar beet as an example), where the first principal component (PC1) explained 61.4% of the variation and the second principal component (PC2) explained 24.4% of the variation, verified that nitrate was primarily found in the juice fractions (GJ, BJ, and WJ; Figure 4).

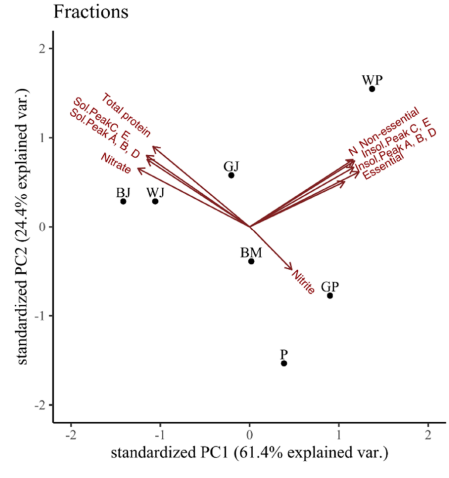

Figure 4. Principal component analysis (PCA) of the content of AAs, nitrogen, nitrate, nitrite, and RuBisCO in the separate fractions in sugar beet, used as one example. Protein components separated by HPLC are denoted Sol. or Insol. Peak A, B, D (RuBisCO containing parts), and Sol. or Insol Peak C, E as described in materials and methods.

A significant part of the nitrogenous compounds in the fractions (30−49%) remained unidentified (Figure 5, Table 2). Most of the unidentified nitrogenous compounds (nitrogenous compounds other than AA, nitrate, and nitrite) were shown to be water-soluble, as the *N* in the protein fractions (GP and

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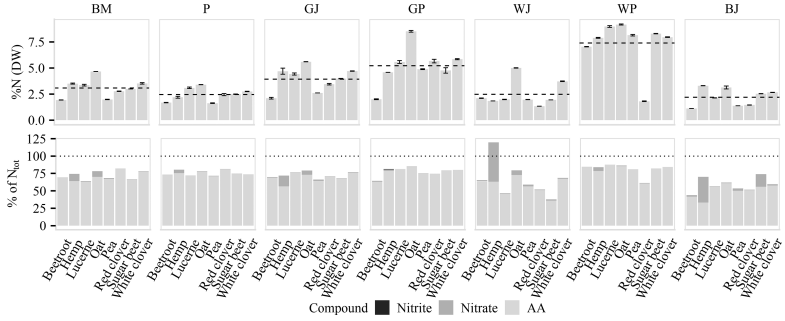

Figure 5. Top: Total *N* content (% dry weight (DW), mean of duplicate measurements) in fractions (unfractionated biomass (BM), pulp (P), green juice (GJ), green protein (GP), white juice (WJ), white protein (WP), and brown juice (BJ) from the different biomass sources. Dashed lines represent the mean of all biomass sources. Bottom: Content of *N* from the nitrogenous compounds (amino acids (AA), nitrate and nitrite; one measurement per sample) as compared to N_{total}. Dotted lines indicate 100% of the *N*.

Table 3. Mean Values and Standard Deviation[a] of Protein Components (mg/g Dry Weight) in Different Fractions and Biomass Sources as Analyzed with SE-HPLC; Peak Regions as in Figure 3: A: 9.5−11.2, B, 13.5−16.5, C, 16.5−19.5, D, 19.5−26.0, E, 26.0−30.0 min[b]

source	soluble peak A, B, D	soluble peak C, E	insoluble peak A, B, D	insoluble peak C, E	total protein
fraction					
BM	123 ± 42.2^{C}	0.58 ± 0.23^{C}	18.8 ± 8.25^{B}	0.06 ± 0.05^{B}	142 ± 46.5^{C}
P	56.0 ± 15.0^{D}	0.32 ± 0.13^{D}	13.8 ± 3.56^{C}	0.06 ± 0.04^{BC}	70.3 ± 16.5^{D}
GJ	166 ± 60.9^{B}	1.00 ± 0.55^{B}	18.4 ± 12.1^{B}	0.06 ± 0.04^{BC}	186 ± 67.6^{B}
GP	78.5 ± 94.0^{D}	0.52 ± 0.59^{CD}	11.6 ± 4.39^{CD}	0.06 ± 0.04^{BC}	90.7 ± 95.0^{D}
WJ	214 ± 59.1^{A}	1.14 ± 0.38^{AB}	8.87 ± 5.75^{D}	0.03 ± 0.03^{BC}	224 ± 60.9^{A}
WP	122 ± 64.2^{C}	0.66 ± 0.41^{C}	24.9 ± 14.1^{A}	0.13 ± 0.14^{A}	148 ± 69.8^{C}
BJ	219 ± 53.4^{A}	1.34 ± 0.74^{A}	3.32 ± 2.87 ^{E}	0.02 ± 0.02^{C}	223 ± 54.3^{A}
biomass source					
beetroot	208 ± 97.1^{A}	1.35 ± 0.68^{A}	16.5 ± 8.45^{B}	0.10 ± 0.05^{AB}	226 ± 93.5^{A}
hemp	124 ± 87.6^{C}	0.90 ± 0.78^{B}	10.5 ± 4.70^{C}	0.07 ± 0.05^{BC}	136 ± 87.0^{C}
lucerne	167 ± 90.4^{B}	1.25 ± 0.55^{A}	15.6 ± 13.7^{B}	0.11 ± 0.14^{A}	184 ± 90.0^{B}
oat	93.9 ± 52.4^{D}	0.63 ± 0.41^{C}	16.1 ± 5.51^{B}	0.05 ± 0.02 ^{CD}	111 ± 53.0^{CD}
pea	84.6 ± 54.0^{D}	0.51 ± 0.37^{C}	8.20 ± 6.21^{C}	0.03 ± 0.03^{CD}	93.3 ± 50.7^{D}
red clover	168 ± 72.6^{B}	0.48 ± 0.23^{C}	15.8 ± 10.1^{B}	0.01 ± 0.01^{E}	184 ± 71.9^{B}
sugar beet	122 ± 58.4^{C}	0.59 ± 0.35^{C}	7.95 ± 4.85^{C}	0.02 ± 0.02^{D}	130 ± 56.5^{C}
white clover	150 ± 71.2^{BC}	0.65 ± 0.35^{C}	24.1 ± 14.9^{A}	0.06 ± 0.04^{BC}	175 ± 72.8^{B}

[a]Total protein is calculated as the sum of both Soluble Peaks A, B, D, C and E and Insoluble Peaks A, B, D, C and E. [b]Values in columns followed by the same letter does not differ significantly ($p < 0.05$) using the Duncan post hoc test.

WP) consisted of up to 80% of AAs while only 50−70% of the *N* in the juices (GJ, WJ, and BJ) consisted of AAs (Table 2). Examples of water-soluble nitrogenous compounds known to be present in plants but not evaluated here are alkaloids, cyanogenic glucosides, glucosinolates,[35] and chlorophyll.

Previous studies have shown that RuBisCO is the most prevalent protein in green leaves and composes up to 50% of their total protein,[14] and the present study, using SE-HPLC, verified the presence of a high number of proteins other than RuBisCO in the samples (Figure 3). Here, using SE-HPLC, the highest amount of soluble and total protein was found in the juice fractions (GJ, WJ, BJ), while the significantly highest amount of insoluble protein was found in the WP (Table 3).

The high content of protein in the juice fractions, as determined by SE-HPLC analyses, was not in accordance with the total *N* and AA contents, where high levels were shown for the GP and WP fractions in relation to the juice fractions (as discussed above). The PCA also verified the lack of correlation between soluble protein (which is the majority of the proteins according to HPLC data; Table 3) and total *N* in the fractions (Figure 4). There are two possible explanations for these results; (i) part of the proteins in the protein fractions may have degraded into peptides and free AAs and (ii) the solubility of the proteins might have decreased. The present study showed that most of the proteins in the fractions were soluble in the 0.05 M phosphate buffer used as extraction buffer, although, a second extraction step with sonication resulted in the solubilization of additionally around 2−20% of protein, with the highest solubility of the proteins in the juice fractions (Table 3). However, the fact that the "insoluble" part of the proteins, to a great extent, had a similar chromatogram profile as the "soluble" part, but with significantly lower absorbance (Figure S-1), indicated the same proteins being present in both parts. Native RuBisCO is water-soluble, although results from the present study indicate that after certain processing conditions, the solubility decreases. Differences in solubility measured by light absorption/scattering might be a result of conformational changes in the proteins.[36]

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Table 4. Scores of Essential Amino Acids, where Values >1 Indicate Sufficient Amounts, in Various Fractions of Green Biomass after Fractionation for Humans, Pigs, and Chickens[a]

fraction		amino acid										
		Try	His	Iso	Leu	Lys	Thr	Val	Met + Cys	Phe + Tyr	Arg	Gly+Ser
human	BM	2.36 ± 0.60	1.18 ± 0.23	1.14 ± 0.26	1.04 ± 0.26	1.12 ± 0.22	1.55 ± 0.28	1.11 ± 0.24	1.24 ± 0.27	1.71 ± 0.40		
	P	2.56 ± 0.29	1.34 ± 0.12	1.35 ± 0.09	1.25 ± 0.10	1.26 ± 0.08	1.77 ± 0.08	1.30 ± 0.10	1.22 ± 0.22	1.95 ± 0.15		
	GJ	2.56 ± 0.34	1.20 ± 0.14	1.40 ± 0.55	0.97 ± 0.40	1.15 ± 0.11	1.65 ± 0.17	1.20 ± 0.13	1.11 ± 0.18	1.89 ± 0.29		
	GP	3.04 ± 0.57	1.31 ± 0.18	1.44 ± 0.22	1.32 ± 0.21	1.22 ± 0.15	1.82 ± 0.12	1.37 ± 0.17	1.18 ± 0.22	2.14 ± 0.34		
	WJ	1.81 ± 0.96	0.85 ± 0.40	0.88 ± 0.43	0.76 ± 0.43	0.80 ± 0.32	1.30 ± 0.46	0.89 ± 0.40	0.83 ± 0.33	1.28 ± 0.58		
	WP	2.95 ± 0.92	1.33 ± 0.37	1.40 ± 0.34	1.29 ± 0.37	1.23 ± 0.24	1.88 ± 0.24	1.37 ± 0.28	1.08 ± 0.30	2.18 ± 0.61		
	BJ	1.54 ± 0.89	0.77 ± 0.35	0.75 ± 0.36	0.65 ± 0.39	0.80 ± 0.32	1.25 ± 0.41	0.81 ± 0.32	0.82 ± 0.31	1.15 ± 0.62		
pig	BM	2.66 ± 0.94	1.67 ± 0.47	2.01 ± 0.63	1.91 ± 0.60	1.61 ± 0.47	1.75 ± 0.51	1.99 ± 0.62	1.39 ± 0.52	2.18 ± 0.65		
	P	2.18 ± 0.75	1.43 ± 0.41	1.79 ± 0.51	1.72 ± 0.51	1.39 ± 0.39	1.51 ± 0.42	1.76 ± 0.48	1.09 ± 0.34	1.91 ± 0.52		
	GJ	3.46 ± 1.36	2.05 ± 0.73	3.07 ± 2.17	1.95 ± 1.00	1.97 ± 0.69	2.24 ± 0.69	2.55 ± 0.87	1.56 ± 0.56	2.89 ± 1.10		
	GP	5.84 ± 2.75	3.13 ± 1.30	4.27 ± 1.80	4.08 ± 1.66	2.96 ± 1.11	3.33 ± 1.27	4.08 ± 1.68	2.30 ± 1.07	4.65 ± 2.05		
	WJ	1.64 ± 1.49	0.94 ± 0.75	1.20 ± 1.06	1.09 ± 1.03	0.92 ± 0.78	1.20 ± 0.90	1.28 ± 1.11	0.76 ± 0.61	1.29 ± 1.15		
	WP	8.42 ± 3.31	4.82 ± 1.85	6.12 ± 2.28	5.96 ± 2.26	4.41 ± 1.58	5.04 ± 1.79	6.00 ± 2.21	3.21 ± 1.29	7.11 ± 2.72		
	BJ	1.01 ± 0.69	0.69 ± 0.35	0.77 ± 0.47	0.68 ± 0.48	0.72 ± 0.38	0.91 ± 0.40	0.91 ± 0.49	0.61 ± 0.38	0.85 ± 0.55		
chicken	BM	2.15 ± 0.76	1.19 ± 0.34	0.97 ± 0.30	1.20 ± 0.38	1.09 ± 0.32	1.07 ± 0.31	1.11 ± 0.35	0.77 ± 0.29	1.08 ± 0.32	0.76 ± 0.26	1.49 ± 0.40
	P	1.76 ± 0.60	1.02 ± 0.30	0.86 ± 0.25	1.08 ± 0.32	0.94 ± 0.26	0.92 ± 0.25	0.98 ± 0.27	0.60 ± 0.19	0.94 ± 0.26	0.67 ± 0.19	1.33 ± 0.31
	GJ	2.80 ± 1.10	1.47 ± 0.52	1.48 ± 1.04	1.23 ± 0.63	1.33 ± 0.47	1.37 ± 0.42	1.42 ± 0.49	0.86 ± 0.31	1.43 ± 0.55	0.99 ± 0.37	1.86 ± 0.59
	GP	4.72 ± 2.23	2.24 ± 0.93	2.05 ± 0.86	2.57 ± 1.05	1.99 ± 0.75	2.03 ± 0.78	2.28 ± 0.94	1.27 ± 0.59	2.31 ± 1.01	1.59 ± 0.67	2.80 ± 1.02
	WJ	1.30 ± 1.20	0.68 ± 0.54	0.58 ± 0.51	0.69 ± 0.65	0.62 ± 0.53	0.73 ± 0.55	0.71 ± 0.62	0.42 ± 0.34	0.64 ± 0.57	0.45 ± 0.38	1.01 ± 0.72
	WP	6.81 ± 2.68	3.45 ± 1.33	2.94 ± 1.09	3.76 ± 1.42	2.97 ± 1.07	3.08 ± 1.09	3.35 ± 1.23	1.78 ± 0.71	3.52 ± 1.34	2.54 ± 0.99	3.94 ± 1.34
	BJ	0.81 ± 0.56	0.49 ± 0.25	0.37 ± 0.23	0.43 ± 0.30	0.49 ± 0.26	0.55 ± 0.25	0.51 ± 0.27	0.34 ± 0.21	0.42 ± 0.27	0.29 ± 0.20	0.77 ± 0.32

[a]Try: Tryptophan. His: Histidine. Iso: Isoleucine. Leu: Leucine. Lys: Lysine. Thr: Threonine. Val: Valine. Met + Cys: Methionine + Cysteine. Phe + Tyr: Phenylalanline + Tyrosine. Arg: Arginine. Gly + Ser: Glycine + Serine.

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However, based on the obtained chromatograms, conformational changes of the proteins seemed an unlikely explanation for the differences in protein content among the fractions. Also, the sample preparation method utilized secured a high protein extraction from samples with the use of the two extraction steps. Thus, the present results indicate that the protein degradation contributed more significantly than the decreased solubility to the higher protein content in the juice fractions (GJ, WJ, and BJ) than in the protein fractions (GP and WP) by HPLC analysis, although further studies are needed.

Utilizing the combined data on all nitrogenous compounds evaluated, the present study clearly showed that (i) the protein fractions (GP and WP) contained the highest amount of *N* and AA but the protein had been partly degraded during the process, and the solubility of the proteins had also been decreased, (ii) the fractionation procedure did not separate proteins with essential and nonessential AAs, as these traveled in a similar way to the different fractions along the pathway, and (iii) nitrate and other nonevaluated water-soluble nitrogenous compounds traveled with the juice fractions and ended up in the BJ.

3.2. Ratio of Variation in Nitrogenous Compounds in Different Fractions. The present study clearly showed a large variation in the amounts of nitrogenous compounds in the different fractions depending on the biomass source evaluated, as verified by the large standard deviations (Tables 1 and 3). Previous studies on leaf fractionation have shown that the *N* content in the fractions is affected by the plant species, the harvest time and year, as well as the extent of biomass disruption during the juice pressing.[19,20,23,24] The present study used different biomass types, harvested on different occasions throughout the year, as replicates for the fractionation of green biomass in a pilot facility. The study could have incorporated several harvests of the same species from various years and seasons, which could have reduced the variation in the content of the nitrogenous compounds in the different fractions. The impact of genotype and environment is known to have an equal magnitude of importance for more or less any compound in the plant, although, their respective size of importance is influenced by how they are selected (genotype might have the largest impact if the plant material is broadly selected, while environment has a larger impact if a broad range is selected).[37] However, despite the large variation in the selection of the green biomass types, this study was able to describe general features for the fate of *N* in fractions along a protein fractionation pathway, as described above.

The setup of the present experiment leaves little room for description of variation in nitrogenous compounds in the different biomass sources (which was neither the aim of the study). However, among the biomass sources, oat showed the highest content of total *N*, and essential, nonessential, and total AA (Table 1), with high levels in the BM fraction, which also correspond with results from previous studies, and in the GP and WP (Figure 5, Table 2).

Since the present study just included one harvest of oat leaves, additional studies are needed to verify these results and eventually the suitability of cereal leaves as a biomass source for protein fractionation. Furthermore, hemp was found as the biomass source with by far the highest amount of nitrate (Table 1), with a high content in several fractions (BM, WJ, BJ, GJ, and WP; Figure 5 and Table 1). If these high values are general for hemp need to be further evaluated, but the important message from this study is that juice fractions (as side streams of protein-rich fractions) that are to be considered to be used as feed would need to be evaluated for toxicity levels of nitrate for livestock.

Differences in protein content and composition measured with SE-HPLC were also found among the biomass sources (Table 3), with the significantly highest amounts of soluble and total protein in beetroot. This might point to differences in biomass sources (or harvest occasion) that influence the degradation and solubility behavior of the proteins, characters that are important to understand in order to secure a well-functioning industrial process of protein fractionation from green biomass.

Also, other compositional differences among the biomasses evaluated here might have had an impact on the HPLC results. One such example is that the red pigments in, e.g., beetroot and red clover, might interfere with light measurements, as might phenolic compounds which form covalent bonds to the RuBisCO and other leaf proteins.[38] In fact, large peaks at late elution times, i.e., after 30 min (most likely as a result of polyphenolic compounds) were present for all oat and sugar beet fractions (including BM; Figure S-1).

3.3. Composition of AA in Different Fractions and Potential for Utilization in Food and Feed. If a protein-rich (extract, concentrate, or isolate) plant-based product (such as GP or WP in the present study) is to be used for human food or animal feed, it is extremely important that the AA composition of the protein meets the dietary requirements. In general, certain AAs are limited in the food/feed for both humans and animals, and therefore, additional protein sources with a good composition of essential AAs are highly desired. Knowledge of the AA limitations of fractions from biomass fractionation is essential to evaluate their potential as human food or animal feed.

In the present study, calculations were carried out to estimate such limitations in the AA composition of each fraction when the intended consumers were humans, pigs, or chickens, as well as suitable AA ratios for lactating cows. However, if green biomass fractions are to be used for food and feed, additional analyses are imperative to further evaluate characteristics such as palatability and biodegradability as well as the content of other nutritional or antinutritional components.

3.3.1. Limitations of Amino Acids: Humans. Out of the different fractionation products in the current study, only the WP[39] and possibly the GP fractions are of relevance as human food, and both of these fractions showed sufficient scores for all AAs essential for human consumption (Table 4). AA scores of >1 are deemed sufficient,[29] however, a rather high variation in the scores for different AAs was obtained (0.65 to 3.04; Table 4). This variation indicates a need to evaluate the AA values for each biomass source, harvest occasion, and year, if green biomass should be utilized in industrial production to produce human protein-rich food alternatives. Levels below the sufficient amount were found, specifically for leucine (Leu) and methionine+cysteine (Met+Cys), in some of the investigated GP and WP samples of the present study. Previous studies have shown that Leu is mainly involved in protein synthesis, energy metabolism, and inhibition of protein degradation.[40] Met is used in the production of important molecules in the human body, e.g., antioxidants, AAs such as Cys, and phospholipids.[41] To meet limitations of Met+Cys in the WP and GP fractions, these might be combined with other

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plant-based protein sources,[42] e.g., cereals, which are normally rich in these AAs and which can also contribute structure to the products.[43] WJ and BJ are less suitable as food, at least in their present form with a high water content (Table S-1), additionally, their AA scores were insufficient for human consumption (Table 4). The BM and *P* are probably not suitable as food due to their high fiber content.

3.3.2. Limitations of Amino Acids: Pigs. For the production of pig feed, GJ and GP are the most relevant fractions; WP might also be useful although it is most likely too expensive due to low recovery rates.[19,21] Similarly as for humans, BM and *P* are not suitable food sources due to their high fiber content. The BJ has the downside of high water content (Table S1) making the transport costly and the feeding process potentially difficult, and other uses for this fraction might be preferable. Corresponding to previous reports,[44] this study showed suitable AA compositions of GJ and GP as pig feed (Table 4). Previous studies on grass protein as a substitute for soybean meal in pig feed, have shown the potential to give sufficient nutrition, reduced cost, and environmental impact.[45,46] Also, red clover, white clover, and lucerne have been shown to have suitable AA profiles, making them potential protein sources for monogastric animals.[47] Thus, protein-rich extracts from these biomass types were suggested as valuable protein sources for pigs, as the content of less digestible fiber-bound proteins was reduced in the feed, resulting in a greater increase in weight as compared to ensiled feed.[48] Here, the variation in the AA score was relatively high for the different GJ and GP, with insufficient levels of Met+Cys in some of them. Previous studies have shown that Met or lysine (Lys) are commonly the limiting AAs in protein used for pig feed.[47] A diet deficient in Met might lead to a decrease in weight gain of the pigs as compared to a diet without AA limitations due to an alteration in the lipid metabolism.[49] As cereals, such as wheat and oats, are fairly high in Met+Cys,[50] locally produced cereals could be used to fortify pig feed based on GJ or GP.

3.3.3. Limitations of Amino Acids: Chicken. Most of the biomass fractions from the present fractionation process, with the exception of GP and WP, have insufficient amounts of several of the AAs required in chicken feed (Table 4). As mentioned above, using WP as feed will most probably be too expensive, but dried GP could also serve as an alternative supplement in the feed for chickens. Besides having inadequate AA scores, the high fiber content of BM and *P* and the high water content of GJ, WJ, and BJ (Table S1) make these fractions unsuitable as chicken feed in their current forms.

For chickens, the main limiting AA group is Met + Cys. Met is important for cell metabolism and acts as a precursor for cysteine.[51] Increased levels of Met in chicken feed may have positive effects on the quality of the chicken meat after slaughter, with increased shelf life and improved color of the meat.[52] Furthermore, a study on elevated content of Met + Cys in the feed showed a correlation with an increase in the weight of broiler chickens.[53] The second limiting AA for chickens is Lys, which is essential for the immune system and digestive tract functionality.[51] Additionally, arginine (Arg) has an impact on the performance of egg-laying hens due to effects on the ovulation and immune system, although excessive Arg impairs the uptake of Lys.[51]

3.3.4. Limitations of Amino Acids: Ruminants. Determination of limiting AAs for ruminants is complicated due to the biology of the different chambers in the stomach and the symbiosis with bacteria in the rumen.[54] Ruminants receive approximately 50% of their AAs from rumen bacteria,[55] and therefore, the ratio of specific AAs has been proven more important than the amounts.[54] The major limiting AAs for milk synthesis in lactating ruminants are Met and Lys,[54] and the ideal ratio (Lys:Met) is 3:1.[32,54,56] A ratio exceeding 3:2 does not affect the milk protein yield, while a lower ratio has a negative impact.[54,56]

In the present study, the Lys:Met ratio was close to or slightly higher than the recommended 3:1 in BM, *P*, GJ, GP, and WP (Table 5), making them all relevant as feed for lactating ruminants. In WJ and BJ, the Lys:Met ratio was considerably higher than 3:1, suggesting that these fractions are not optimal for this purpose. The most useful fraction for cattle feed, except the original BM, is probably *P*, as it contains an adequate Lys:Mat ratio and a high amount of fiber, which is suitable for ruminants. The use of *P* as feed for ruminants, with or without ensiling, has also been verified in previous studies.[57]

Table 5. Lysine/Methionine Ratio for Lactating Ruminants, where Values around 3 are Optimal

	fraction	ratio Lys/Met
ruminant	BM	3.84 ± 0.73
	P	3.71 ± 0.49
	GJ	3.95 ± 0.35
	GP	3.33 ± 0.50
	WJ	5.38 ± 1.24
	WP	3.68 ± 1.29
	BJ	6.89 ± 1.63

3.4. Impact of the Fractionation Process: From the Laboratory Scale to Industrial Settings. *3.4.1. Extraction of N.* The highest *N* content was found in the GP and WP, with average values of 5.2 and 7.4%, respectively (Table 1, Figure 5). The values obtained in the present pilot process correspond well with values reported in earlier studies using a directly comparable lab scale process, with *N* levels in WP of 7% for lucerne and 9% for beetroot.[19] Others have reported white clover protein concentrates with 7.2% *N*,[47] utilizing a laboratory-scale process resulting in a combined GP and WP fraction. However, higher levels of *N* in protein fractions have been reported, e.g., sugar beet WP with 14.8% *N*,[58] although achieved using a more elaborate method aiming at reaching pure RuBisCO. Thus, results from both the present and earlier studies indicate opportunities for reaching higher *N* levels, although the methodology for sustainable up-scaling of processes resulting in high *N* levels is limited and needs additional research.

Similarly, *N* yield in the protein fractions (GP and WP) as compared to the BM, using methodology available for up-scaled processes, is low in the present study (results not shown) as well as in previous studies.[19,20] The low *N* yield is partly a result of low protein extraction in the first juice-pressing step, as a large part of the *N* remains in the *P* fraction.[19,21] The literature suggests that the digestibility of protein, in the digestive tract, is suppressed in the presence of soluble dietary fiber,[47,59] due to the gel-forming characteristics of this dietary fiber, or the presence of tannins.[60] This might also, at least partly, explain the poor protein extractability during biomass fractionation. Thus, applying methods for disrupting fiber-protein interactions to increase the protein yield might be a prospective for the future. In general, higher *N* recovery already in juice pressing is a prerequisite for reaching

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sufficient *N* yield in the GP and WP, which in turn is of utmost importance for a sustainable process. Thus, future studies should focus on developing scalable methodologies reaching higher *N* extraction rates from *P*.[21]

3.4.2. Oxidation and Degradation. Previous studies have reported protein oxidation and enzymatic degradation as common problems in leaf protein extraction processes.[61] As discussed in section 3.1, the solubility of the protein in GP and WP, produced in the current pilot-scale system, was impaired and severe degradation of protein into free AAs had occurred.

A laboratory-scale procedure utilizing the same methodology as used here, reported RuBisCO as the main component of WP and protein solubility up to 68%,[17] indicating that issues with oxidation and degradation of the proteins are process-related. Based on the studies carried out here, industrial processes for protein fractionation of green biomass need to focus on methodologies to extract GP and WP without degrading the proteins. Opportunities for protein fractionation without protein degradation have been discussed in previous studies, and suggestions are to combine a reduced temperature with an efficient cell disruption, which can be solved by a careful choice of pressing/juicing equipment.[61] Another possible method is reverse micellar systems which have been successfully used for recovering functional proteins from other plant material.[62] To conclude, every industrial facility for green biomass protein fractionation should carefully evaluate the degree of protein degradation in their process.

3.4.3. Process Scale Conditions. Up-scaled processes might be more sensitive to system errors than processes on a laboratory scale. Here, a low *N* content in WJ (1.3%) and in the WP (1.8%) was received for red clover (Figure 5). This indicates that most of the protein precipitated at the heating step, hence ending up in the GP fraction, as a result of an error, causing an increased temperature during processing. This points at the importance of using optimal processing conditions for each biomass source to obtain a high protein yield, while for industrial processing, the use of the same parameters might be optional. In this study, which was carried out at a pilot scale, the same processing conditions for all biomass sources were followed based on what is most beneficial from a biorefinery and industrial point of view. The selected conditions have in previous studies under lab conditions been shown sufficient for such a concept.[19]

3.5. Impact of Fractionation Methodology on Target Product Characteristics. *3.5.1. RuBisCO Content in Biomass Fractions.* The HPLC results of the present study indicated that all types of leaf proteins (RuBisCO and all other types) seemed to be fractionated in a manner similar to the pilot scale fractionation process adopted here. As can be seen in Figures 3 and 4, peak regions A, B, and D (including RuBisCO peaks) proteins (soluble and insoluble) were highly correlated with the other types of proteins in the regions C and E (soluble and insoluble, respectively). Thus, the GP and WP fractions did not consist of pure RuBisCO protein but of a mixture of leaf proteins, in a similar composition as in the BM. However, the fact that GP and WP consist of a mixture of proteins seems to have little impact on the functionality of these fractions, as demonstrated in a recent study where similar air−water interfacial properties were obtained for WP extracted from various biomass sources.[17] Thus, a high extraction rate of the proteins seems more important than the purification of certain proteins from green biomass if these should be used in a protein fractionation process.

3.5.2. Nutritional and Antinutritional Constituents. As the present study focused on the fate of the *N* obtained after fractionation of green biomass, no chemical analyses to identify all potential nutritional and antinutritional compounds were carried out. However, as green biomass is known to contain a range of components of nutritional and antinutritional value,[63] these compounds are expected to end up and possibly accumulate in some of the fractions. Examples of nutritional compounds are vitamins, minerals, and essential fatty acids, and examples of antinutritional compounds are nitrate, phytates, tannins, and oxalates.[60,63] Previous studies have shown that, e.g., phenolic compounds are present in all fractions.[21] In general, water-soluble compounds are expected to end up in the juice fractions, with the highest accumulation in the BJ (as also discussed above). The possible accumulation of certain compounds in this fraction makes it exceptionally interesting for the further evaluation of additional fractionation and uses. Compounds bound to either polysaccharides or proteins might be accumulated in *P* and in GP and WP, respectively, which might be either beneficial or nonbeneficial depending on the nutritional/antinutritional value of the compound. Hence, additional analyses are required to understand the accumulation of various compounds in the BJ and also in other fractions and what opportunities or obstacles this brings.

A factor to consider for feed products is the level of nitrate and nitrite, as these compounds may have adverse effects on the animals. A generally recommended safe level in feed for livestock is currently lacking, and the safe level depends on animal tolerance, the conversion rate of nitrate into nitrite in the digestive tract, and environmental conditions.[64] Also, nonruminants are considered more susceptible to nitrate poisoning than ruminants.[65,66] Although, built on single replicates, the present study indicates variation among biomass sources in the nitrate content in *P* (0.08 and 4.7 mg/g) and GP (0.04 and 3.7 mg/g) (Table 1), which according to the analyses on limiting AAs should be suitable as feed sources for ruminants and for pigs and chickens, respectively (Tables 4 and 5). The highest content of nitrate was found in hemp *P* and GP, with high values also in BM (Figure 5). Hence, based on nitrate content, the *P* and GP of most of the biomass sources evaluated here are useful as feed sources, although those of, e.g., hemp might be considered for other uses.

3.5.3. Other Possible Uses for the Fractions. Some of the fractions obtained in this pilot process might not be useful directly for feed and food purposes due to the nutritional content or water content. All of the juices contain over 90% water (Table S-1), which might result in difficulties in storing and handling these fractions. However, for the utilized fractionation methodology to be a feasible part of the circular bioeconomy, it is required that all side streams are valorized, especially the BJ.[19,21] Thus, the BJ needs to be further evaluated for the content of interesting compounds. Microwave- and ultrasonic-assisted extraction has previously been shown successful for the fractionation of phenolic compounds in, e.g., sugar beet leaves.[67] Furthermore, cost-benefit analyses of the uses of the different fractions need to be carried out, as each of them has many potential uses. *P* is rich in cellulose and associated compounds,[39,61] and could serve as a substrate for pyrolysis,[68] anaerobic digestion for production of biofuel, and raw material for lignocellulose extraction.[69] *P* could also be used to remove pollutants from wastewater[70] or as a substrate for biogas production.[39] GJ can be used for biofuel

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production,[71] or as a source of active compounds for skin care products.[72] BJ can be used to produce dietary fiber for feed,[46] as a sugar source for PHA production[73] biofuel[74,75] or as a biostimulant for plants.[76,77] Thus, there are several possibilities for valorizing all the fractions.

The results of this study clearly showed the large potential of using green leafy biomass in a protein fractionation process to produce food and feed. In general, the proteins of the green biomass were water-soluble, although only around 50% of the proteins were extracted from the biomass to the green juice. Thus, a need was indicated to change the protein extraction procedure for protein extraction from biomass in pilot and industrial settings to obtain a feasible protein yield. All types of proteins (including RuBisCO) from the green biomass were extracted in a similar manner. However, the fact that protein fractions from green biomass contain a mixture of proteins seems not to be negative for the functional properties of these fractions.[17] Amino acids were the major *N* component in all the fractions. The fact that some of the proteins are degraded or oxidized by the fractionation process was verified here. Thus, this risk has to be taken into account and the methodology developed to minimize degradation in pilot/industrial settings for protein fractionation of green biomass. Also, the fractionation of the proteins had an impact on the solubility of the proteins, with decreased solubility in the protein-rich fractions, a measure that needs to be taken into account in feasibility studies. In the concept of using the fractions for food and feed, the presence of antinutritional components has to be investigated. The content of nitrate was found here to vary highly between the biomass source and fraction, with the highest content in hemp and juice fractions. The high content of nitrate has a significantly negative impact on the usefulness of the biomass/fraction as a food/feed. Furthermore, additional utilization areas of the brown juice are a necessity for the feasibility of the whole fractionation process of green biomass. The *P*, GP, and GJ/GP fractions are good sources of protein for feed to ruminants, pigs, and chickens, respectively, apart from some biomass sources high in nitrate, e.g., hemp. Thus, the use of green biomass to produce protein to feed animals does not only offer a climate-friendly option,[7] but has also the potential to provide nutrition to animals. Both the GP and WP were found as good sources of protein for human food, independent of the biomass source. However, some of the fractions from some of the biomasses were found with Met-Cys as the limiting AAs. Thus, proteins from green biomass should on some occasions be complemented with proteins from cereals, known to be rich in Met-Cys, and also have the ability to cross-link and build structures.[43]

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsfoodscitech.3c00426.

> Water content for all fractions (with biomass sources combined); representative chromatograms for all fractions from all evaluated biomass sources, with integration lines added for the RuBisCO elements (PDF)

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■ ABBREVIATIONS

BM	unfractionated biomass
P	pulp
GJ	green juice
GP	green protein
WJ	white juice
WP	white protein
BJ	brown juice
AA	amino acid
N	nitrogen

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Green leaves represent an underutilised but abundant biomass source for extracting proteins, phenolic compounds (flavonoids and phenolic acids) and dietary fibre. This thesis used a biorefinery process, yielding fractions suitable as either food (WP), feed (GP and WP) or biogas production (P and BJ). Findings demonstrated that both harvest time and fertilisation influenced the protein extractability. The systematic utilisation of green leafy biomass through biorefinery approaches offers promising potential as a sustainable supplement to global food production systems.

Emilia Berndtsson received her graduate education at the department of Plant Breeding, and her MSc degree in Upper Secondary Education, with specialisation in Biology and Science studies, from Lund University and Kristianstad University, Sweden.

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