Extraction of plant protein from green leaves: Biomass composition and processing considerations

Sara Pérez-Vila[1, 2], Mark A. Fenelon[1, 2], James A. O'Mahony[2], Laura G. Gómez-Mascaraque[1*]

[1]Teagasc Food Research Centre, Moorepark, Fermoy, Co. Cork, Ireland

[2]School of Food and Nutritional Sciences, University College Cork, Cork, Ireland

*Corresponding author: Tel.: +353 76 111 22 41

E-mail address: laura.mascaraque@teagasc.ie (L. G. Gómez-Mascaraque)

ABSTRACT

There is an increasing need to explore alternative sources of proteins for food applications. Green leaves contain high levels of the enzyme RuBisCO, representing a source of protein with good functional and nutritional properties. However, the optimal conditions for extraction and purification of RuBisCO at a large scale have not yet been defined. This review discusses the main factors affecting the extraction of proteins from green leaves, from plant composition in terms of protein content and other compounds that affect the yield and quality of extractable protein, to the essential steps and challenges faced during extraction and purification, including considerations for achieving food-grade ingredient status. There are some key factors to consider when developing a protein concentrate for human consumption. The first step is the selection of an optimal raw material; plant tissues are complex matrices that require thorough characterization, including non-protein nitrogen and other undesirable compounds. The effect of the extraction and purification process on functionality, oxidation and proteolytic stability should also be considered. Moreover, the appropriate removal of undesired compounds must be considered to obtain plant protein concentrates suitable for food products.

KEYWORDS

Forage, leaf protein, green protein, RuBisCO, sustainability, human nutrition

1 Introduction

The continuous growth in global population, which is expected to reach 10 billion people by 2050, is contributing to the increased demand for proteins as essential nutrients (Nadathur, Wanasundara, & Scanlin, 2017). Additionally, more consumers are shifting to flexitarian, vegetarian or vegan diets (Pojić, Mišan, & Tiwari, 2018) in recent years, in some cases motivated by ethical and environmental concerns related to meat production. There is also an increased demand for local products with reduced waste associated with their production (Prade, et al., 2021). To address these changes and consumer preferences, there is a need for new sources of proteins (Di Stefano, Agyei, Njoku, & Udenigwe, 2018), to achieve a more sustainable healthy diet (Day, 2013).

Plants have been proposed as one of the preferred alternative sources of proteins. The conversion of plant to animal proteins is intrinsically inefficient, so it has been suggested that using plants as protein sources for human consumption can reduce environmental impact and water requirements compared to meat production (Aiking, 2011; Day, 2013; Dijkstra, Linnemann, & van Boekel, 2003).

Among plant proteins, soy protein has traditionally been the most commonly used of the plant proteins to replace protein in meat and dairy products; however, it is mainly grown outside Europe (Nynäs, 2018; Stødkilde, Damborg, Jørgensen, Lærke, & Jensen, 2019). Considering that transportation over long distances can have the same environmental impact as meat production (Jungbluth, Tietje, & Scholz, 2000; Santamaría-Fernández & Lübeck, 2020), an endogenous plant-based crop would be desirable (Santamaría-Fernández, et al., 2017).

As early as 1942, green leaves were proposed as a source of protein for feeding the population during World War II (Pirie, 1942). RuBisCO (ribulose-1,5-bisphosphate

carboxylase/oxygenase) is the main protein in green leaves and is the most abundant protein in nature (Ellis, 1979). It is a photosynthetic enzyme common in most autotrophic organisms (Andersson & Backlund, 2008). RuBisCO has been reported to possess a good amino acid profile, ranking better than other plant proteins. In particular, it has higher amounts of sulfur-containing amino acids, which are usually deficient in other plant proteins, such as soy protein (Chiesa & Gnansounou, 2011; Fiorentini & Galoppini, 1983; Hermansen, et al., 2017). Moreover, while one of the limitations of most plant proteins is their low solubility and, thus, their poor techno-functional properties, RuBisCO has been reported to exhibit good functional properties in comparison with other established proteins, such as soy or whey protein (Barbeau & Kinsella, 1988; Martin, Castellani, de Jong, Bovetto, & Schmitt, 2019).

Bearing this in mind, green leaves are a promising protein source for food applications with the potential for improving the long-term sustainability of the global food production system. Wildman and Bonner (1947) reported, for the first time, the extraction of soluble proteins from spinach leaves, and several researchers have studied it since (Barbeau, et al., 1988; Hojilla-Evangelista, Selling, Hatfield, & Digman, 2016; Martin, et al., 2019; Martin, Nieuwland, & de Jong, 2014; Tenorio, Gieteling, de Jong, Boom, & van der Goot, 2016). However, plant tissues are complex matrices, so several factors determine their composition and protein extractability (Hermansen, et al., 2017).

On the other hand, the incorporation of soluble leaf proteins in food products requires that their functional properties are preserved during extraction and processing to prevent denaturation (Barbeau, et al., 1988; Jiménez-Munoz, Tavares, & Corredig, 2021). In addition, food manufacturers need to understand the impact that other plant components, such as antinutritional compounds or pigments, have on the nutritional value of proteins and overall consumer acceptability. These are some of the reasons why leaf proteins are

still underutilized in the food industry (Di Stefano, et al., 2018), due to both the challenges faced during extraction and the economic cost associated with enrichment (Day, 2013). However, although a number of reviews have been published recently on the extraction and functionality of other types of plant proteins, a comprehensive review on the parameters affecting leaf protein extractability and quality has not yet been performed.

This review aims to examine the different factors that affect biomass composition in leaves and critically discuss the importance of the challenges faced during extraction and purification of protein from green leaves to facilitate more targeted scientific and technical advancement in the field.

2 Biomass source and composition

Several factors determine the composition of plants and, therefore, the amount and quality of extractable protein from their leaves. These have to be considered when selecting plant material for protein isolation in a large-scale application (Nielsen, Stødkilde, Jørgensen, & Lærke, 2021; Pirie, 1966; Stødkilde, Lashkari, Eriksen, & Jensen, 2021). The main factors that affect the protein composition of plants include species, growing conditions, and stage of growth.

Plant species are classified as C3, C4 or CAM depending on the carbon fixation pathway. RuBisCO from C3 plants has the lowest carbon fixation efficiency, and to compensate, these plants possess higher levels of the enzyme in their leaves (Ku, Schmitt, & Edwards, 1979). These species are best adapted to cold climates and are more abundant in northern latitudes and higher elevations (Barbehenn, Chen, Karowe, & Spickard, 2004). They include forage species such as alfalfa, perennial ryegrass, barley, sugar beets, soybean, spinach and tobacco. In alfalfa, for instance, RuBisCO constitutes about 25% of the total

leaf protein (Barbeau, et al., 1988) and 65% of the soluble leaf protein fraction (de Jong & Nieuwland, 2011; Ellis, 1979; Huffaker & Miller, 1978; Kobbi, et al., 2017). Apart from the content of soluble protein in the leaves, other compositional factors affect the amount of protein that can be extracted from the plant, with significant interspecies variation; for instance, the presence of polyphenols, which can lead to oxidation reactions, or fibre, which determines plant toughness, also influence suitability of plants as a source of protein (Hermansen, et al., 2017; Pirie, 1966). These factors are addressed in greater detail in Section 4.

Agronomic factors are also important in influencing plant composition; during a plant's growth, environmental conditions, especially light intensity and nitrogen availability, determine protein concentration in the leaf. Long periods of darkness induce plant senescence, which correlates with a reduction in soluble nitrogen content. This nitrogen can be fractionated into: thylakoids, which increase at low radiance conditions; soluble proteins (RuBisCO mainly), which reach a peak at high irradiance; and nucleic acids and other compounds whose proportions remain largely unchanged by light exposure (Evans & Seemann, 1989). The timing and rate of nitrogen fertilization also affect protein concentration in leaves. An early supply of nitrogen increases the concentration of RuBisCO to a greater extent than a late application (Barbeau, et al., 1988). Nevertheless, the application of ammonia or nitrate fertilization can decrease the proportion of true/crude protein due to an increase in assimilation products (Mangan, 1982). To fully understand the impact of the different variables, more field work is required to set up a farming system for controlled, steady crop production for leaf protein extraction (Nielsen, et al., 2021), as long suggested by Pirie (1966).

The age of the plant is also a critical factor in influencing composition. The soluble protein content in green leaves varies throughout the plant's life (Huffaker, et al., 1978;

Stødkilde, et al., 2021). Over the maturation process, grasses increase the proportion of stem, and consequently, the lignified tissue in the plant, lowering the availability of nutrients (Stone, 1994). The protein content decreases from about 19 to 10% of dry weight as it matures, but this process is typically delayed by repeated cutting, as the regrowth proportion of the plant maintains a reasonably constant composition (Mangan, 1982). Generally, the content of soluble protein reaches its highest when the leaves are fully expanded; after that stage, the content of RuBisCO decreases to its lowest levels during senescence (Barbeau, et al., 1988).

Seasonality also has an impact on the composition of plants. Previous work has shown a decline in the content of protein in plants across the spring (Solati, Jørgensen, Eriksen, & Søegaard, 2017a; Stødkilde, et al., 2021). The ability to attain maximum soluble protein content is a function of harvesting time and plant type (e.g., the optimum stage for harvesting alfalfa is pre-flowering (Fiorentini, et al., 1983; Muneer, et al., 2021), and will determine protein yield during production (de Jong, et al., 2011; Solati, et al., 2017a; Solati, Jørgensen, Eriksen, & Søegaard, 2017b; Stødkilde, et al., 2021).

Due to the numerous factors affecting protein content in leafy biomass, the range of protein values reported in the literature for different plants varies considerably and is dependent on the growing conditions of each study (Table 1) (Kiskini, Vissers, Vincken, Gruppen, & Wierenga, 2016; Muneer, et al., 2021). These studies estimate the protein content at different stages of the process, referring to different protein fractions (assessed in the section 2.1). Furthermore, even within the same study, variations were observed when the growing conditions or the maturation state of the plant changed, highlighting the importance of reporting the plant species and the detail of their growing conditions and state of growth to allow accurate and reproducible comparisons between studies.

2.1 Protein and other nitrogen-containing compounds in leaves

The nitrogen content of leaves is classified according to different criteria in the literature, as summarized in Figure 1. Previous research has assessed protein quality according to its availability for animals using the Cornell Net Carbohydrate and Protein System (CNCPS) (Licitra, Hernandez, & Van Soest, 1996; Solati, et al., 2017a, 2017b). CNCPS classifies nitrogen or crude protein components based on their solubility (Figure 1a). According to this system leaf nitrogen is classified into: fraction A, which includes the non-protein nitrogen (discussed in detail in Section 2.1.3); fraction B, which is described as the true protein fraction and is further divided into three sub-fractions, based on decreasing solubility, B1, B2 and B3; and fraction C, which is the unavailable protein because of being bound to other cell structures. Therefore, the soluble fractions that are directly extractable are A, B1 and B2, but just B1 and B2 are defined as extractable true protein (Licitra, et al., 1996; Solati, et al., 2017a, 2017b).

Other authors have also classified the nitrogen based on role in cell metabolism (Figure 1b). Nitrogen can be divided into the fraction involved in photosynthesis (about 50%; and therefore is associated with the chloroplasts), the fraction that is involved in the rest of the metabolism pathways (about 30%), and other forms of nitrogen, which are mainly structural (20%) (Houles, Guerif, & Mary, 2007). At the same time, the leaf nitrogen involved in photosynthesis can be sub-divided into thylakoid proteins, cytoplasmic proteins and other nitrogen compounds (Figure 1c). Thylakoid proteins, also known as green proteins, originate in the chloroplasts and are considered integral membrane proteins that carry out light-dependent reactions of photosynthesis (Xie, 2017). They are associated with chlorophyll, carotenoids and lipids (Chiesa, et al., 2011), having a dark

green colour and grassy flavour (Fiorentini, et al., 1983). The cytoplasmic proteins, also called white proteins, include mainly RuBisCO, together with a small fraction of other chloroplast enzymes (Evans, et al., 1989). These are stable soluble proteins, odourless, tasteless and with a clear colour (Fiorentini, et al., 1983).

2.1.1 RuBisCO

Research on leaf protein extraction is mainly focussed on soluble proteins because they constitute the major fraction of the total protein in leaves (Dotsenko & Lange, 2017), are easy to extract, and are considered to have excellent nutritional value (Barbeau, et al., 1988). In addition, due to their solubility, they exhibit enhanced techno-functional properties compared to insoluble proteins. Among soluble proteins, RuBisCO is the most abundant and widely studied.

RuBisCO, or ribulose biphosphate carboxylase/oxygenase, is a photosynthetic enzyme that catalyzes the first step of carbon-fixation (Barbeau, et al., 1988). Due to its inefficient activity, plants accumulate large amounts of RuBisCO in their leaves (Di Stefano, et al., 2018; Ku, et al., 1979), constituting about 50% of the soluble protein under optimal conditions (Huffaker, et al., 1978), with levels varying depending on a number of factors that determine the composition of the plant, as discussed in Section 2. In addition, its presence in most autotrophic organisms makes RuBisCO a highly abundant protein on Earth (Andersson, et al., 2008; Ellis, 1979).

RuBisCO is present in nature in four different forms (Di Stefano, et al., 2018), all of which are multimeric with a combination of large and small subunits. The most common form is Form I (Andersson, et al., 2008), which has a molecular weight of about 560 kDa, and is composed of eight large and eight small subunits with approximate molecular

weights of 56 and 16 kDa, respectively (Mangan, 1982). In its hydrated state, it is a spherical molecule with a three-dimensional structure typical of globular proteins (Barbeau, et al., 1988). The amino acid sequence of RuBisCO is very similar in all plants (Table 2) (Fiorentini, et al., 1983; Hermansen, et al., 2017; Stødkilde, et al., 2019). Indeed, proteins extracted from green leaves have minimal variations in amino acid composition, even for different harvesting times and fertilizer conditions (Gerloff, Lima, & Stahmann, 1965). The main variations between species are related to the small subunit, while the large subunit has almost the same amino acid composition in all plants (Barbeau, et al., 1988; Mangan, 1982).

RuBisCO meets the FAO/WHO requirements for essential amino acids (Chiesa, et al., 2011; de Jong, et al., 2011; Di Stefano, et al., 2018; Hermansen, et al., 2017). Furthermore, some studies have highlighted its potential as a source of bioactive compounds (Di Stefano, et al., 2018; Udenigwe, et al., 2017). RuBisCO also exhibits desirable techno-functional properties (Section 4.4), making it suitable for a range of food applications (Kobbi, et al., 2017). However, its functional properties are highly dependent on the extraction process, which determines the extent of denaturation, solubility and composition of the isolated proteins (Di Stefano, et al., 2018; Lamsal, Koegel, & Gunasekaran, 2007; Martin, et al., 2014). Overall, RuBisCO is very promising as a valuable source of protein for food product development. However, more research work is needed to develop extraction processes suitable for scale-up (Di Stefano, et al., 2018).

2.1.2 Other leaf proteins

Polyphenol oxidases (PPO) are a group of enzymes widely distributed in vascular plants. They are responsible for the so-called browning process that occurs when plant tissue is

disrupted, as they catalyze the first reaction of this process, in which polyphenols are oxidized to quinones. These quinones have electrophilic sites that react with other compounds such as proteins (Jones, Hatfield, & Muck, 1995). Previous studies have compared the PPO activity of different crops, showing different rates of activity depending on the species (Lee, Olmos Colmenero, Winters, Scollan, & Minchin, 2006; Winters, Minchin, Merry, & Morris, 2003), as well as other factors including pH, temperature, oxygen availability and concentration of active PPO and phenolic compounds (Parveen, Threadgill, Moorby, & Winters, 2010). Browning reactions may compromise protein extraction, functionality and nutritional value, having a considerable impact on protein quality (Amer, Juul, Møller, Møller, & Dalsgaard, 2021; Di Stefano, et al., 2018), and this phenomenon is addressed in more detail in Section 4.1.

Mitochondrial enzymes constitute less than 5% of total leaf protein. These enzymes take part in the tricarboxylic acid cycle (Mangan, 1982). Various other enzymes are also present, constituting up to 25% of leaf protein in a very complex mixture (Mangan, 1982). Notably, some of them have proteolytic activity with adverse effects on the stability of other proteins, including RuBisCO (Koschuh, et al., 2004), and this is described in more detail in Section 4.1.

Regarding insoluble proteins, extensine is the main protein present in plant cell walls. It is a glycoprotein with a molecular weight of about 230 kDa with a high content of hydroxyproline, lysine and serine; this protein is tightly bound to cellulose, and, thus, it is classified as an unavailable protein (Mangan, 1982). It constitutes around 10% of total nitrogen in some forages, such as perennial ryegrass. However, there is little information about the seasonal changes in this fraction (Hoekstra, Schulte, Struik, & Lantinga, 2007).

The free amino acid content of green leaves represents about 15-20% of the soluble nitrogen. But it varies considerably with the species, stage of maturity, fertilizer treatments or environmental conditions such as light or temperature (Mangan, 1982). This fraction may also increase during the process of extraction, as some protein hydrolysis takes place (Koschuh, et al., 2004).

2.1.3 Non-Protein nitrogen

The non-protein nitrogen (NPN) fraction is well-defined as the difference between the crude protein nitrogen and true protein nitrogen. Its accurate quantification depends on the precipitation of true protein by a suitable precipitant. Several precipitants can be used for this purpose, with tungstic acid and trichloroacetic acid the most utilized for analysis of feed and food (Licitra, et al., 1996; Solati, et al., 2017a).

This fraction is composed of different nitrogen-containing compounds, which are not clearly defined, with the most important being nitrate. Forage plants reduce nitrate to ammonia during its assimilation; however, nitrate accumulates in the plant when the rate of uptake is greater than the rate of assimilation (i.e., under conditions of high temperature and low light). Therefore, nitrate and ammonia concentrations can fluctuate greatly during the growing season (Mangan, 1982).

Another type of nitrogen-containing compound present in all live cells is nucleic acids. In 1982, a report by Mangan raised awareness of the lack of scientific data on the amount of nitrogen from plant tissue that corresponds to nucleic acids (Mangan, 1982). More scientific investigation is required to understand the concentration and profile of the NPN fraction in more detail.

Chlorophyll is a green pigment present in plant cells, and it has an essential role in photosynthesis. Given that the chlorophyll molecule contains four nitrogen atoms (Figure 2), the authors consider that it should also be included in the NPN fraction. As chlorophyll is released during the extraction process, it is discussed in greater detail in Section 2.4.

It is worth noting that in previous studies on the extraction of soluble proteins from green leaves, protein content is estimated by measuring total nitrogen content (Amer, et al., 2021; Hernandez, Martinez, & Alzueta, 1989; Kobbi, et al., 2017; Stødkilde, et al., 2021; Tenorio, et al., 2016). However, as discussed, the information available on the NPN fraction in leaves is limited and can vary widely (Prade, et al., 2021; Walgenbach, Marten, & Blake, 1981). This suggests that the true protein content may have been over-estimated in previous reports and highlights the importance of explicitly determining the NPN fraction in addition to the total nitrogen content.

2.2 Other macronutrients

Plant leaves do not tend to accumulate lipids, as their metabolism mainly focuses on the synthesis of carbohydrates (Chapman, Dyer, & Mullen, 2013), with their low lipid content concentrated in the chloroplast structures, such as pigments and lipid membranes. Therefore, when green leaves are processed, the lipids are found mainly in the green fraction (Tenorio, et al., 2016) together with the insoluble proteins (refer to Section 3.2). The vegetation state is one of the most critical factors affecting fat content and fatty acid composition. In particular, α-linolenic acid, the main component of the lipid fraction, decreases during senescence (Glasser, Doreau, Maxin, & Baumont, 2013), when less membrane lipids are produced in favour of fibre and the storage fraction for the development of the grain. However, the fat content does not show major differences

between species (Glasser, et al., 2013). If part of the lipid fraction is present within the final protein extracts, it can increase oxidative instability and the nutritional value of the final product (Dijkstra, et al., 2003), highlighting the importance of α-linolenic acid as an essential fatty acid.

Leaf carbohydrates can be broadly classified into soluble and insoluble, with the latter being separated with the pulp in the protein extraction process (Section 3). This fibre fraction comprises plant cell walls containing cellulose, hemicellulose and lignin (Dotsenko, et al., 2017), making it poorly digestible by monogastric mammals. However, the fibre can be used as feed for ruminant animals (Tenorio, Kyriakopoulou, Suarez-Garcia, van den Berg, & van der Goot, 2018). Regarding the soluble carbohydrates, their content in the leaves varies from less than 5% to almost 40% (McGrath, 1988). Leaves reduce carbon to synthetize sucrose, which is utilized in plant metabolism, or stored as starch (Chapman, et al., 2013). In general, the content of carbohydrates is negatively correlated with nitrogen concentration (Humphreys, 1989); therefore, the optimal forage material for protein extraction would have a low carbohydrate content.

2.3 Micronutrients

Phenolic compounds are the main secondary metabolites in vascular plants, being a large diverse category that encompasses over eight thousand different compounds, such as phenols, tannins, flavonoids and lignin. The escort micronutrient profile varies with species, tissue and developmental stage of the plant and are generally more abundant in adult green tissues (Wang, Tai, & Chen, 2008). Phenolic compounds are controversial constituents because they have been attributed to several health benefits, linked to their antioxidant properties, reducing oxidative stress related to cellular damage (Durairaj,

Hoda, Shakya, Babu, & Rajagopalan, 2014). However, some limitations include decreased digestibility and palatability (Rathore, 2010), representing antinutritional factors at high concentrations (Makkar, 1993) (Section 4.2). Moreover, polyphenols participate in the previously mentioned browning reactions, being oxidized to quinones by PPO (Jones, et al., 1995), with quinones then reacting with proteins, compromising their extractability, functionality and nutritional value (Amer, et al., 2021; Di Stefano, et al., 2018; Martin, et al., 2014) (Section 4.1).

The mineral content of plant leaves varies as a function of the exposure of the plant to micronutrients from the soil, and the availability of these micronutrients is conditioned by environmental factors such as temperature, moisture and pH (Fageria, Baligar, & Clark, 2002; Lindström, Frankow-Lindberg, Dahlin, Wivstad, & Watson, 2013). It also depends on plant species, with greater differences between species than between general groups such as grasses, legumes or forbs (Lindström, et al., 2013; Stone, 1994). Although the mineral content in plants is difficult to measure, the presence of the potentially toxic heavy metals, cadmium and lead in green leaves is lower than the European Commission's limits (Fageria, et al., 2002; Lindström, et al., 2013). Furthermore, minerals present in plants often have low bioavailability, owing to antinutritional factors such as oxalic acid or phytic acid, which interferes with the utilization of some nutrients like iron, zinc, magnesium or calcium (Natesh, Abbey, & Asiedu, 2017; Popova & Mihaylova, 2019).

Vitamins are organic molecules that are essential for the normal functioning and metabolism of live cells. Since humans cannot synthetize many of them in the required quantities, most vitamins must be obtained through the diet. The levels of vitamins in forages are highly variable, making it difficult to give a precise estimation (Ballet, Robert, & Williams, 2000). Vitamin A and E are fat-soluble compounds, which can be present in the diet as precursors in the form of carotenoids and tocopherols. Although forages appear

to be a good source of those compounds, they are generally overestimated by analytical methods, and there is a lack of data about their bioavailability (Ballet, et al., 2000). Leaves also have high levels of vitamin C; however, it is easily oxidized during the extraction process with associated loss of bioactivity (Pirie, 1942). On the other hand, plants, especially green leaves, have significant levels of folic acid (which belongs to the vitamin B group) and are thus a primary source thereof for humans (Natesh, et al., 2017). They are also considered a good source of vitamin K (Pirie, 1942), meeting the recommended dietary allowance (RDA), albeit more information about lability during the extraction process and subsequent bioavailability on ingestion are required (Ferland & Sadowski, 1992).

2.4 Chlorophyll

Chlorophyll is a green pigment (representative of all vegetal pigments) located in the chloroplast, where it takes part in photosynthesis (Tenorio, Boom, & van der Goot, 2017). Chlorophyll affects the efficiency of photosynthesis, and thus, cell metabolism. Originally, quantification of chlorophyll was used as a rapid means of estimating nitrogen fertilization requirements (Houles, et al., 2007), since its content is associated with nitrogen status (Xie, 2017) and soluble protein content (Kupke, 1962).

Some studies aimed at extraction of leaf protein focussed on obtaining white protein fractions by removing chlorophyll (Jwanny, Montanari, & Fantozzi, 1993; Martin, et al., 2019; Tenorio, et al., 2016), on the basis that the green colour may result in lower consumer acceptance of food products (Merodio, Martin, & Sabater, 1983). However, recent studies describe a positive relationship between green colour and consumer perception of a natural product, which is considered a symbol of health (Plasek, Lakner,

& Temesi, 2020; Wąsowicz, Styśko-Kunkowska, & Grunert, 2015). Pirie (1966) also showed that consumers accepted the novel appearance of products containing green leaf protein extracts only after some weeks, making the decolourization process unnecessary for certain applications of the final product. For example, products such as the "Organic Supergreens blend" and "Organic wheatgrass powder" produced by *Alesto* and commercialized by Lidl U.K. are green powders sold as food ingredients to prepare beverages for human consumption and are labelled as rich in fibre, protein and iron, with protein content of 13-40%, respectively.

In fact, there is an increasing commercial demand to substitute artificial additives with natural compounds. Therefore, chlorophyll and its derivative compounds have a wide range of technological applications as food colorants, which are strictly regulated (Fernandes, Pinheiro Nass, Oliveira, & Queiroz Zepka, 2020). Nevertheless, during the extraction process, special care must be taken to avoid its transformation to compounds with less attractive colour. Moreover, the potential applications of chlorophyll as a food additive are not just limited to its colour properties. Some studies suggest chlorophyll and derivate compounds may provide health benefits, including reducing the genotoxic effects of several known toxicants and other biological functions such as antioxidant or anti-inflammatory properties (Fernandes, et al., 2020; Queiroz Zepka, Jacob-Lopes, & Roca, 2019).

3 Protein extraction from green leaves

There are limitations to using some green leaves in their native form as sources of proteins to be introduced directly in the human diet, since they contain non-digestible fibre and other undesirable compounds (Rathore, 2010; Stødkilde, et al., 2019; Xie, 2017). Hence,

extraction and purification processes are necessary to produce food-grade proteins to develop structured food systems or as ingredients incorporated within protein-enriched food products. Moreover, the protein extraction process generates coproducts, which can be utilized to obtain high-value materials in the rapidly expanding bio-economy sector, such as biogas or roughage (Fog, Ytting, & Lübeck, 2017).

Green leaves are primarily produced for animal feed, and the extraction and purification of leaf protein for food applications would provide important opportunities for adding value (de Jong, et al., 2011). The most extensively investigated leaves for protein extraction are alfalfa (Colas, Doumeng, Pontalier, & Rigal, 2013; Fiorentini, et al., 1983; Hojilla-Evangelista, et al., 2016; Knuckles & Kohler, 1982; Kobbi, et al., 2017; Lamsal & Koegel, 2005; Lamsal, et al., 2007; Nynäs, 2018; Nynäs, Newson, & Johansson, 2021; Santamaría-Fernández, et al., 2017; Walgenbach, et al., 1981), spinach (Martin, et al., 2014; Nynäs, et al., 2021), tobacco (Sheen & Sheen, 1985), sugar beet (Jwanny, et al., 1993; Kiskini, et al., 2016; Martin, et al., 2019; Nynäs, et al., 2021; Tenorio, et al., 2017; Tenorio, et al., 2016), broccoli (Nynäs, et al., 2021; Prade, et al., 2021), kale (Nynäs, et al., 2021; Prade, et al., 2021) and grasses (Amer, et al., 2021; Bals, Dale, & Balan, 2012; Bray & Humphries, 1979; Damborg, Jensen, Weisbjerg, Adamsen, & Stødkilde, 2020; Fog, et al., 2017; Gerloff, et al., 1965; Kammes, Bals, Dale, & Allen, 2011; Koschuh, et al., 2004; Nielsen, et al., 2021; Stødkilde, et al., 2019; Stødkilde, Damborg, Jørgensen, Laerke, & Jensen, 2018).

Different methods have been developed for the extraction of soluble protein from green leaves (Hernandez, et al., 1989; Jwanny, et al., 1993; Martin, et al., 2019; Martin, et al., 2014; Stødkilde, et al., 2018; Tenorio, et al., 2017), and some basic steps are common in all of them (Figure 3): plant tissue disruption to separate the leaf juice from the fibrous pulp, separation of the soluble proteins from unwanted compounds, and protein

concentration or purification. Each of these methods are explained in detail below and should be tailored depending on the particular properties (including biochemistry) of the raw material and the intended application for the final product (Dijkstra, et al., 2003; Stødkilde, et al., 2021). Indeed, depending on the intended application requirements, the extraction process may need to be adapted to ensure the obtained protein extract has the desired techno-functional properties, appropriate sensorial characteristics, and optimal nutritional value.

3.1 Plant tissue disruption

The first step in extracting protein from green leaves is the disruption of the plant cell walls to release intracellular content, where the water-soluble proteins are located. An efficient method for this is screw pressing (Nynäs, 2018; Stødkilde, et al., 2018), resulting in a green juice containing the cytosol and a press-cake consisting of a fibrous matrix (Dijkstra, et al., 2003). The juice obtained has a lower percentage of dry matter and total dietary fibre than the leaves, with higher proportions of protein and ash on a dry basis (Tenorio, et al., 2016). Some studies have calculated the degree of cell disruption, defined by the ratio of protein nitrogen in the juice to protein nitrogen in the unground material (Bals, et al., 2012), to estimate the effectiveness of the pulping process. This ratio reflects protein partitioning between the two fractions in this first step. Different strategies can be applied during the cell disruption step to increase protein recovery, with the principal strategies discussed below.

The pH of the system is one of the most significant factors that determine protein extractability. The isoelectric point of RuBisCO, where it does not have a net charge and is, therefore, less soluble, is between 4.4 and 5.5, with the exact value being species-

specific (Barbeau, et al., 1988; Martin, et al., 2019). This agrees with studies on the solubility of RuBisCO, which is minimum around that pH range (Hojilla-Evangelista, et al., 2016; Kobbi, et al., 2017; Lamsal, et al., 2007; Van de Velde, Alting, & Pouvreau, 2011). Hence, increasing the pH to values in the range 8 to 10.5 generally increases the amount of protein extracted (Bals, Teachworth, Dale, & Balan, 2007). Moreover, raising the pH enhances chloroplast disruption (Nynäs, 2018). However, it may also increase the solubility of other compounds, such as polyphenols, potentially leading to increased oxidation (Section 4.1). These implications of pH should also be considered when adding other compounds to the system. For instance, the addition of certain sulphur compounds, such as sodium metabisulphite ($Na_2S_2O_5$), to prevent oxidative reactions (refer to Section 4.1), might result in reduced protein extractability, as it decreases the pH. On the other hand, the addition of sodium sulphite (Na_2SO_3) increases the pH and results in higher protein recovery (Amer, et al., 2021).

Enzymatic treatments have also been proposed to enhance protein yield. For example, carbohydrate degrading enzymes weaken plant cell walls, facilitating separation of the cytosol, containing the soluble proteins (Tenorio, et al., 2018). However, the activity of carbohydrases is limited to acidic pH, at which the solubility of the leaf proteins is low. It explains the small effect of these enzymatic treatments on protein recovery observed by Sari, Mulder, Sanders, and Bruins (2015). Alternatively, proteases have also been suggested to improve protein extraction by reducing protein size, facilitating the extraction of additional proteins from the cell walls (Pojić, et al., 2018; Sari, et al., 2015). Nevertheless, hydrolyzing proteins can impact techno-functional properties, and this should be considered. Moreover, the use of enzymes to assist with protein extraction requires careful adjustment of the parameters¸ involving time-consuming procedures, high energy consumption, all adding cost (Pojić, et al., 2018).

Another way to promote cell disruption is the application of pre-treatments such as microwave or ultra-sonication. Microwave radiates electromagnetic waves of frequency between 300 MHz and 300 GHz. This energy disrupts hydrogen bonds, increasing porosity of the biological matrix, resulting in greater extractability of compounds, but may also affect the secondary structure of the proteins and, thus, their functional properties (Contreras, et al., 2019; Pojić, et al., 2018). The application of microwave technology has been shown to increase the extraction yield for other sources of plant biomass, such as soybean (Choi, Choi, Chun, & Moon, 2006). However, no data is available on the effect this pre-treatment technique may have on protein extraction yield from leafy biomass.

Ultra-sonication propagates ultrasound pressure waves, causing cavitation. It generates macro-turbulence, i.e., inter-particular collisions at high velocity, which accelerates diffusion and exposes new surfaces that increase mass transfer. Xu, et al. (2017) reported an increase in cell disruption of homogenized cauliflower leaf materials by ultra-sonication, leading to increased protein recovery_ENREF_94. Pre-treatment of the leaves with harsh conditions, such as high temperatures or extreme pH conditions, has also been applied to extract proteins for animal feeding. However, these treatments affect the functional and nutritional quality of the extracted protein, limiting their use for food-grade applications (Chiesa, et al., 2011).

3.2 Separation of chlorophyll and insoluble proteins (green proteins)

The green juice obtained by pressing leaves is rich in plant cell components such as proteins, chlorophyll, membrane fragments and other undesirable compounds (Nynäs, 2018). The extracted protein consists of two different fractions, as discussed in Section

2.1: cytoplasmic or soluble proteins and thylakoid or insoluble proteins (Fiorentini, et al., 1983). Thus, it is a complex mixture that requires further processing to obtain a protein concentrate free of undesirable compounds (Hernandez, et al., 1989; Tenorio, et al., 2016).

The different physicochemical properties of both types of protein can be exploited to fractionate them (Fiorentini, et al., 1983). Furthermore, ultracentrifugation can separate the insoluble proteins together with the chlorophyll and other debris components from the soluble fraction (Amer, et al., 2021), although this process is non-scalable (Merodio, et al., 1983). Therefore, a heating step is commonly applied to achieve aggregation of these insoluble proteins, facilitating their removal using standard centrifugation (de Jong, et al., 2011). However, to preserve protein functionality and minimize co-precipitation of the soluble proteins, high temperatures of 80-82 ℃ should be avoided (Di Stefano, et al., 2018; Tenorio, et al., 2016). Previous studies commonly used temperatures of 50-60 ℃ (Chiesa, et al., 2011; Dijkstra, et al., 2003; Knuckles, et al., 1982; Lamsal, et al., 2005; Nynäs, 2018; Udenigwe, et al., 2017), followed by a centrifugation step to obtain a green protein fraction (insoluble protein and cell debris containing chlorophyll) in the pellet, and a white protein fraction (soluble proteins) in the supernatant. However, in practice, both cytoplasmic and chloroplast proteins can be found in both fractions, as heat precipitation is not highly selective (Chiesa, et al., 2011; Merodio, et al., 1983; Tenorio, et al., 2016).

Tenorio, et al. (2016) analyzed the presence of RuBisCO subunits after the fractionation of the green and white protein fractions and found that 70% of the proteins in the green fraction were soluble proteins. Moreover, when attempting to re-suspend the soluble proteins from the green pellet, just a small proportion of those soluble proteins could be recovered due to their strong interactions with other molecules that rendered them

insoluble. Considering this, although the white protein fraction, composed mainly of soluble proteins, has higher nutritional and functional value, the green fraction is also a source of protein worth valorizing. Incomplete valorization of the plant biomass could be a limiting factor for economic viability (Tenorio, et al., 2016), in addition to negatively impacting on overall environmental and sustainability indices.

Other methods have been proposed as alternatives to completely remove the chlorophyll and obtain a decoloured juice, adding activated carbon in one such approach. The activated carbon absorbs the chlorophyll, and then it can be separated by any solid-liquid separation technique leaving a dechlorophyllized juice (Van de Velde, et al., 2011). The addition of flocculants has also been investigated for this purpose; for instance, Superfloc A150 (which is approved for some food uses by the US Food and Drug Administration) has been used in previous studies (Bray, et al., 1979; Fiorentini, et al., 1983).

3.3 Concentration and purification of soluble proteins (white proteins)

After removing the green protein fraction, juice containing soluble proteins needs to be concentrated and may require an additional purification step to remove undesired compounds. Those compounds, such as thylakoid fragments, phenols and off-flavours, can affect the techno-functional and sensorial properties of the final product (Di Stefano, et al., 2018; Nynäs, 2018). Moreover, as discussed in Section 2.3, the presence of polyphenols and polyphenol oxidase in that fraction triggers browning reactions that can compromise the functionality and nutritional value of the proteins (D'Alvise, Lesueur-Lambert, Fertin, Dhulster, & Guillochon, 2000; Di Stefano, et al., 2018; Martin, et al., 2014). Concentration and purification steps can remove these undesirable compounds, improving the stability of the protein while increasing its concentration (Xie, 2017). The

concentration and purification method must be chosen carefully, given that it can impact the functional properties of the proteins obtained (de Jong, et al., 2011; Lamsal, et al., 2007). Sometimes more than one approach is combined to increase the purity of the final protein (Di Stefano, et al., 2018).

Protein precipitation is the most straightforward approach to isolate proteins; these are aggregated and then collected in the precipitate. This can be carried out by reducing the steric repulsions or by denaturalizing the protein structure. Thermal precipitation involves the unfolding of protein by thermal denaturation, exposing hydrophobic sites that interact and coagulate, facilitating isolation by filtration or centrifugation. This can be achieved by heat treatments between 70 to 100 °C through steam injection or direct heat treatment (Bals, et al., 2012; Dijkstra, et al., 2003). However, such a process can cause the proteins to lose their native structure, become less soluble, and digestible (Bals, et al., 2012), limiting their application in the food industry (Lamsal, et al., 2005; Xie, 2017). Therefore, this method is not ideal if the intended application of the resulting protein extract requires the retention of protein functionality.

Another way to trigger protein precipitation is by adjusting the pH close to the isoelectric point. In the absence of electrical charge, protein-protein interactions are favoured over protein-water interactions, leading to protein aggregation and precipitation (Böcker, et al., 2021). Different pH values, generally in the range 3-3.5, have been used to precipitate soluble proteins from leaves (Fiorentini, et al., 1983; Hernandez, et al., 1989; Hojilla-Evangelista, et al., 2016; Kobbi, et al., 2017; Merodio, et al., 1983; Stødkilde, et al., 2018). Unlike the previous method, this aggregation can be partially reversed (Böcker, et al., 2021), and the protein can be re-dissolved by increasing pH (Dijkstra, et al., 2003; Hojilla-Evangelista, et al., 2016). As a result, a protein isolated with this approach can have good functional properties, such as emulsification (Hojilla-Evangelista, et al., 2016).

This approach has also been used as an alternative strategy to separate soluble proteins from the green fraction, by acidifying the green juice directly. In this case, chlorophyll and other cell debris precipitated together with the proteins, but only the soluble protein fraction was re-dissolved when the pH was readjusted to 8 (Merodio, et al., 1983).

Chromatographic techniques have also been used to isolate RuBisCO with high purity (Martin, et al., 2014), by selecting chromatography columns with low affinity for RuBisCO and high affinity for polyphenols. Several options are available depending on the plant source because each plant has its own polyphenol profile (de Jong, et al., 2011). However, chromatography is a time-consuming and expensive method. Although it is very effective for laboratory research, it is challenging and expensive to apply to large-scale production (Barbeau, et al., 1988; de Jong, et al., 2011; Di Stefano, et al., 2018).

Ultrafiltration is an alternative method to enrich soluble proteins without affecting their structure. Based on the membrane pore size, proteins are retained and concentrated, whereas water and smaller solutes, including some antinutritional factors, are discarded with the permeate fraction (Bals, et al., 2012; Xie, 2017). Although promising, ultrafiltration processes still require optimization to address some of their main limitations. One of the problems associated with membrane filtration is fouling, i.e., the membrane retains some of the filtered solutes, forming deposits, referred to as secondary membranes. This alters the selectivity and efficiency of the membrane and associated filtration performance, such as the amount of permeate passing through the membrane per unit of membrane area and time (permeate flux) and the size of particles that are retained by the membrane (Blais, Ho, Murphy, Schroën, & Tobin, 2021; Lamsal, et al., 2005). Another limitation associated with membrane filtration is enzymatic protein degradation since the duration of the process, and the working temperature (usually room temperature), increase proteolytic activity (Koschuh, et al., 2004; Ostrowski, 1979).

Nevertheless, the use of ultrafiltration for enrichment of extracted leaf protein presents some distinct benefits compared with other techniques. The protein obtained is in its native state, so it has high solubility, with functional and nutritional properties retained (D'Alvise, et al., 2000; Dijkstra, et al., 2003; Martin, et al., 2019; Ostrowski, 1979). Moreover, it has shown to be an efficient method for large-scale production (D'Alvise, et al., 2000; Di Stefano, et al., 2018). Some authors have suggested using membranes in series with different pore sizes to isolate and successively concentrate/enrich soluble proteins from the green fraction (Dijkstra, et al., 2003; Knuckles, et al., 1982; Martin, et al., 2019).

The final step of drying the enriched protein is required to achieve a product sufficiently stable for transport and trade (Bals, et al., 2012). The most commonly used method in studies published to date is freeze-drying, but the economic cost of this process makes it prohibitive for industrial production. An alternative method suitable and well-established at large-scale would be spray-drying, which involves spraying the fluid into a hot drying medium to convert it into a dried powder. However, there is lack of information of how this process affects the microstructure plant proteins and its effect on techno-functional properties and digestibility (Rivera del Rio, et al., 2020). Furthermore, since the drying technique impacts the solubility of the final dried product, studies on powder reconstitution properties are necessary.

3.4 Valorization of fibrous pulp coproducts

After pressing the leaves to extract green juice, the press cake obtained is high in fibre (Fiorentini, et al., 1983) and contains half of the crude protein of the leaves, often bound to the fibre (Damborg, et al., 2020; Dotsenko, et al., 2017; Hermansen, et al., 2017). The

extraction of this protein fraction has been studied by Dotsenko, et al. (2017), who suggested that free proteases remaining in the press cake, facilitate up to 30% of the protein recovery by aqueous extraction at pH 8, which is conducive to plant protease activity; with the obtained proteins having a similar amino acid composition to RuBisCO (Dotsenko, et al., 2017). Alternatively, the application of ammonia fibre expansion has been proposed as a technique to extract the protein bound to the cell walls (Solati, et al., 2017b). However, the complex composition of this matrix requires a broad range of extraction conditions to separate the heterogeneity of proteins bound to the membranes. To address this, Tenorio, et al. (2018) suggested using solvents and reagents employed for proteomic analysis, such as chloroform, methanol and trichloroacetic acid, which provide optimal conditions to purify specific proteins, although with poor yield and restricted applicability in food-grade products.

The press cake fraction has also been proposed for alternative applications, such as feed for ruminant animals (Damborg, et al., 2020; Fiorentini, et al., 1983; Solati, et al., 2017b; Stødkilde, et al., 2021), recovery of 5-carbon sugars (Dotsenko, et al., 2017), cellulose extraction (Liu, et al., 2006) or biofuels like biogas, methanol and ethanol by fermentation (Fiorentini, et al., 1983). Exploiting a sustainable source of proteins in combination with the valorization of all derived coproducts to maximize value would contribute towards the development of more sustainable processes.

4 Current challenges in the extraction of proteins from green leaves

Although the existing processes to extract soluble proteins from leafy biomass described in the previous section consist of relatively simple steps, some challenges need to be

addressed to ensure the quality of the final protein extracts while maximizing the protein yield on extraction.

4.1 Oxidation and proteolytic reactions

Several undesirable reactions can occur during the extraction process, including lipid and polyphenol oxidation (Dijkstra, et al., 2003) or proteolysis (Koschuh, et al., 2004). These have the potential to negatively affect the nutritional value, sensorial characteristics and functional properties of the protein extracts; therefore, the processing conditions should be carefully controlled (Dijkstra, et al., 2003). Polyphenol oxidases and phenolic compounds (previously mentioned in Sections 2.1.2 and 2.3) are located in different cell compartments within intact leaf cells (chloroplasts and vacuoles, respectively) (Vissers, et al., 2017). When the plant tissue is disrupted during protein extraction, both components are mixed in the green juice (Li, et al., 2018), initiating oxidative reactions. In the presence of oxygen, polyphenol oxidase catalyzes the oxidation of monophenols to orto-diphenols and further to orto-quinones. Those orto-quinones are highly reactive with functional groups of proteins and amino acids (Amer, et al., 2021), binding covalently to proteins, leading to the formation of melanin pigments (Lee, et al., 2006). Moreover, throughout the extraction and purification process, phenolic compounds are enriched principally in the white protein fraction (Prade, et al., 2021). The resulting protein is less soluble, with lower functionality and nutritional value (Amer, et al., 2021; Di Stefano, et al., 2018; Nynäs, 2018). Previous studies have shown decreased protein digestibility *in vitro* and *in vivo* when the oxidation process is not prevented (Amer, et al., 2021). In addition, the formation of such protein-quinone complexes also reduces the extractability of protein, and thus the yield of extracted protein (Fiorentini, et al., 1983; Nynäs, 2018).

Moreover, melanin pigments also modify the colour of the protein extracts and give rise to off-flavours (such as bitterness) that can alter the taste and aroma of the final product (Hermansen, et al., 2017), affecting the sensorial properties and consumer acceptance. Hence, the extraction process requires a step to prevent oxidative reactions to obtain a protein with optimal quality. The integrity of the polyphenols, which could also be recovered as valuable by-products, is also maintained.

The activity of PPO is plant species specific; for example, red clover shows higher PPO activity than white clover, perennial ryegrass, alfalfa and other forage species, although the oxidative reactions that take place are the same (Belanche, Lee, Moorby, & Newbold, 2013). For most of them, the optimal pH range for the enzyme is between 5 and 7 (Winters, et al., 2003). On the other hand, the phenolic compounds are also found in different amounts in leaves, depending on the species. For example, spinach is one of the green leaves with the lowest polyphenol content, often making it suitable for research purposes (de Jong, et al., 2011). In general, for most green leaves, the polyphenol oxidase activity and the phenolic content increases with the age of the plant (Vissers, et al., 2017), and there is a relationship between PPO activity/phenolic content and enzymatic browning (Chutichudet, Chutichudet, & Kaewsit, 2011). However, a recent study suggested that enzymatic browning cannot be predicted without considering the presence of other reducing compounds in the leaves, such as ascorbic acid and glutathione (Vissers, et al., 2017).

Several strategies have been proposed to prevent the enzymatic browning incurred during protein extraction from green leaves. The addition of sulphur-containing compounds, mainly sodium metabisulphite, is the most commonly used (Amer, et al., 2021; de Jong, et al., 2011; Fiorentini, et al., 1983; Narváez-Cuenca, Kuijpers, Vincken, de Waard, & Gruppen, 2011). The mechanism of action of these compounds is based on their covalent

688 binding to PPO, inhibiting the enzyme irreversibly, and thus, reducing the oxidation of
689 polyphenols (Amer, et al., 2021; Narváez-Cuenca, et al., 2011). However, the addition of
690 sodium metabisulphite decreases the juice's pH, with the potential to negatively impact
691 yield by reducing protein solubility. Other approaches to reduce PPO activity include
692 minimizing contact with oxygen, decreasing temperature (Nynäs, 2018) or adding other
693 reducing compounds such as ascorbic acid (Vissers, et al., 2017; Winters, et al., 2003).

694 An alternative to reducing the PPO activity is to reduce the reactivity of phenolic
695 compounds. This can be achieved by shifting the pH to within a range lower than the
696 optimal activity for PPO, which is usually pH 5-7 (Winters, et al., 2003), binding them
697 with polyvinylpolyprrolidone (de Jong, et al., 2011), or removing them by adsorptive
698 resins or chromatographic separation (Nynäs, 2018); however, such strategies are not
699 practical or economically viable at large scale. Furthermore, the unique added nutritional
700 value of phenolic compounds in their non-oxidized state should be considered (Amer, et
701 al., 2021).

702 Proteolytic reactions during the extraction process are another challenge to consider.
703 During the tissue disruption step, endogenous proteases present in the plant cells get in
704 contact with proteins and hydrolyze them, with the activity of these enzymes being
705 especially high at room temperature. Koschuh, et al. (2004) studied the degradation of
706 RuBisCO in green leaf juice at different temperatures and reported that only 8% of the
707 initial RuBisCO remained intact after 12 h when stored at 20 °C, and only 2% remained
708 intact after 24 h. However, this reaction can be retarded by decreasing temperature. The
709 same authors showed that up to 65% of the initial RuBisCO remained intact after 24 h at
710 4 °C, and 80% at 0 °C. Therefore, temperature control during the extraction and
711 purification steps would be advisable. To avoid friction-induced heating of the sample
712 during the tissue disruption step, the use of appropriately engineered screw-presses or

juicers whose operation mechanism prevents heating of the moving parts is recommended.

4.2 Antinutritional and other undesired compounds

The nutritional value of RuBisCO, like other plant proteins, is adversely affected by antinutritional compounds present in the plant tissues (Barbeau, et al., 1988; Gupta & Wagle, 1988). These compounds limit the assimilation of nutrients, particularly the digestibility of proteins, reducing their nutritional value. Moreover, some antinutritional compounds may interfere with human metabolism, responsible for some adverse effects on human health (Chiesa, et al., 2011; Di Stefano, et al., 2018).

Phenolic compounds are described as antinutrients because they may decrease the digestibility of food at high concentrations (Makkar, 1993; Rathore, 2010). Moreover, they take part in browning reactions, which can also compromise the functionality and nutritional value of proteins (see Section 4.1). Tannins are the main group of phenolic compounds in food and beverages (Popova, et al., 2019), and thus are water-soluble, heat-stable and have a molecular weight range of 0.5-3 kDa (Gemede & Ratta, 2014; Makkar, 1993; Sarwar Gilani, Wu Xiao, & Cockell, 2012), which make them easily extractable with the soluble protein fraction of plants. Tannins precipitate proteins by forming tannin-protein complexes, and consequently, they decrease protein digestibility (Gemede, et al., 2014; Samtiya, Aluko, & Dhewa, 2020), and also affect the palatability of food due to precipitation of saliva proteins (Makkar, 1993), increasing the associated astringent sensory perception. Moreover, it has been suggested that tannins also decrease bioavailability of carbohydrates, minerals and vitamins (Makkar, 1993). However, phenolic compounds have antioxidant properties (Durairaj, et al., 2014), and are

737 considered valuable bioactive compounds. Therefore, the balance between their
738 advantages and disadvantages in food, and the possibility of finding an optimal
739 concentration that would not compromise efficient nutrient assimilation should be further
740 investigated.

741 Phytic and oxalic acid are also categorized as antinutrients. They have negatively charged
742 functional groups in their structures, which bind micronutrients such as calcium,
743 magnesium, iron or zinc, reducing their bioavailability (Gemede, et al., 2014; Samtiya, et
744 al., 2020). Diets containing high levels of these compounds have been associated with
745 mineral deficiencies (Gemede, et al., 2014; Popova, et al., 2019). Moreover, oxalates can
746 form irreversible complexes in the urinary tract, leading to the accumulation of kidney
747 stones, with their subsequent detrimental effects on health (Popova, et al., 2019).

748 Other antinutritional compounds affect the nutritional value of plant proteins by inhibiting
749 the activity of enzymes such as proteases or α-amylases. Protease inhibitors have been
750 related to decreased growth rate caused by reducing protein digestion and amino acid
751 availability (Gemede, et al., 2014; Samtiya, et al., 2020). In addition, diets with high
752 levels of protease inhibitors induce an increase in the secretion of pancreatic enzymes,
753 leading to pancreatic hypertrophy (Popova, et al., 2019; Samtiya, et al., 2020).
754 Nevertheless, they have also been associated with positive health effects such as
755 anticarcinogenic activity (Gemede, et al., 2014). In addition, α-amylase inhibitors have
756 been proposed as a strategy for treating carbohydrate disorders such as type 2 diabetes or
757 obesity (Popova, et al., 2019; Samtiya, et al., 2020).

758 Within the enzyme inhibitors, saponins reduce the activity of digestive enzymes, decrease
759 protein digestibility, and the bioavailability of other nutrients (Gemede, et al., 2014).
760 They are also related to hemolysis effects and digestion disorders (Samtiya, et al., 2020).

761 Moreover, saponins reduce food intakes, given that they confer bitter taste and
762 astringency (Gemede, et al., 2014). Nevertheless, it has been suggested that diets rich in
763 saponins could have significant benefits such as reducing plasma cholesterol,
764 immunostimulatory and anticarcinogenic properties (Gemede, et al., 2014; Popova, et al.,
765 2019).

766 Given the health benefits attributed to the presence of some antinutritional compounds,
767 they might not always be harmful; it depends on their concentration, time of exposition
768 and interaction with other dietary compounds (Chiesa, et al., 2011; Gemede, et al., 2014).
769 Hence, some researchers consider that the antinutritional effect is not intrinsic to a
770 compound and is mainly determined by the diet pattern and the processing method before
771 consumption (Natesh, et al., 2017). According to this consideration, a purification process
772 to completely remove antinutrient compounds might not be strictly required (Chiesa, et
773 al., 2011). The absence of regulations about the maximum safe levels for these
774 compounds also complicates decision-making on processing steps required to reduce
775 levels of, or remove, such antinutrients (Sarwar Gilani, et al., 2012). However, to reduce
776 antinutritional effects, the selection of appropriate species for protein extraction should
777 be considered. For instance, selecting forages with low saponin content has been shown
778 to improve the nutritional quality of such forages for livestock (Dijkstra, et al., 2003).
779 Other studies propose applying heat treatments to reduce antinutritional effects by
780 inactivating protease inhibitors (Gemede, et al., 2014; Sarwar Gilani, et al., 2012).
781 However, the potential loss of protein functionality should again be considered (Dijkstra,
782 et al., 2003). Taking all this into account, mild purification treatments, like ultrafiltration
783 or activated carbon (Section 3.3), may be used to recover proteins with sufficiently low
784 levels of antinutritional factors (Dijkstra, et al., 2003).

Apart from the antinutritional factors, the removal of other undesired compounds that could affect consumer acceptance, such as pigments (Martin, et al., 2014) (see Section 2.4), off-flavours (de Jong, et al., 2011; Kinsella & Melachouris, 1976) or compounds that confer astringency, should also be considered. The need to remove these compounds should be aligned with the requirements of the specific intended use and, if a decision is made to separate them during the protein purification process, their valorization as value-added byproducts should be considered (Tenorio, et al., 2017).

4.3 Maximizing extraction yield

The yield of protein during extraction is one of the main factors that determine the commercial viability of the industrial-scale processes. Despite the abundance of RuBisCO in leafy biomass, its yield on extraction is generally low (Kobbi, et al., 2017) using the processes available to date, with reported yield values of 0.09-6% (Prade, et al., 2021; Tenorio, et al., 2016). A number of factors contribute to this, including ineffective cell disruption, poorly selective separation methods, the occurrence of oxidation and proteolytic reactions that result in protein losses, and the application of demanding purification steps to remove unwanted compounds without negatively affecting the functionality of the proteins. Several strategies have been reviewed herein to improve cell disruption (Section 3.1) and avoid unwanted reactions (Section 4.1), as well as the need to carefully consider the extent of purification required for the intended application of the protein extracts (Section 4.2). Therefore, further research is still required to develop an efficient and affordable extraction process (Di Stefano, et al., 2018), which can also be scalable to isolate large amounts of proteins for food-grade applications (Hermansen, et al., 2017).

809 The first step that determines the yield is the choice of the raw material. As discussed in
810 Section 2, the plant composition varies widely, and so does the protein content and its
811 extractability. Therefore, selection of green leaves species, optimizing growing
812 conditions and harvest time, directly affect protein recovery (Barbeau, et al., 1988; Di
813 Stefano, et al., 2018; Fiorentini, et al., 1983; Pirie, 1942). Secondly, the purity of the final
814 fraction is an essential factor that determines the yield of the process; higher purity
815 requires increasing the number of purification steps, increasing the protein losses along
816 the process (Tenorio, et al., 2017; Tenorio, et al., 2018). Tenorio, et al. (2018) studied the
817 relationship between those three factors, concluding that the differences between
818 traditional crops and green biomasses require new technologies to achieve protein purity
819 and yield from green leaves. To obtain a food-grade ingredient with a high protein
820 recovery, a lower purity may be accepted. Some researchers consider it an advantage, due
821 to the presence of bioactive compounds such as chlorophyll, carotene or polyphenols in
822 such less pure protein extracts (Tenorio, et al., 2016).

823 The target and achievable extraction yield will naturally impact on the economic
824 feasibility of any process designed to develop leaf protein extracts. However, previous
825 studies that evaluated the balance of cost and purity for protein fractionation processes
826 concluded that their cost and revenues largely depend on the final intended use of the
827 product. Although some factors such as harvesting time, nitrogen application or
828 intercropping with legumes can increase the protein content in leaves (Muneer, et al.,
829 2021), the economic feasibility of the process depends mainly on: 1) the development of
830 added value sub-products, for example by valorisation of the fibre fraction obtained after
831 the juicing step, which contains more than 50% of the protein; 2) increasing protein
832 recovery in the white protein fraction; and 3) decreasing the feedstock cost by selecting
833 as a raw material residual leaves that are discarded in the current food production system

such as kale, broccoli leaves (Prade, et al., 2021), cabbage leaves, beetroot leaves, sugar beet leaves, carrot leaves, (Nynäs, et al., 2021) quinoa leaves, yacon leaves, or potato leaves.

4.4 Relationship between protein enrichment and functional properties

The importance of proteins in food systems relies not only on their nutritional value but also on their functional and food structuring properties (de Jong, et al., 2011; Jiménez-Munoz, et al., 2021; Kumar, et al., 2021). Therefore, treatments applied during extraction should ideally preserve protein functionality, both in nutritional value and techno-functional properties (Chiesa, et al., 2011; Martin, et al., 2014). RuBisCO has shown good foaming properties (Barbeau, et al., 1988; Hojilla-Evangelista, et al., 2016; Lamsal, et al., 2007), even greater than egg white, whey protein or other plant proteins such as soy protein isolate (Martin, et al., 2019; Sheen, et al., 1985). Moreover, RuBisCO was used to produce emulsion systems with good stability over time (Knuckles, et al., 1982; Martin, et al., 2019), becoming a promising ingredient for application in dispersed systems. It also forms strong gels with faster gelation and higher gel strength at lower concentration than other food proteins (Martin, et al., 2014). However, there are some discrepancies in the literature regarding the results for leaf protein functionality due to the purification process applied and the purity achieved (de Jong, et al., 2011). Indeed, the extraction process has a significant impact on the physical state (i.e., denaturation/aggregation) of the proteins, and therefore, on their solubility (see Section 3), which also considerably influences their functional properties (Lamsal, et al., 2007). Overall, RuBisCO displays good solubility when isolated by mild treatments such as ultrafiltration (Lamsal, et al., 2007; Martin, et

al., 2019) or acid precipitation (Hojilla-Evangelista, et al., 2016; Lamsal, et al., 2007; Sheen, et al., 1985).

5 Concluding remarks and future trends

Plant leaves demonstrate considerable potential as an important source of protein for food applications. Although they have long been proposed as an alternative source of other nutritional compounds, most species are still underutilized for food applications. However, when aiming to develop a protein concentrate for human consumption, some considerations must be addressed. First of all, green leaves show considerable variation in their composition; therefore, more effort is required to characterize those variations and correlate them with relevant environmental factors during growth, as they determine protein extractability, and consequently yield and economic viability of extraction processes. New technologies, such as aeroponics or hydroponics, present opportunities to control the growing conditions and address some of the sustainability challenges with traditional farming systems.

Moreover, further studies should be conducted on the non-protein nitrogen fraction to better characterize the nitrogen compounds extracted. Secondly, to obtain concentrated plant proteins suitable for use in food products, more work is needed to develop a scalable method and also to investigate its influence on the functional properties and nutritional value, given that these are greatly influenced by the physical state of RuBisCO and the presence of unwanted compounds such as chlorophyll and polyphenols. Finally, the incomplete exploitation of the plant biomass would limit the economic sustainability of the process. Hence the valorization of the derived coproducts would contribute to developing more sustainable processes and waste reduction.

FUNDINGS

Sara Perez-Vila is a recipient of a Walsh Scholarship from Teagasc, the Irish Agricultural and Food Development Authority (Ref 2020032).

DECLARATION OF INTEREST: None

REFERENCES

Aiking, H. (2011). Future protein supply. *Trends in Food Science & Technology, 22*(2), 112-120.

Amer, B., Juul, L., Møller, A. H., Møller, H. S., & Dalsgaard, T. K. (2021). Improved solubility of proteins from white and red clover – inhibition of redox enzymes. *International Journal of Food Science & Technology, 56*(n/a), 302-311.

Andersson, I., & Backlund, A. (2008). Structure and function of Rubisco. *Plant Physiology and Biochemistry, 46*(3), 275-291.

Ballet, N., Robert, J., & Williams, P. (2000). Vitamins in forages. In D. I. Givens, E. Owen, H. M. Omed & A. R. F. E. (Eds.), *Forage evaluation in ruminant nutrition* (Vol. Vitamins in forages, pp. 399-431): CABI.

Bals, B., Dale, B. E., & Balan, V. (2012). Recovery of Leaf Protein for Animal Feed and High-Value Uses. In C. Bergeron, D. J. Carrier & S. Ramaswamy (Eds.), *Biorefinery Co-Products: Phytochemicals, Primary Metabolites and Value-Added Biomass Processing* (pp. 179-197): John Wiley & Sons, Ltd.

Bals, B., Teachworth, L., Dale, B., & Balan, V. (2007). Extraction of proteins from switchgrass using aqueous ammonia within an integrated biorefinery. *Appl Biochem Biotechnol, 143*(2), 187-198.

Barbeau, W. E., & Kinsella, J. E. (1988). Ribulose bisphosphate carboxylase/oxygenase (rubisco) from green leaves-potential as a food protein. *Food Reviews International, 4*(1), 93-127.

Barbehenn, R. V., Chen, Z., Karowe, D. N., & Spickard, A. (2004). C3 grasses have higher nutritional quality than C4 grasses under ambient and elevated atmospheric CO2. *Global Change Biology, 10*(9), 1565-1575.

Belanche, A., Lee, M. R. F., Moorby, J. M., & Newbold, C. J. (2013). Comparison of ryegrass and red clover on the fermentation pattern, microbial community and efficiency of diet utilisation in the rumen simulation technique (Rusitec). *Animal Production Science, 53*, 1052-1064.

Blais, H., Ho, Q. T., Murphy, E. G., Schroën, K., & Tobin, J. T. (2021). A cascade microfiltration and reverse osmosis approach for energy efficient concentration of skim milk. *Journal of Food Engineering, 300*, 110511.

Böcker, L., Bertsch, P., Wenner, D., Teixeira, S., Bergfreund, J., Eder, S., Fischer, P., & Mathys, A. (2021). Effect of Arthrospira platensis microalgae protein purification on emulsification mechanism and efficiency. *Journal of Colloid and Interface Science, 584*, 344-353.

Bray, W. J., & Humphries, C. (1979). Preparation of white leaf protein concentrate using a polyanionic flocculant. *Journal of the Science of Food and Agriculture, 30*(2), 171-176.

Chapman, K. D., Dyer, J. M., & Mullen, R. T. (2013). Commentary: Why don't plant leaves get fat? *Plant Sciences, 207*, 128-134.

Chiesa, S., & Gnansounou, E. (2011). Protein extraction from biomass in a bioethanol refinery – Possible dietary applications: Use as animal feed and potential extension to human consumption. *Bioresource technology, 102*(2), 427-436.

Choi, I., Choi, S. J., Chun, J. K., & Moon, T. W. (2006). Extraction yield of soluble protein and microstructure of soybean affected by microwave heating. *Journal of Food Processing and Preservation, 30*(4), 407-419.

Chutichudet, B., Chutichudet, P., & Kaewsit, S. (2011). Influence of developmental stage on activities of Polyphenol Oxidase, internal characteristics and colour of lettuce cv. Grand Rapids. *American Journal of Food Technology, 6*(3), 215-225.

Colas, D., Doumeng, C., Pontalier, P. Y., & Rigal, L. (2013). Twin-screw extrusion technology, an original solution for the extraction of proteins from alfalfa (Medicago sativa). *Food and Bioproducts Processing, 91*(2), 175-182.

Contreras, M. d. M., Lama-Muñoz, A., Gutiérrez-Pérez, J. M., Espínola, F., Moya, M., & Castro, E. (2019). Protein extraction from agri-food residues for integration in biorefinery: Potential techniques and current status. *Bioresource technology, 280*, 459-477.

D'Alvise, N., Lesueur-Lambert, C., Fertin, B., Dhulster, P., & Guillochon, D. (2000). Removal of Polyphenols and Recovery of Proteins from Alfalfa White Protein Concentrate by Ultrafiltration and Adsorbent Resin Separations. *Separation Science and Technology, 35*(15), 2453-2472.

Damborg, V. K., Jensen, S. K., Weisbjerg, M. R., Adamsen, A. P., & Stødkilde, L. (2020). Screw-pressed fractions from green forages as animal feed: Chemical composition and mass balances. *Animal Feed Science and Technology, 261*, 114401.

Day, L. (2013). Proteins from land plants – Potential resources for human nutrition and food security. *Trends in Food Science & Technology, 32*, 25-42.

de Jong, A., & Nieuwland, M. (2011). *Literature study on the properties of Rubisco*: TNO.

Di Stefano, E., Agyei, D., Njoku, E. N., & Udenigwe, C. C. (2018). Plant RuBisCo: An Underutilized Protein for Food Applications. *Journal of the American Oil Chemists' Society, 95*(8), 1063-1074.

Dijkstra, D. S., Linnemann, A. R., & van Boekel, T. A. J. S. (2003). Towards sustainable production of protein-rich foods: appraisal of eight crops for Western Europe. PART II: Analysis of the technological aspects of the production chain. *Critical Reviews in Food Science and Nutrition, 43*(5), 481-506.

Dotsenko, G., & Lange, L. (2017). Enzyme Enhanced Protein Recovery from Green Biomass Pulp. *Waste Biomass Valorization, 8*(4), 1257-1264.

Durairaj, V., Hoda, M., Shakya, G., Babu, S. P. P., & Rajagopalan, R. (2014). Phytochemical screening and analysis of antioxidant properties of aqueous extract of wheatgrass. *Asian Pacific journal of tropical medicine, 7*, S398-S404.

Ellis, R. J. (1979). The most abundant protein in the world. *Trends in Biochemical Sciences, 4*(11), 241-244.

Evans, J. R., & Seemann, J. R. (1989). The allocation of protein nitrogen in the photosynthetic apparatus: costs, consequences, and control. *Photosynthesis, 8*, 183-205.

Fageria, N. K., Baligar, V. C., & Clark, R. B. (2002). Micronutrients in crop production. In *Advances in agronomy* (Vol. 77, pp. 185-268): Elsevier.

FAO. (2011). Dietary protein quality evaluation in human nutrition. In F. a. a. o. o. t. u. nations (Ed.). Auckland, New Zealand.

Ferland, G., & Sadowski, J. A. (1992). Vitamin K1 (phylloquinone) content of green vegetables: effects of plant maturation and geographical growth location. *Journal of Agricultural and Food Chemistry, 40*(10), 1874-1877.

974 Fernandes, A. S., Pinheiro Nass, P., Oliveira, Á., & Queiroz Zepka, L. (2020). Chlorophylls as Food
975 Additives. In *Pigments from Microalgae Handbook* (pp. 391-420): Springer Nature
976 Switzerland.

977 Fiorentini, R., & Galoppini, C. (1983). The proteins from leaves. *Plant Foods for Human Nutrition,*
978 *32*(3), 335-350.

979 Fog, E., Ytting, N. K., & Lübeck, M. (2017). Biorefining of proteins from grass clover as an
980 innovative solution to a truly sustainable organic production. In *Innovative Research for*
981 *Organic Agriculture 3.0*. New Delhi, India.

982 Gemede, H. F., & Ratta, N. (2014). Antinutritional factors in plant foods: Potential health benefits
983 and adverse effects. *International Journal of Nutrition and Food Sciences, 3*(4), 284-289.

984 Gerloff, E. D., Lima, I. H., & Stahmann, M. A. (1965). Amino Acid Composition of Leaf Protein
985 Concentrates. *Journal of Agricultural and Food Chemistry, 13*(2), 139-143.

986 Glasser, F., Doreau, M., Maxin, G., & Baumont, R. (2013). Fat and fatty acid content and
987 composition of forages: A meta-analysis. *Animal Feed Science and Technology, 185*(1),
988 19-34.

989 Gupta, K., & Wagle, D. S. (1988). Nutritional and antinutritional factors of green leafy vegetables.
990 *Journal of Agricultural and Food Chemistry, 36*(3), 472-474.

991 Hermansen, J. E., Jørgensen, U., Lærke, P. E., Manevski, K., Boelt, B., Jensen, S. K., Weisbjerg, M.
992 R., Dalsgaard, T. K., Danielsen, M., Asp, T., Amby-Jensen, M., Claus, Grøn Sørensen, C.
993 A., Vestby Jensen, M., Gylling, M., Lindedam, J., Lübeck, M., & Fog, E. (2017). *Green*
994 *biomass-protein production through bio-refining*: DCA-Nationalt Center for Fødevarer
995 og Jordbrug.

996 Hernandez, A., Martinez, C., & Alzueta, C. (1989). Effects of alfalfa leaf juice and chloroplast-free
997 juice pH values and freezing upon the recovery of white protein concentrate. *Journal of*
998 *Agricultural and Food Chemistry, 37*(1), 28-31.

999 Hoekstra, N. J., Schulte, R. P. O., Struik, P. C., & Lantinga, E. A. (2007). Pathways to improving the
1000 N efficiency of grazing bovines. *European Journal of Agronomy, 26*(4), 363-374.

1001 Hojilla-Evangelista, M. P., Selling, G. W., Hatfield, R., & Digman, M. (2016). Extraction,
1002 composition, and functional properties of dried alfalfa (Medicago sativa L.) leaf protein.
1003 *Journal of the Science of Food and Agriculture, 97*, 882-888.

1004 Houles, V., Guerif, M., & Mary, B. (2007). Elaboration of a nitrogen nutrition indicator for winter
1005 wheat based on leaf area index and chlorophyll content for making nitrogen
1006 recommendations. *European Journal of Agronomy, 27*(1), 1-11.

1007 Huffaker, R. C., & Miller, B. L. (1978). Reutilization of ribulose bisphosphate carboxylase. *Basic*
1008 *Life Sciences, 11*, 139-152.

1009 Humphreys, M. O. (1989). Water-soluble carbohydrates in perennial ryegrass breeding. ni.
1010 Relationships with herbage production, digestibility and crude protein content. *Grass*
1011 *and Forage Science, 44*(4), 423-430.

1012 Jiménez-Munoz, L. M., Tavares, G. M., & Corredig, M. (2021). Design future foods using plant
1013 protein blends for best nutritional and technological functionality. *Trends in Food*
1014 *Science & Technology, 113*, 139-150.

1015 Jones, B. A., Hatfield, R. D., & Muck, R. E. (1995). Screening legume forages for soluble phenols,
1016 polyphenol oxidase and extract browning. *Journal of the Science of Food and Agriculture,*
1017 *67*(1), 109-112.

Jungbluth, N., Tietje, O., & Scholz, R. W. (2000). Food purchases: Impacts from the consumers' point of view investigated with a modular LCA. *The International Journal of Life Cycle Assessment, 5*(3), 134-142.

Jwanny, E. W., Montanari, L., & Fantozzi, P. (1993). Protein production for human use from sugarbeet: Byproducts. *Bioresource technology, 43*(1), 67-70.

Kammes, K. L., Bals, B. D., Dale, B. E., & Allen, M. S. (2011). Grass leaf protein, a coproduct of cellulosic ethanol production, as a source of protein for livestock. *Animal Feed Science and Technology, 164*(1), 79-88.

Kinsella, J. E., & Melachouris, N. (1976). Functional properties of proteins in foods: a survey. *Critical Reviews in Food Science & Nutrition, 7*(3), 219-280.

Kiskini, A., Vissers, A., Vincken, J.-P., Gruppen, H., & Wierenga, P. A. (2016). Effect of Plant Age on the Quantity and Quality of Proteins Extracted from Sugar Beet (Beta vulgaris L.) Leaves. *Journal of Agricultural and Food Chemistry, 64*(44), 8305-8314.

Knuckles, B. E., & Kohler, G. O. (1982). Functional properties of edible protein concentrates from alfalfa. *Journal of Agricultural and Food Chemistry, 30*(4), 748-752.

Kobbi, S., Bougatef, A., Le flem, G., Balti, R., Mickael, C., Fertin, B., Chaabouni, S., Dhulster, P., & Nedjar, N. (2017). Purification and Recovery of RuBisCO Protein from Alfalfa Green Juice: Antioxidative Properties of Generated Protein Hydrolysate. *Waste and Biomass Valorization, 8*(2), 493-504.

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

Ku, M. S. B., Schmitt, M. R., & Edwards, G. E. (1979). Quantitative Determination of RuBP Carboxylase–Oxygenase Protein in Leaves of Several C3 and C4 Plants. *Journal of Experimental Botany, 30*(1), 89-98.

Kumar, M., Tomar, M., Potkule, J., Reetu, Punia, S., Dhakane, J., Singh, S., Dhumal, S., Chandra Pradhan, P., Bhushan, B., Anitha, T., Alajil, O., Alhariri, A., Amarowicz, R., & Kennedy, J. F. (2021). Functional characterization of plant-based protein to determine its quality for food applications. *Food Hydrocolloids*, 106986.

Kupke, D. W. (1962). Correlation of a Soluble Leaf Protein with Chlorophyll Accumulation. *Journal of Biological Chemistry, 237*(10), 3287-3291.

Lamsal, B. P., & Koegel, R. G. (2005). Evaluation of a dynamic ultrafiltration device in concentrating soluble alfalfa leaf proteins. *Transactions of the ASAE, 48*, 691-701.

Lamsal, B. P., Koegel, R. G., & Gunasekaran, S. (2007). Some physicochemical and functional properties of alfalfa soluble leaf proteins. *LWT-Food Science and Technology, 40*(9), 1520-1526.

Lee, M. R. F., Olmos Colmenero, J. d. J., Winters, A. L., Scollan, N. D., & Minchin, F. R. (2006). Polyphenol oxidase activity in grass and its effect on plant-mediated lipolysis and proteolysis of Dactylis glomerata (cocksfoot) in a simulated rumen environment. *Journal of the Science of Food and Agriculture, 86*(10), 1503-1511.

Li, X., Tian, J., Zhang, Q., Jiang, Y., Wu, Z., & Yu, Z. (2018). Effects of mixing red clover with alfalfa at different ratios on dynamics of proteolysis and protease activities during ensiling. *Journal of Dairy Science, 101*(10), 8954-8964.

Licitra, G., Hernandez, T. M., & Van Soest, P. J. (1996). Standardization of procedures for nitrogen fractionation of ruminant feeds. *Animal Feed Science and Technology, 57*(4), 347-358.

Lindström, B. E. M., Frankow-Lindberg, B. E., Dahlin, A. S., Wivstad, M., & Watson, C. A. (2013). Micronutrient concentrations in common and novel forage species and varieties grown on two contrasting soils. *Grass and Forage Science, 68*(3), 427-436.

Liu, C. F., Xu, F., Sun, J. X., Ren, J. L., Curling, S., Sun, R. C., Fowler, P., & Baird, M. S. (2006). Physicochemical characterization of cellulose from perennial ryegrass leaves (Lolium perenne). *Carbohydrate Research, 341*(16), 2677-2687.

Makkar, H. P. S. (1993). Antinutritional factors in foods for livestock. *Animal Production in Developing Coun, 16*, 69-85.

Mangan, J. (1982). The Nitrogenous Constituents of Fresh Forages. *Forage Protein in Ruminant Animal Production, 6*, 25-40.

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

Martin, A. H., Nieuwland, M., & de Jong, G. A. H. (2014). Characterization of Heat-Set Gels from RuBisCO in Comparison to Those from Other Proteins. *Journal of Agricultural and Food Chemistry, 62*(44), 10783-10791.

McGrath, D. (1988). Seasonal variation in the water-soluble carbohydrates of perennial and Italian ryegrass under cutting conditions. *Irish Journal of Agricultural Research, 27*(2-3), 131-139.

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

Muneer, F., Hovmalm, H. P., Svensson, S.-E., Newson, W. R., Johansson, E., & Prade, T. (2021). Economic viability of protein concentrate production from green biomass of intermediate crops: A pre-feasibility study. *Journal of Cleaner Production, 294*, 126304.

Nadathur, S. R., Wanasundara, J. P. D., & Scanlin, L. (2017). Proteins in the Diet: Challenges in Feeding the Global Population. In S. R. Nadathur, J. P. D. Wanasundara & L. Scanlin (Eds.), *Sustainable Protein Sources* (pp. 1-19). San Diego: Academic Press.

Narváez-Cuenca, C.-E., Kuijpers, T. F. M., Vincken, J.-P., de Waard, P., & Gruppen, H. (2011). New Insights into an Ancient Antibrowning Agent: Formation of Sulfophenolics in Sodium Hydrogen Sulfite-Treated Potato Extracts. *Journal of Agricultural and Food Chemistry, 59*(18), 10247-10255.

Natesh, H. N., Abbey, L., & Asiedu, S. K. (2017). An overview of nutritional and antinutritional factors in green leafy vegetables. *Horticulture International Journal, 1*(2), 00011.

Nielsen, C. K., Stødkilde, L., Jørgensen, U., & Lærke, P. E. (2021). Effects of Harvest and Fertilization Frequency on Protein Yield and Extractability From Flood-Tolerant Perennial Grasses Cultivated on a fen Peatland. *Frontiers in Environmental Science, 9*, 47.

Nynäs, A.-L. (2018). White proteins from green leaves in food applications. In (Vol. 1). Alnarp, Sweden: Faculty of Landscape Architecture, Horticulture and Crop Production Science.

Nynäs, A.-L., Newson, W. R., & Johansson, E. (2021). Protein Fractionation of Green Leaves as an Underutilized Food Source—Protein Yield and the Effect of Process Parameters. *Foods, 10*(11), 2533.

Ostrowski, H. T. (1979). Membrane filtration for isolation, fractionation and purification of food- and feed-grade proteins from pasture herbage. *Journal of Food Processing and Preservation, 3*(1), 59-84.

Parveen, I., Threadgill, M. D., Moorby, J. M., & Winters, A. (2010). Oxidative Phenols in Forage Crops Containing Polyphenol Oxidase Enzymes. *Journal of Agricultural and Food Chemistry, 58*(3), 1371-1382.

Pirie, N. W. (1942). Green leaves as a source of proteins and other nutrients. *Nature, 149*(3774), 251.

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

Plasek, B., Lakner, Z., & Temesi, Á. (2020). Factors that Influence the Perceived Healthiness of Food-Review. *Nutrients, 12*.

Pojić, M., Mišan, A., & Tiwari, B. (2018). Eco-innovative technologies for extraction of proteins for human consumption from renewable protein sources of plant origin. *Trends in Food Science & Technology, 75*, 93-104.

Popova, A., & Mihaylova, D. (2019). Antinutrients in plant-based foods: A review. *The Open Biotechnology Journal, 13*(1), 68-76.

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.

Queiroz Zepka, L., Jacob-Lopes, E., & Roca, M. (2019). Catabolism and bioactive properties of chlorophylls. *Current opinion in food science, 26*, 94-100.

Rathore, M. (2010). Leaf protein concentrate as food supplement from arid zone plants. *Journal of dietary supplements, 7*(2), 97-103.

Rivera del Rio, A., Opazo-Navarrete, M., Cepero-Betancourt, Y., Tabilo-Munizaga, G., Boom, R. M., & Janssen, A. E. M. (2020). Heat-induced changes in microstructure of spray-dried plant protein isolates and its implications on in vitro gastric digestion. *LWT - Food Science and Technology, 118*, 108795.

Samtiya, M., Aluko, R. E., & Dhewa, T. (2020). Plant food anti-nutritional factors and their reduction strategies: an overview. *Food Production, Processing and Nutrition, 2*(6).

Santamaría-Fernández, M., & Lübeck, M. (2020). Production of leaf protein concentrates in green biorefineries as alternative feed for monogastric animals. *Animal Feed Science and Technology, 268*, 114605.

Santamaría-Fernández, M., Molinuevo-Salces, B., Kiel, P., Steenfeldt, S., Uellendahl, H., & Lübeck, M. (2017). Lactic acid fermentation for refining proteins from green crops and obtaining a high quality feed product for monogastric animals. *Journal of Cleaner Production, 162*, 875-881.

Sari, Y. W., Mulder, W. J., Sanders, J. P. M., & Bruins, M. E. (2015). Towards plant protein refinery: Review on protein extraction using alkali and potential enzymatic assistance. *Biotechnology Journal, 10*(8), 1138-1157.

Sarwar Gilani, G., Wu Xiao, C., & Cockell, K. A. (2012). Impact of antinutritional factors in food proteins on the digestibility of protein and the bioavailability of amino acids and on protein quality. *British Journal of Nutrition, 108* S315-S332.

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

Solati, Z., Jørgensen, U., Eriksen, J., & Søegaard, K. (2017a). Dry matter yield, chemical composition and estimated extractable protein of legume and grass species during the spring growth. *Journal of the Science of Food and Agriculture, 97*(12), 3958-3966.

Solati, Z., Jørgensen, U., Eriksen, J., & Søegaard, K. (2017b). Estimation of extractable protein in botanical fractions of legume and grass species. *Grass and Forage Science, 73*, 572-581.

Stødkilde, L., Damborg, V. K., Jørgensen, H., Lærke, H. N., & Jensen, S. K. (2019). Digestibility of fractionated green biomass as protein source for monogastric animals. *Animal, 13*(9), 1817-1825.

Stødkilde, L., Damborg, V. K., Jørgensen, H., Laerke, H. N., & Jensen, S. K. (2018). White clover fractions as protein source for monogastrics: dry matter digestibility and protein digestibility-corrected amino acid scores. *J Sci Food Agric, 98*(7), 2557-2563.

Stødkilde, L., Lashkari, S., Eriksen, J., & Jensen, S. K. (2021). Enhancing protein recovery in green biorefineries through selection of plant species and time of harvest. *Animal Feed Science and Technology, 278*, 115016.

Stone, B. A. (1994). Prospects for improving the nutritive value of temperate, perennial pasture grasses. *New Zealand Journal of Agricultural Research, 37*(3), 349-363.

Tenorio, A. T., Boom, R. M., & van der Goot, A. J. (2017). Understanding leaf membrane protein extraction to develop a food-grade process. *Food chemistry, 217*, 234-243.

Tenorio, A. T., Gieteling, J., de Jong, G. A. H., Boom, R. M., & van der Goot, A. J. (2016). Recovery of protein from green leaves: Overview of crucial steps for utilisation. *Food chemistry, 203*, 402-408.

Tenorio, A. T., Kyriakopoulou, K. E., Suarez-Garcia, E., van den Berg, C., & van der Goot, A. J. (2018). Understanding differences in protein fractionation from conventional crops, and herbaceous and aquatic biomass-Consequences for industrial use. *Trends in Food Science & Technology, 71*, 235-245.

Udenigwe, C. C., Okolie, C. L., Qian, H., Ohanenye, I. C., Agyei, D., & Aluko, R. E. (2017). Ribulose-1, 5-bisphosphate carboxylase as a sustainable and promising plant source of bioactive peptides for food applications. *Trends in Food Science & Technology, 69*, 74-82.

Van de Velde, F., Alting, A. C., & Pouvreau, L. (2011). Process for isolating a dechlorophyllized rubisco preparation from a plant material. In *Patent Cooperation Treaty* (Vol. WO2011078671).

Vissers, A., Kiskini, A., Hilgers, R., Marinea, M., Wierenga, P. A., Gruppen, H., & Vincken, J.-P. (2017). Enzymatic Browning in Sugar Beet Leaves (Beta vulgaris L.): Influence of Caffeic Acid Derivatives, Oxidative Coupling, and Coupled Oxidation. *Journal of Agricultural and Food Chemistry, 65*(24), 4911-4920.

Walgenbach, R. P., Marten, G. C., & Blake, G. R. (1981). Release of Soluble Protein and Nitrogen in Alfalfa. I. Influence of Growth Temperature and Soil Moisture 1. *Crop Science, 21*(6), 843-849.

Wang, W., Tai, F., & Chen, S. (2008). Optimizing protein extraction from plant tissues for enhanced proteomics analysis. *Journal of Separation Science, 31*(11), 2032-2039.

Wąsowicz, G., Styśko-Kunkowska, M., & Grunert, K. G. (2015). The meaning of colours in nutrition labelling in the context of expert and consumer criteria of evaluating food product healthfulness. *Journal of Health Psychology, 20*(6), 907-920.

Wildman, S., & Bonner, J. (1947). The proteins of green leaves. 1. Isolation, enzymatic properties and auxin content of spinach cytoplasmic proteins. *Arch. Biochem., 14*, 381-413.

Winters, A. L., Minchin, F. R., Merry, R. J., & Morris, P. (2003). Comparison of polyphenol oxidase activity in red clover and perennial ryegrass. *Aspects of Applied Biology, 70*, 121-128.

Xie, R. (2017). *Evaluation of leaf protein content, extraction, and purification in Napiergrass (Pennisetum purpureum Schumach.).* Texas A&M University.

Xu, Y., Li, Y., Bao, T., Zheng, X., Chen, W., & Wang, J. (2017). A recyclable protein resource derived from cauliflower by-products: Potential biological activities of protein hydrolysates. *Food chemistry, 221*, 114-122.