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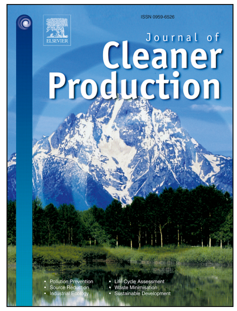

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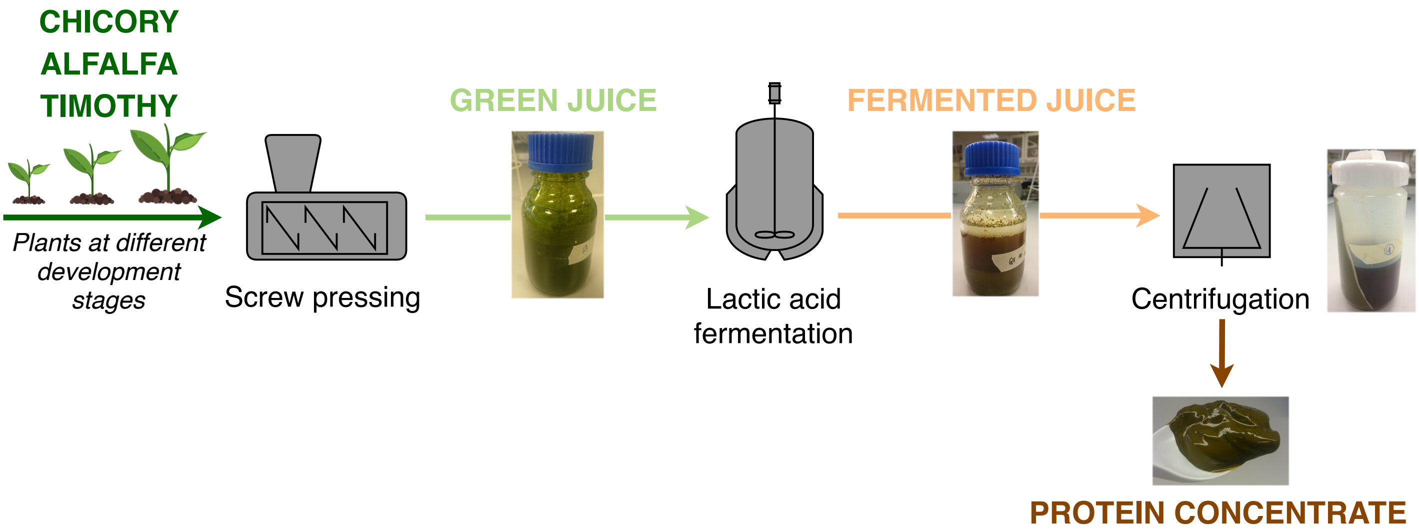
CHICORY
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Plants at different development stages
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Abstract

The extraction of leaf proteins from perennial forage crops within a green biorefinery concept represents a promising approach to face the increasing demand for protein arisen in the organic farming sector to feed monogastric animals. Given the background, the present research aims at assessing the protein extractability from three plant species i.e. chicory, red clover and timothy, at different development stages and investigating lactic acid fermentation as the key method for the extraction of leaf proteins. Based on our results, up to 86% of the proteins in the green juice were recovered in the leaf protein concentrate (LPC) by means of lactic acid fermentation. Red clover presented the highest protein content and resulted in the extraction of 65-98 kg crude proteins per ton dry matter and the production of 186-235 kg dry LPC per ton dry matter. The plants development stage significantly influenced the process figures i.e. protein extractability and production of protein concentrate were reduced with maturity. Accordingly, the maturity of the plants should be addressed when utilized as feedstock for producing protein concentrates for animal feeding in order to optimize the process yields.

Keywords:

Biorefinery; leaf protein concentrate; organic feed; lactic acid bacteria; amino acid

Introduction

In the recent years, the interest on leaf protein is increasing considerably. The enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase, known as Rubisco, can represent up to 50% of the proteins in plant leaves and indeed, Rubisco is the most abundant protein in the world (Martin et al., 2014). Therefore, the extraction of proteins from forage crops such as alfalfa, clover or grass is a potential process for the production of leaf protein concentrates (LPC), which can be utilized as feed or food, but also hydrolyzed into amino acids for the cosmetics or pharma industries (Kromus et al., 2004). This is one of the targets of the green biorefinery (GBR), which aims at developing sustainable processes for the efficient utilization of green biomass into a variety of products like proteins but also lactic acid, fibers, specialty products or energy in the form of biogas (Kromus et al., 2004), which are produced in a single facility thus avoiding the production of any waste stream (Kamm et al., 2010). According to a techno-economic assessment, the establishment of GBRs might also represent a profitable alternative for the utilization of grasslands (Höltinger et al., 2014), which are widely available in Europe but less and less utilized as consequence of the restructuration of agriculture and reduction of livestock farming (Kamm et al., 2010).

GBR processes involve an initial mechanical treatment for disruption of the plants tissue, followed by protein precipitation and protein concentration (Tamayo Tenorio et al., 2016). The initial fractionation is mostly performed with a screw press in order to remove the liquid from the plant fibrous structure, and resulting in a fiber-rich press cake (PC) and nutrient-rich green juice (GJ) (King et al., 2012a). The green juice contains several valuable compounds like proteins, lipids, glycoproteins, lectins, sugars, free amino acids, dyes, hormones, enzymes, minerals, and other substances (Kromus et al., 2008).

The precipitation of proteins can be achieved by different methods. Heat coagulation is the most widespread method for the precipitation of proteins and it can be conveniently performed at 75-80°C by direct stem injection in the green juice according to Morrison and Pirie (1961). Collins (1986) also utilized steam injection at 80°C to coagulate proteins and produce protein concentrates from alfalfa, red clover and birdsfoot trefoil. Besides, sequential heating of the green juice at 60°C firstly, and then at 80°C allowed the separation of a green protein fraction and a white protein fraction in a pilot plant (Edwards et al., 1975). Acid precipitation might also be efficient for the isolation of leaf proteins since the minimum protein solubility is achieved at the isoelectric point i.e. between pH 3.2-3.7 for soybean leaf proteins (Betschart and Kinsella, 1973) or at pH 4.0 for spinach leaf proteins (Merodio et al., 1983). For instance, protein concentrates obtained from alfalfa juice by acidification pH 3.5 presented the highest content of essential amino acids and solubility compared with concentrates obtained by heat coagulation at 85°C or by addition of cationic or anionic flocculants (Baraniak, 1990). Besides, ultrafiltration of the green juice was proposed alternatively to heat coagulation in order to obtain protein concentrates with higher solubility, but protein degradation during the ultrafiltration process was significant (Koschuh et al., 2004). Recently, lactic acid fermentation of the green juice was studied for decreasing the pH of the green juice without addition of any inorganic acid in order to efficiently use the available sugars and to produce a protein product that can be utilized in organic farming (Santamaría-Fernández et al., 2017).

Currently, soybeans are the most common protein-sources utilized for feeding monogastric animals in the organic sector because of their high nutritional value. Indeed, soybeans are an essential ingredient in diets for organic laying hens and might be difficult to replace with other vegetal protein sources (Steenfeldt and Hammershøj, 2015). Alternatively, LPC extracted according to the organic guidelines from forage crops could supplement or substitute the use of soybeans and other grains legumes in organic farming (Santamaría-Fernández et al., 2017). Apart from a suitable amino acid composition, the LPCs are good sources of β-carotenes, vitamins E and K and unsaturated fatty acids (Arkcoll and Festenstein, 1971). However, some limitations of the LPC include a low content of sulphur containing amino acids (cysteine and methionine), with methionine being often the first limiting amino acid. Collins (1986) found a slightly higher methionine concentration in LPC from alfalfa than red clover suggesting the importance of the plant species in this regards.

Up to date, not much research has focused on the agricultural practices required to optimize the protein extraction within a green biorefinery context. For instance, Arkcoll and Festenstein (1971) studied in detail some agronomic factors affecting the production and extraction of leaf protein like plant species and variety, soil fertility, age at harvest, climate and plant density. However, their study focuses on the extraction of proteins from the crops into the juice by means of a lab-scale pulper together with a press but it does not include the production of leaf protein concentrates. Some of their results pointed out that the protein yield is affected by both the plants age and growth stage and that most protein can be extracted if harvesting is performed just before the end of the vegetative growth. A recent study also focused on the amount of extractable protein, from some legume and grass species during spring growth (Solati et al., 2017).

Legumes resulted in higher amount of extractable protein per kg dry matter compared with grasses and the authors concluded that delaying the harvest time had a negative impact on the amount of extractable proteins (Solati et al., 2017). In their study, the amount of extractable protein was estimated based on fractionation of the crude protein into different protein fractions that were separately analyzed for N but no protein extraction procedure was performed. Therefore, there is a lack of knowledge relating how the plant species and their development stage might affect the whole biorefining process for the production of LPC. Moreover, the quality of the green juice in terms of dry matter, proteins and sugars as result of the composition of the plants at harvest is crucial for the accomplishment of the proposed biorefinery process via lactic acid fermentation.

The aim of this study was to investigate the effect of the plant species and their development stage on protein extractability and production of leaf protein concentrate using the lactic acid fermentation method in order to identify the optimal harvesting times. This included investigating how plant species and harvest time affects the quality of the green juice and hence, the lactic acid evolution. The three plant species chicory, red clover and timothy are all growing in Northern Europe, and typically utilized as forage crops, and were selected for this study due to their large difference in composition. Chicory is a herbaceous plant with relatively low fiber; red clover is a leguminous plant able to accumulate N as a result of fixing atmospheric N; and timothy is a fast-growing grass with relatively high fiber. The plants were processed at different harvesting times in order to ensure morphological changes due to development stage.

Material and Methods

2.1. Plant species and growth conditions

Three different plant species were selected for this study i.e. chicory (*Cichorium intybus* `Spandora´), red clover (*Trifolium pretense* `Milvus´) and timothy (*Phleum pretense* `Dolina´). Plants were seeded in 2014 and 2015 in an organic farmed experimental plot at the University of Copenhagen's research facility in Taastrup, Denmark (55°40´90.35"N, 12°18´24.84"E), 23 m above sea level. In 2014, plants were undersown with spring barley on 5th May in plots (3 X 10 m)**.** Plants were fertilized with 30 kg-S ha^{-1} in the form of Patentkali ® and 100 kg-N ha^{-1} of organic nitrogen (only chicory and timothy) in the form of Biogrow (Danish Agro A.m.b.a). Sulphur fertilization was applied 24th March 2015 (only plants seeded in 2014) and 7th April 2016. Nitrogen fertilizer was applied 25th March 2015 (only plants seeded in 2014) and 8 April 2016. Plants seeded in 2015 were row harrowed on 27th September 2015 and hand-weeded during April 2016. Row distance was 24 cm for all plants.

2.2. Plants harvesting and processing

Plants were hand-harvested 5 cm above ground level, to avoid the presence of soil in the harvested plant material, on different dates between May 20 and June 7, 2016 at three (chicory and red clover) or two (timothy) distinct phonological development stages (DS) (Table 1). Identification of the development stage of the plants at harvesting was performed based on the BBCH scale. For each species and development stage, three replicate samples were harvested and processed separately.

Freshly harvested plants (approx. 1 kg per sample) were stored at 5°C in sealed plastic bags for a maximum of two days before screw pressing on a twin gear stainless steel Angelia 8500S juicer, which operates at low turning speed creating almost no heat and friction and resulting a nutrient-rich juice (Angel Juicer Co., Queensland, Australia). Similar equipment has also been utilized for pressing and extracting proteins from sugar beet leaves (Tamayo Tenorio et al., 2016). Green juice samples were kept cold immediately after the screw pressing, frozen within two hours and stored until further use.

2.3. Green juice fermentation and protein extraction

The methodology utilized for the green juice fermentation and protein extraction was based on the process patented by Kiel et al. (2015) for providing functional proteins from a plant material. Green juice samples were unfrozen and lactic acid fermentation was run in triplicates for each plant species and development stage. The lactic acid fermentation of the green juice was performed alike previously described for the production of protein concentrates from red clover, clover grass, alfalfa and oilseed radish (Santamaría-Fernández et al., 2017). Between 150-200 g of green juice were inoculated with 5% (v/v) *Lactobacillus salivarius* BC 1001 pure culture. The lactic acid fermentation was performed at 38±1°C and 150 rpm. . During the lactic acid fermentation, 1.5-ml samples were taken every 2 hours in order to monitor the evolution of the fermentation. The lactic acid fermentation was stopped after 10 hours, when the pH had dropped to the proteins isoelectric point ca pH 4.0. Afterwards, the extraction of proteins was performed by centrifugation at 3800 rpm for 10 minutes which resulted in two fractions: the precipitated proteins (leaf protein concentrate, LPC) and the liquid fermented juice (brown juice, BJ).

2.4. Chemical analyses

Fresh plants samples were analyzed in terms of dry matter (DM) by drying the biomass at 80°C for two days. The N content was determined in the dried and finely grounded plant biomass samples by dynamic flash combustion (modified Dumas method) using a Flash 2000 CHNS-O Organic Elemental Analyzer (Thermo Fisher Scientific, Cambridge, UK). Green juice samples and protein concentrate samples were analyzed for pH, dry matter (DM), ash content, and Total Kjeldahl Nitrogen (TKN) according to APHA (2005). DM was determined by drying the samples at 105°C overnight. Ash content was determined by burning the samples at 550°C for 3 hours. TKN was determined by digestion of the samples in concentrated sulfuric acid with a catalyst, followed by separation of the ammonia into a boric acid solution by steam distillation and quantification of the ammonia by acid-base titration. The crude protein (CP) content was estimated based on the measured N content and a conversion factor of 6.25 for mass-N to mass-protein. Sugars and lactic acid were determined in the green juices and protein concentrates by HPLC on a Dionex Ultimate 3000-LC system with an Aminex® HPX-87H column coupled to a refractive index detector. H_2SO_4 (4 mmol L^{-1}) was used as mobile phase, with a flow rate of 0.6 ml min^{-1} at 60°C. Samples taken during the fermentation were also measured for pH, sugars and lactic acid, as previously described.

The amino acid composition was determined at Department of Animal Science, Aarhus University, Denmark, using the method adopted by the EC Regulation No 152/2009. This method determines free (synthetic and natural) and total (peptide bound and free) amino acids in feed, using a Biochrom 30 Amino Acid Analyzer (Biochrom Ltd.). The "true" protein content (TP) was calculated by adding up the concentration of all amino acids tested.

2.5. Calculations

The following equations (Eq. 1-3) were utilized in order to estimate the process yields:

$$\text{CP recovery (\%)} = (CP_{fraction} \times FW_{fraction}) / (CP_{input} \times FW_{input}) \times 100 \quad (1)$$

$$\text{Protein extractability (g-CP LPC/kg-DM plant)} = (CP_{LPC} \times YIELD_{LPC}) / DM_{plant} \quad (2)$$

$$\text{LPC production (g-dry LPC/kg-DM plant)} = (YIELD_{LPC} \times DM_{LPC}) / DM_{plant} \quad (3)$$

Where FW is fresh weight; input refers to the plant before screw pressing and to green juice before fermentation-centrifugation, respectively. In the overall CP recovery, input refers to the total plant CP. The $YIELD_{LPC}$ refers to the amount of LPC produced from 100 g plant (on wet weight basis).

2.6. Statistical analysis

Data were subjected to statistical analysis consisting of analysis of variance (ANOVA) followed by mean multi-comparison analysis using Tukey´s test to determine significant effects due to different plant species and development stages. Statistical analyses were performed with RStudio software (Version 1.0.136).

Results and discussion

3.1. Plant development stage and composition

The development stage (DS) and composition of the plants at harvesting are presented in Table 1. The dry matter (DM) content ranged between 11-14% for chicory plants, 16-27% for red clover plants and 26-31% for timothy plants. These values are in agreement with the DM contents previously detailed in the literature for red clover (16.4%), clover grass (18.7%) or alfalfa (15.4%) (Santamaría-Fernández et al., 2017), as well as for the leaves of various legume plants including red clover (9.4-16.5%) or white clover (9.6-12.5%) (Byers and Sturrock, 1965). The DM content in the plants at harvesting was significantly influenced by the plant species and DS at harvesting. As expected, a significant increase in the DM content with increasing maturity was observed, because of the accumulation of biomass during the plants' growth. Increasing DM concentrations and DM yields (in terms of ton DM ha^{-1}) with subsequent harvests were also found for five common grass species, including timothy (King et al., 2012b). Besides, the DM yield (ton DM ha^{-1}) significantly increased from the first to the last harvest dates for several legume and grass species, with a more pronounced increment in grasses than in legumes (Solati et al., 2017). In our study, the greatest DM increase was observed for red clover suggesting a higher productivity in red clover compared with chicory and timothy, and which could probably indicate the need for higher fertilization requirements in chicory and timothy plants.

The CP content also varied significantly among the three different plant species, being in a range between 10-15% DM in chicory, 16-22% DM in red clover and 8-10% DM in timothy. The significant effect of the plant species on the CP content is in agreement with observations found by Donnelly et al. (1983) for white clover, ryegrass, alfalfa and a mixture of ryegrass/white clover. In our study, red clover presented the highest CP content in all harvests, as expected when comparing legume species with non-legume species (Elgersma et al., 2014) or with grasses (Solati et al., 2017). Moreover, the CP decreased with increasing maturity in the three plants. Indeed, there was a meaningful reduction in the CP content from the first to the last harvest date for chicory, from 14% DM to 10% DM and for red clover, from 22% DM to 16% DM. A decline in the CP content with increasing maturity was also observed in several grasses and legumes by Solati et al. (2017), but opposite to our results, the CP decrease was larger for the grass species compared to legumes. According to Solati et al. (2017), the decreased CP content with increasing maturity shows changes in the protein fractions, in the content of soluble protein and, it could be related with the lower proportion of leaves, which have higher protein content. Several legumes, including red and white clover, also showed a decrease in the N content with increasing age (Byers and Sturrock, 1965). Further, the CP content decreased in five common grass species with advancing maturity and in particular, the greatest decrease was found between elongation and reproductive growth stages, which is in agreement with our results (King et al., 2012b).

Certainly, the composition of the plants at harvesting was significantly affected by the DS with a significant decrease in the CP with increasing maturity. Therefore, the DS of the plants at harvesting is a crucial factor to consider in order to maximize the extraction of proteins in a green biorefinery context, as also discussed by Solati et al. (2017). The plant species also showed an important effect on the plants composition but however, agricultural practices could probably contribute to minimize such effect e.g. fertilization rate or use of different plant varieties more suited for the protein extraction purpose. Nevertheless, legume species are advantageous compared to grass species because of their ability to fix atmospheric N, in association with rhizobia, reducing thus the need for N fertilizers.

3.2. Green juice production and composition

The screw pressing of chicory, red clover and timothy resulted in GJ wet weight yields of 71-74%, 61-72% and 46-54%, respectively (Fig. 1A). The proportion of GJ pressed out from the plants markedly depended on the DM content in the plants at harvesting. Indeed, a sharp decline in the GJ yield was observed with increasing DM in the plants (Fig. 1B) and accordingly, with increasing plant maturity or later harvesting. The amount of green juice being pressed out from the plants during screw pressing is crucial for the release of proteins i.e. the higher GJ yield, the also higher CP recovery in the GJ, as later discussed in Section 3.4. The GJ yields obtained in the present study were higher (Vodnar et al., 2010) or lower (Tamayo Tenorio et al., 2016) than reported in previous studies. As previously mentioned, the GJ yield depends on the DM content in the plant but the screw press itself is also a very important factor determining the GJ yield. In this study, a small lab-scale juicer was used, and it may be difficult to obtain similar amounts of GJ using larger scale screw presses, which often result in GJ yields between 40-50% according to our experience.

The composition of the different GJ is shown in Table 2. The CP content was in the range of 13-16% DM for chicory, 26-30% DM for red clover and 12-15% DM for timothy (i.e. 9-11 g L^{-1} for chicory, 31-37 g L^{-1} for red clover and 16-21 g L^{-1} for timothy). Therefore, the CP content was significantly larger in red clover GJ compared with chicory and timothy, as consequence of the large CP content in red clover together with the high GJ yields achieved. According to our results, green juices with higher CP content are expected to be produced from plants with larger CP content and not so high DM content. Consequently, GJ produced from grasses or from legume-grass mixtures might have reduced CP compared with GJ produced from legumes alone. For instance, Santamaría-Fernández et al. (2017) found that GJ from a clover grass mixture presented a lower CP content of 17% DM (based on total N x 6.25). Nevertheless, clover grass GJ with a CP content of 26% DM have been reported (Andersen and Kiel, 2000). Dietz et al. (2016) also reported clover grass GJ with CP contents in a range between 12-25% DM (based on total N x 6.25 and DM in the juices). Besides, the CP content in the GJ decreased with increasing maturity for the three plant species, similarly to the CP content in the plants. The largest decrease in the CP content was observed for red clover GJ, which also presented the greatest decline in CP in the plants. This highlights the fact that the GJ composition is highly determined by the composition of the plants at harvesting, as expected. Moreover, it is worth mentioning that the CP concentration in the GJ (in terms of g L^{-1}) was significantly increased from 31 g L^{-1} at DS1 to 37 g L^{-1} at DS3 for red clover while it was significantly decreased from 21 g L^{-1} at DS1 to 16 g L^{-1} at DS3 for timothy as a consequence of the increasing DM content in the GJ with maturity. Accordingly, it is likely that more proteins are fiber-bound both in the plant and in the GJ, with increasing plant maturity.

The content of sugars in the GJ, mainly glucose and fructose, was between 41-48% DM in chicory, 43-45% DM in red clover and 40-44% DM in timothy (i.e. 28-31 g L^{-1}, 46-63 g L^{-1} and 59-60 g L^{-1}, respectively). Hence, the different GJ presented very similar content of sugars on dry matter basis, regardless of the plant species or development stage. However, the concentration of sugars (in terms of g L^{-1}) varied significantly between plant species and DS, likely because of the different DM contents in the GJ, which might have an influence on the performance of the lactic acid fermentation. For instance, significantly higher sugar concentrations were found in red clover and timothy GJ compared with chicory GJ. In previous works, alfalfa green juices with between 5-12 g L^{-1} glucose and between 6-10 g L^{-1} fructose were described by Papendiek and Venus (2014) and, clover green juices presented concentrations of glucose between 3-12 g L^{-1} and of fructose between 2-16 g L^{-1} (Dietz et al., 2016).

3.3. Lactic acid fermentation in green juices

Lactic acid fermentation in the GJ was carried out for 10 hours, as shown in Fig. 2. After 10 hours, the lactic acid concentration in the fermented juices was between 10-11 g L^{-1} for chicory, 14-21 g L^{-1} for red clover and 19-23 g L^{-1} for timothy. Some differences in the lactic acid concentration at the end of the fermentation were observed for red clover and timothy juices. The lactic acid concentration increased 1.5-fold and 1.2-fold from DS1 to DS3 in red clover and timothy juices, probably because of the increased glucose concentration in those GJ with increasing maturity. Likewise, the lower lactic acid concentration achieved in chicory juices is probably related with the lower glucose concentration found in those juices. Dietz et al. (2016) attributed the differences observed during the lactic acid fermentation of three different GJ to the varying N content in the GJ i.e. higher substrate conversion was related with higher ammonia concentration. However, those differences were only observed after 10 hours of lactic acid fermentation and therefore, N limitations were not likely in our case.

During the lactic acid fermentation of the GJ, the pH dropped as a consequence of the lactic acid production (Fig. 2). The pH evolution was very similar in all the different juices and after 10 hours, the pH in the fermented juices was within 3.8-4.0. The low pH achieved at the end of the fermentation indicates the good performance of the lactic acid bacteria facilitating the precipitation of proteins. Ajibola (1984) pointed out pH 4.5 as the point where a clear separation between the supernatant (brown juice) and the green precipitate (protein concentrate) can be made. In the present study, around 6 hours of fermentation were required to reach a pH of around 4.5 but however, the lactic acid fermentation was carried to a lower pH (3.8-4.0). The idea was to drop the pH in the juice close to the isoelectric point of the leaf proteins to precipitate as many proteins as possible. For instance, the solubility of proteins from spinach leaves was minimal at pH 4.0 with around 75% of protein precipitation at such pH (Merodio et al., 1983). However, the isoelectric point can differ between the different proteins found in a specific GJ as well as between the proteins found in GJ produced from different plants. Moreover, some other soluble components found in the GJ could also precipitate at a lower pH, which might later influence the protein content in the leaf protein concentrate. Indeed, the minimum chlorophyll solubility occurred between pH 3.7 and 4.0 in spinach leaves (Merodio et al., 1983). Therefore, further research should be carried out to identify optimal final pH in order to maximize the precipitation of proteins while minimizing the precipitation of other plant components.

3.4. Protein recoveries along the process

The screw pressing of the plants resulted in significantly different CP recoveries in the GJ i.e. 45-48% for chicory, 50-58% for red clover and 28-35% for timothy (Fig. 3A). The highest CP recoveries were achieved for red clover GJ, with more than half of the plant proteins extracted into the GJ after screw pressing, which is probably related with a higher proportion of soluble proteins as well as with the high CP content found in red clover plants. Previous studies described CP recoveries in the GJ after screw pressing for red clover, clover grass and alfalfa to be 25%, 33% and 39%, respectively (Santamaría-Fernández et al., 2017). Otherwise, Digman et al. (2013) concluded that only 30% of the total leaf proteins could be recovered after screw pressing alfalfa leaves. The relatively high CP recoveries obtained in our study for all GJ were probably achieved due to the elevated efficiency of the screw press compared to other studies. Besides, a significant decline in the CP recovery was observed for red clover and timothy GJ with increasing plant maturity, which is probably related with the composition of the plants in terms of DM and CP. It is likely that the large DM increase in red clover and timothy plants found between the first and last harvest dates is the reason for the decrease in the CP recoveries in the GJ with increasing plant maturity. More proteins were held in the fiber-rich press cake with the less availability of water in the plants. Indeed, the low DM content in chicory favored the protein extraction compared with timothy, which had twice as much DM but significantly lower CP recovery in GJ. Therefore, the water content in the plants plays an important role favoring the release of proteins during screw pressing and should be always considered for such purpose. Moreover, ensuring large CP recoveries in the GJ during screw pressing is crucial to achieve high CP recoveries in the LPC for the overall process so the efficiency of the screw press is also important.

On the other hand, only minor differences were observed regarding the CP recovery in the LPC after the lactic acid fermentation and centrifugation of the GJ, with between 72-86% of the CP in the GJ recovered in the LPC (Fig. 3B). Specifically, the CP recoveries accounted for 79-83% in chicory LPC, 72-80% in red clover LPC and 76-86% in timothy LPC. The fermented juices presented really similar pH values at the end of the lactic acid fermentation and hence, the alike proportion of precipitated proteins and the alike CP recoveries in the final LPC. Lower CP recoveries in the LPC (i.e. 67% for red clover, 52% for clover grass, 39% for alfalfa and 44% for oilseed radish) were previously reported (Santamaría-Fernández et al., 2017), probably because of the slightly higher pH obtained after the lactic acid fermentation (pH 4.1-4.3) compared to this study (pH 3.8-4.0).

Relatively high CP recoveries from the plants into the LPCs were achieved overall in this study i.e. between 36-40% for chicory, 40-42% for red clover and 24-26% for timothy plants (Fig. 3C). As it was expected, red clover resulted in the highest overall CP recoveries, but closely followed by chicory. However, significantly lower overall CP recoveries were observed for timothy, which is probably related with the high DM of timothy plants (26-31%), as already discussed. Actually, cellulose was reported as the main factor hampering protein extractability during alkali protein extraction from diverse biomasses (Sari et al., 2015). Accordingly, timothy grass might not be suitable for producing LPC because of the high content of fibers, which likely increases with maturity. Previous research using the same methodology as in the present study resulted in lower CP recoveries in the LPC i.e. 23% from red clover, 17% from clover grass, 15% from alfalfa and 12% from oilseed radish (Santamaría-Fernández et al., 2017). Such differences in the CP recoveries with previous work can be related to differences in the efficiency of the screw pressing machinery and to the performance of the fermentation, as previously mentioned. At this point it is worth mentioning the strong influence of the CP recovery in the GJ (Fig. 3A) on the overall CP recoveries in the LPC (Fig. 3C) observed in the present study, which highlights the importance of the composition of plant and the screw pressing efficiency in order to achieve good process yields, especially in terms of proteins. Even though any significant difference in the overall CP reveries was observed with varying DS, there was a decreasing trend with increasing plant maturity, especially for red clover and timothy, mainly caused by differences from the screw pressing.

3.5. Leaf Protein Concentrate: an organic protein feed

The composition of the leaf protein concentrates (LPC) is presented in Table 3. The DM content in LPC was in the range of 16-24% and the CP represented between 29-33% DM in chicory LPC, 35-42% DM in red clover LPC and 19-23% DM in timothy LPC. The LPC presented CP contents comparable to previous studies (Santamaría-Fernández et al., 2017); however, the CP content represented less than 50% DM in all the LPC and was particularly low in timothy LPC. According to our results, the LPC produced from red clover presented the highest CP content suggesting that red clover LPC presented the best quality to be used as protein feed for monogastric animals compared to chicory and timothy LPC. Previous studies have also found a significant effect of the plant species on crude nitrogen content in the LPC (Donnelly et al., 1983). Further, the CP content was slightly reduced with maturity in chicory and red clover LPC, which might relate with an increased fiber content in the LPC and hence, with a reduced quality of the LPC with increasing maturity of the plants. The proportion of TP relative to CP in red clover LPC also decreased with plant maturity suggesting that the protein N to non-protein N ratio decreases with plants age, as also discussed by Arkcoll and Festenstein (1971), which could reduce the quality of the LPC as well.

Apart from proteins, the LPC contained free sugars (7-21% DM), lactic acid (4-9% DM) due to the lactic acid fermentation, dietary fibers (non-starch polysaccharides) (7-12 % DM), fat (6-10%), lignin (10-14% DM), and inorganic material (5-10% DM) probably from the presence of soil in the plants at harvesting. The presence of free sugars in the LPC, despite their low pH (3.9-4.0), might not be beneficial since sugars are easily fermentable substrates for some microorganisms, which could hinder the stability and quality of the LPC. For instance, some fungi like *Mucor racemosus* are not inhibited at the low pH conditions and might be responsible for microbial spoilage of the LPC (Arkcoll, 1973). In addition, Maillard reactions between reducing sugars and lysine could take place during thermal processing limiting lysine availability and decreasing the nutritional value of the proteins (Gilani et al., 2012). Indeed, greater losses of available lysine were observed in legumes with larger amounts of reducing sugars after heat treatment (Almas and Bender, 1980). The lipid fraction of the LPC, which may represent between 20-30% according to Arkcoll (1973), can also oxidize rapidly causing co-oxidation of sulfur-amino acids and thereby, limiting their availability and reducing the nutritional quality of the LPC (Arkcoll, 1973). Consequently, the LPC processing in terms of heat drying, freeze-drying or vacuum packing is crucial for preserving the quality and stability of the LPC product.

The concentration of amino acids was analyzed only for the three LPC produced from red clover (Table 4). The amino acid composition is important for the suitability and quality of the LPC as an organic protein source for monogastric animals such as poultry. Actually, a deficit in a single essential amino acid triggers a generalized protein deficiency (Blair, 2008). In general, the concentration of amino acids in the LPC decreased significantly with plant maturity, following the same trend observed for the CP in the LPC (Table 3). Indeed, the concentration of each amino acid was between 1.1-fold and 1.4-fold higher in the LPC at DS1 than at DS3. The proportion of true protein (TP) relative to the CP represented 84% in red clover LPC at DS1 while it was reduced to 81% at DS2 and DS3. Therefore, the quality of the red clover LPC in terms of amino acids and true protein contents was slightly reduced with increasing maturity. Nevertheless, the proportion of essential amino acids relative to TP was very similar amongst the LPC (Fig. 4).

Mostly, the LPC from red clover showed a balanced and suitable amino acid concentration compared with soybeans (Steenfeldt and Hammershøj, 2015) and lupines (Hammershøj and Steenfeldt, 2005). For instance, the content of some essential amino acids i.e. isoleucine, leucine, methionine, phenylalanine, threonine and valine was larger in the LPC than in soybeans while all essential amino acids, except for arginine were more abundant in the LPC compared with lupine. In legume grains, there is usually a relative deficit in sulfur amino acids (methionine and cysteine) and tryptophan. In particular, methionine is crucial for the feather forming process (van de Weerd et al., 2009) and its deficiency might affect hens growth and egg production (Hammershøj and Steenfeldt, 2005). The methionine content in the LPC was up to 1.4-fold higher than in soybeans and up to 3.3-fold higher than in lupine. Indeed, a lack of methionine in lupine for organic layer diets was already reported by Hammershøj and Steenfeldt (2005). Lysine is also a valuable amino acid in poultry feeds for optimal egg production (Hammershøj and Steenfeldt, 2005) and it is the most limiting amino acid in most animal feeds. Lysine in the LPC was in similar concentration compared with soybeans and in higher concentration compared to lupine. On the other hand, the concentration of all amino acids in the red clover LPC was slightly lower compared with red clover LPC previously produced (Santamaría-Fernández et al., 2017). Further, a recent review focusing on alfalfa leaf protein separation technology detailed the content of amino acids in alfalfa leaf protein (Zhang et al., 2017). The content of all essential amino acids in the alfalfa leaf protein was lower than in the LPC produced from red clover in our study, probably due to a different methodology for the protein precipitation and separation. Overall, the LPC proved suitable organic protein feed for poultry diets in terms of amino acid composition. However, the digestibility and presence of anti-nutritional factors (ANFs) should be also considered for a complete evaluation of the nutritive value of the LPC (Hussein et al., 1999).

3.6. Overall figures for the green biorefinery

The overall figures for the green biorefinery process are summarized in Table 5. The protein extractability (i.e. amount of CP extracted per kg DM plant) ranged between 41-56 g CP per kg DM for chicory, 65-98 g CP per kg DM for red clover, and 16-32 g CP per kg DM for timothy. Therefore, red clover resulted in the largest protein extractability and moreover, a decreasing trend in the protein extractability with increasing plant maturity was observed for the three plant species. In particular, the protein extractability was markedly reduced by 1.5-fold for red clover between DS1 and DS3. In general, the increased DM content and the reduced CP content observed for the plants with increasing maturity explain such decline in the protein extractability. Besides, the LPC production (i.e. amount of dry LPC produced per kg DM plant) was in a range of 142-173 g dry LPC per kg DM for chicory, 186-235 g dry LPC per kg DM for red clover, and 69-167 g dry LPC per kg DM for timothy. Accordingly, large differences in the LPC production were found between plants, with red clover resulting also in the greatest LPC production. The LPC production was also reduced with increasing plant maturity for the three plant species, but especially for red clover and timothy.

The protein extractability and the LPC production differed significantly between the three plant species studied. Best results were obtained for red clover, which as a leguminous plant presented the highest CP content compared with chicory and timothy. The use of legumes like clover or alfalfa in this type of green biorefineries is preferred because of their advantageous process yields as seen in the present study, which might also encourage their cultivation and use in crop rotations. Moreover, the use of legumes is related with lower requirements for N fertilization due to their ability to fix atmospheric N and thus, a more sustainable agricultural production. Nevertheless, chicory also resulted in relatively high figures in terms of LPC production, despite its lower CP content compared with red clover. Therefore, the selection of the plant species is important in this type of green biorefinery. However, agricultural practices including the selection of varieties for a specific plant species or fertilization rates are also important and could diminish the differences found between plants in terms of process recoveries. On the other hand, the development stage of the plants is crucial for optimizing the figures for this type of green biorefinery, as shown in our study. Changes in the plants composition with maturity e.g. increasing DM and fiber contents as well as decreasing CP content negatively affected the CP recoveries along the process, in particular during screw pressing. Indeed, achieving high CP recoveries during screw pressing, favored by relatively high water content in the plants, positively contributes to large overall CP recoveries. According to our results, the decrease in the protein extractability and LPC production was more pronounced between DS2 and DS3 suggesting that plants should be harvested before flowering when utilized as feedstock for protein extraction in a green biorefinery concept.

Conclusions

Overall, between 24-42% of the plant proteins were extracted into the leaf protein concentrates produced in this study. The high protein recoveries (72-86%) achieved after the lactic acid fermentation and centrifugation of the green juices indicates that the fermentation can be efficiently applied for the precipitation of proteins in a less energy-demanding and cleaner process compared to conventional methods like heat coagulation or acidification. Protein extractability was close to 2-fold higher in red clover compared with chicory, and up to 4-fold higher compared with timothy for a particular development stage; while the LPC production from red clover was between 1.3-2.7 times greater than from chicory or timothy at the same development stage. Such significant differences observed between plant species highlight the importance of utilizing N-rich and low-fiber plant species like legumes for a protein green biorefinery. Agricultural practices should also be studied in this regards in order to maximize the protein content in the plants and probably use a varied range of plant species. Besides, the development stage of the plants is crucial since significant reductions in protein extractability and LPC production were observed for the plants with increasing maturity, which is related with the accumulation of dry matter, especially fiber hindering the extraction of proteins. An early harvesting in the plant development will probably benefit the process yield, as we have proven. Outcomes from the present study are relevant for the establishment of green biorefineries contributing to a more sustainable agricultural development.

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References

Ajibola, O.O., 1984. Physical, chemical and microbiological changes during green plant juice fermentation. Food Chem. 13, 181–192. https://doi.org/10.1016/0308-8146(84)90072-4

Almas, K., Bender, A.E., 1980. Effect of Heat Treatment of Legumes on Available Lysine. J. Sci. food Agric. 31, 448–452.

Andersen, M., Kiel, P., 2000. Integrated utilisation of green biomass in the green biorefinery. Ind. Crops Prod. 11, 129–137. https://doi.org/10.1016/S0926-6690(99)00055-2

APHA, 2005. Standard Methods for the Examination of Water and Wastewater, twenty-first ed. American Public Health Association, American Water Works Association and Water Environment Federation, Washington DC

Arkcoll, D.B., 1973. The preservation and storage of leaf protein preparations. J. Sci. Food Agric. 24, 437–445. https://doi.org/10.1002/jsfa.2740240412

Arkcoll, D.B., Festenstein, G.N., 1971. A preliminary study of the agronomic factors affecting the yield of extractable leaf protein. J. Sci. Food Agric. 22, 49–56. https://doi.org/10.1002/jsfa.2740220202

Baraniak, B., 1990. The effect of flocculant applied in the process of fractionating alfalfa juice on the chemical composition of the obtained protein concentrates. Anim. Feed Sci. Technol. 31, 305–311. https://doi.org/10.1016/0377-8401(90)90135-U

Betschart, A., Kinsella, J.E., 1973. Extractability and Solubility of Leaf Protein. J. Agric. Food Chem. 21, 60–65. https://doi.org/10.1136/bmj.283.6299.1121-a

Blair, R. (Ed.), 2008. Nutrition and feeding of organic poultry. CABI, Wallingford. https://doi.org/10.1079/9781845934064.0000

Byers, M., Sturrock, J.W., 1965. The yields of leaf protein extracted by large-scale processing of various crops. J. Sci. food Agric. 16, 341–355.

Collins, M., 1986. Composition of Leaf Protein Concentrate and Deproteinized Juice from Alfalfa, Red Clover, and Birdsfoot Trefoil. Agron. J. 78, 1018–1022. https://doi.org/10.2134/agronj1986.00021962007800060017x

Dietz, D., Schneider, R., Papendiek, F., Venus, J., 2016. Leguminose green juice as an efficient nutrient for L(+)-lactic acid production. J. Biotechnol. 236, 26–34. https://doi.org/10.1016/j.jbiotec.2016.07.008

Digman, M.F., Runge, T.M., Shinners, K.J., Hatfield, R.D., 2013. Wet fractionation for improved utilization of Alfalfa leaves. Biol. Eng. Trans. 6, 29–42.

Donnelly, P.E., McDonald, R.M., Rattray, P. V., 1983. Protein extraction from pasture: Effect of crop species and of a reducing agent on the quality of extracted protein. J. Sci. Food Agric. 34, 828–838. https://doi.org/10.1002/jsfa.2740340809

EC 152/2009. Commission Regulation (EC) No 152/2009 of 27 January 2009 laying down the methods of sampling and analysis for the official control of feed. Annex III Methods of analysis to control the composition of feed materials and compound feed. Off. J. Eur. Union, 26.2.2009, L54/1-130, 23-32.

Edwards, R.H., Miller, R.E., De Fremery, D., Knuckles, B.E., Bickoff, E.M., Kohler, G.O., 1975. Pilot plant production of an edible white fraction leaf protein concentrate from alfalfa. J. Agric. Food Chem. 23, 620–626. https://doi.org/10.1021/jf60200a046

Elgersma, A., Søegaard, K., Jensen, S.K., 2014. Herbage dry-matter production and forage quality of three legumes and four non-leguminous forbs grown in single-species stands. Grass Forage Sci. 69, 705–716. https://doi.org/10.1111/gfs.12104

Gilani, G.S., Xiao, C.W., 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. Br. J. Nutr. 108, S315–S332. https://doi.org/10.1017/S0007114512002371

Hammershøj, M., Steenfeldt, S., 2005. Effects of blue lupin (*Lupinus angustifolius*) in organic layer diets and supplementation with foraging material on egg production and some egg quality parameters. Poult. Sci. 84, 723–733. https://doi.org/10.1093/ps/84.5.723

Hussein, L., el-Fouly, M.M., el-Baz, F.K., Ghanem, S. a, 1999. Nutritional quality and the presence of anti-nutritional factors in leaf protein concentrates (LPC). Int. J.

Food Sci. Nutr. 50, 333–343. https://doi.org/10.1080/096374899101067

Höltinger, S., Schmidt, J., Schönhart, M., Schmid, E., 2014. A spatially explicit techno-economic assessment of green biorefinery concepts. Biofuels, Bioprod. Biorefining 8, 325–341. https://doi.org/10.1002/bbb

Kamm, B., Hille, C., Schönicke, P., 2010. Green biorefinery demonstration plant in Havelland (Germany). Biofuels, Bioprod. Biorefining 6, 253–262. https://doi.org/10.1002/bbb

Kiel, P., Andersen, M., Lübeck, M., 2015. A method for providing functional proteins from a plant material. PCT DK2015 050185. WO2015197078.

King, C., McEniry, J., O'Kiely, P., Richardson, M., 2012a. The effects of hydrothermal conditioning, detergent and mechanical pressing on the isolation of the fibre-rich press-cake fraction from a range of grass silages. Biomass and Bioenergy 42, 179–188. https://doi.org/10.1016/j.biombioe.2012.03.009

King, C., McEniry, J., Richardson, M., O'Kiely, P., 2012b. Yield and chemical composition of five common grassland species in response to nitrogen fertiliser application and phenological growth stage. Acta Agric. Scand. Sect. B Soil Plant Sci. 62, 644–658. https://doi.org/10.1080/09064710.2012.687055

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 sativa subsp. sativa*). Comparison between heat coagulation/centrifugation and ultrafiltration. Desalination 163, 253–259. https://doi.org/10.1016/S0011-9164(04)90197-X

Kromus, S., Wachter, B., Koschuh, W., Mandl, M., Krotscheck, C., Narodoslawsky, M., 2004. The green biorefinery Austria-development of an integrated system for green biomass utilization. Chem. Biochem. Eng. Q. 18, 7–12.

Kromus, S., Kamm, B., Kamm, M., Fowler, P., Narodoslawsky, M., 2008. Green Biorefineries: The Green Biorefinery Concept - Fundamentals and Potential, Biorefineries-Industrial Processes and Products: Status Quo and Future Directions. https://doi.org/10.1002/9783527619849.ch12

Martin, A.H., Nieuwland, M., Jong, G.A.H. De, 2014. Characterization of Heat-Set Gels from RuBisCO in Comparison to Those from Other Proteins. J. Agric. Food Chem. 62, 10783–10791. https://doi.org/10.1021/jf502905g

Merodio, C., Martin, M., Sabater, B., 1983. Improved Separation of Green and Soluble Leaf Proteins by pH Shift. J. Agric. Food Chem. 31, 957–959. https://doi.org/10.1021/jf00119a009

Morrison, J.E., Pirie, N.W., 1961. The large-scale production of protein from leaf extracts. J. Sci. Food Agric. 12, 1–5. https://doi.org/10.1002/jsfa.2740120101

Papendiek, F., Venus, J., 2014. Cultivation and fractionation of leguminous biomass for lactic acid production. Chem. Biochem. Eng. Q. 28, 375–382. https://doi.org/10.15255/CABEQ.2013.1854

RStudio Team, 2018. RStudio: Integrated Development for R. RStudio, Inc., Boston, MA. http://www.rstudio.com/.

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

Sari, Y.W., Syafitri, U., Sanders, J.P.M., Bruins, M.E., 2015. How biomass composition determines protein extractability. Ind. Crops Prod. 70, 125–133.

https://doi.org/10.1016/j.indcrop.2015.03.020

Solati, Z., Jørgensen, U., Eriksen, J., Søegaard, K., 2017. Dry matter yield, chemical composition and estimated extractable protein of legume and grass species during the spring growth. J. Sci. Food Agric. 97, 3958–3966. https://doi.org/10.1002/jsfa.8258

Steenfeldt, S., Hammershøj, M., 2015. Organic egg production. I: Effects of different dietary protein contents and forage material on organic egg production, nitrogen and mineral retention and total tract digestibility of nutrients of two hen genotypes. Anim. Feed Sci. Technol. 209, 186–201. https://doi.org/10.1016/j.anifeedsci.2015.08.001

Tamayo Tenorio, A., 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 Chem. 203, 402–408. https://doi.org/10.1016/j.foodchem.2016.02.092

van de Weerd, H.A., Keatinge, R., Roderick, S., 2009. A review of key health-related welfare issues in organic poultry production. Worlds Poult. Sci. J. 65, 649–684. https://doi.org/Doi 10.1017/S0043933909000464

Vodnar, D.C., Venus, J., Schneider, R., Socaciu, C., 2010. Lactic acid production by Lactobacillus paracasei 168 in discontinuous fermentation using lucerne green juice as nutrient substitute. Chem. Eng. Technol. 33, 468–474. https://doi.org/10.1002/ceat.200900463

Zhang, W., Grimi, N., Jaffrin, M.Y., Ding, L., Tang, B., 2017. A short review on the research progress in alfalfa leaf protein separation technology. J. Chem. Technol. Biotechnol. https://doi.org/10.1002/jctb.5364

Figure legends:

Figure 1. (A) Green juice yield for chicory, red clover and timothy at DS1 (white), DS2 (light grey) and DS3 (dark grey) after screw pressing; (B) correlation between the DM content in the plants at harvesting and the green juice yield for chicory (rhombus), red clover (circle) and timothy (square) at DS1 (white), DS2 (light grey) and DS3 (dark grey).

Figure 2. Evolution of pH (grey), glucose (white) and lactic acid (black) during fermentation of green juices from chicory (A), red clover (B) and timothy (C) at DS1 (circle), DS2 (square) and DS3 (triangle). Bars show standard deviation for all results.

Figure 3. CP recovery in the GJ after screw pressing (A), in the LPC after fermentation-centrifugation (B) and overall CP recovery (C) for chicory, red clover and timothy at DS1 (white), DS2 (light grey) and DS3 (dark grey). Bars show standard deviation.

Figure 4. Content of essential amino acids (% of true protein) in the LPC produced from red clover at DS1 (white), DS2 (light grey) and DS3 (dark grey). Bars show standard deviation.

Table 1. Development stage (DS) and composition of the plants at harvest. Standard deviation is shown in brackets.

Plant	**Development stage (DS)**	**Harvest date**	**DM (%)**	**CP (%DM)**
Chicory	*DS1* *Elongation* – Main shoot begins to elongate	23 May	11.5 (1.2) A*	14.5 (1.0) AB
	DS2 *Elongation* – 5 to 9 internodes elongated	23 May	11.1 (1.3) A	11.8 (0.4) AC
	DS3 *Inflorescence emergence* – 1st individual flowers of secondary inflorescence visible (still closed)	2 June	14.2 (1.9) AB	9.6 (2.7) C
Red clover	*DS1* *Elongation* – 40 % of full height	20 May	16.4 (0.7) BC	21.7 (0.4) D
	DS2 *Inflorescence emergence* – Full length, buds visible in some plants	27 May	19.6 (0.9) C	17.4 (1.0) B
	DS3 *Flowering* – halfway to full flowering	7 June	26.5 (0.7) D	16.4 (1.1) B
Timothy	*DS1* *Elongation* – 2nd internode detectable	16 May	25.9 (0.2) D	10.0 (0.1) C
	DS3 *Earing* – Ear 70 % passed	3 June	31.0 (1.3) E	8.1 (0.1) C

*For each column, mean values with different alphabet letter indicate significantly different values ($p < 0.05$).

Table 2. Composition of the green juices. Standard deviation is shown in brackets.

Plant and development stage		**DM** (%)	**CP** (%DM)	**Glucose** (%DM)	**Fructose** (%DM)	**Sugars*** (%DM)
Chicory	*DS1*	6.9 (0.3) A**	16.4 (0.4) AB	19.1 (0.9) A	19.8 (2.5) A	41.3 (3.4) A
	DS2	6.5 (0.5) A	16.1 (1.9) AB	21.5 (0.9) A	24.2 (1.1) A	47.7 (1.6) A
	DS3	7.0 (0.5) A	13.2 (0.9) AC	19.7 (0.7) A	22.9 (0.8) A	43.8 (1.4) A
Red clover	*DS1*	10.4 (0.2) B	29.9 (1.6) D	26.9 (0.5) B	9.7 (0.2) B	44.0 (0.6) A
	DS2	12.4 (0.2) C	27.9 (0.2) DE	26.3 (0.9) BC	9.2 (0.3) B	42.8 (1.3) A
	DS3	14.1 (0.5) D	26.2 (0.7) E	27.3 (1.0) B	11.4 (0.9) B	44.9 (1.7) A
Timothy	*DS1*	14.9 (0.6) D	15.1 (0.6) BC	14.5 (1.3) D	24.8 (4.5) A	40.4 (5.5) A
	DS3	13.6 (0.8) CD	12.3 (1.0) C	22.6 (2.9) AC	19.8 (2.8) A	43.7 (5.7) A

*Sum of glucose, fructose, arabinose and cellobiose.

**For each column, mean values with different alphabet letter indicate significantly different values ($p < 0.05$).

Table 3. Composition of the leaf protein concentrates. Standard deviation is shown in brackets.

Plant and development stage		**DM** (%)	**CP** (%DM)	**Sugars*** (%DM)	**Lactic Acid** (%DM)	**Inorganics** (%DM)
Chicory	*DS1*	20.2 (1.3) AC**	31.5 (3.2) AB	7.4 (0.4) A	4.4 (0.5) A	10.2 (1.1) A
	DS2	18.4 (0.6) AB	32.8 (2.4) AC	8.8 (0.2) AB	5.6 (0.4) AB	7.1 (1.1) BC
	DS3	16.3 (1.7) B	28.7 (3.2) AB	11.3 (1.5) ABC	6.0 (1.3) AB	8.2 (0.6) ACD
Red clover	*DS1*	21.5 (0.6) C	41.5 (1.4) D	12.0 (0.8) BC	5.5 (0.4) AB	5.0 (1.3) B
	DS2	22.0 (0.4) C	40.5 (2.1) DC	13.9 (0.8) CD	6.4 (0.4) BC	5.0 (0.3) B
	DS3	23.5 (0.7) C	35.0 (5.9) AD	14.7 (0.2) CD	7.0 (0.3) BC	6.0 (0.7) BD
Timothy	*DS1*	21.5 (0.3) C	19.3 (1.5) E	21.4 (2.6) E	7.9 (0.6) CD	6.7 (0.4) BD
	DS3	21.4 (0.5) C	23.2 (2.1) BE	19.3 (2.4) E	9.2 (0.4) D	8.3 (0.3) ACD

*Sum of glucose, fructose, arabinose and cellobiose.

**For each column, mean values with different alphabet letter indicate significantly different values ($p < 0.05$).

Table 4. True protein and amino acids content in the LPC produced from red clover at DS1, DS2 and DS3. Standard deviation is shown in brackets.

	DS1	**DS2**	**DS3**	**Soybeans**[a]	**Lupine**[b]
TP (%DM)	34.7 (0.3) A*	32.7 (0.8) A	28.2 (0.7) B		
Essential amino acids (g/kg DM)					
Arg	23.0 (0.2) A	21.3 (0.5) A	18.1 (0.5) B	31.4	35.9
His	9.2 (0.0) A	8.5 (0.1) A	7.3 (0.2) B	10.1	8.8
Ile	19.9 (0.3) A	18.5 (0.4) A	16.1 (0.6) B	18.5	13.6
Leu	34.0 (0.2) A	32.3 (0.7) A	28.0 (0.7) B	29.3	21.4
Lys	23.6 (0.3) A	22.0 (0.5) A	19.4 (0.5) B	26.2	15.1
Met	7.0 (0.1) A	6.5 (0.2) A	5.5 (0.1) B	5.2	2.2
Phe	23.1 (0.2) A	21.5 (0.7) A	17.8 (0.4) B	19.7	12.5
Thr	17.9 (0.1) A	17.0 (0.3) A	14.9 (0.4) B	15.6	11.3
Val	24.2 (0.1) A	22.8 (0.5) A	19.9 (0.6) B	18.0	13.1
Non-essential amino acids (g/kg DM)					
Ala	22.7 (0.2) A	21.1 (0.6) A	18.2 (0.5) B	16.9	10.9
Asp	43.9 (1.0) A	41.3 (1.3) A	35.4 (0.5) B	43.8	33.2
Cys	2.1 (0.0) A	1.7 (0.0) AB	1.5 (0.1) B	5.8	5.0
Glu	41.6 (0.4) A	38.9 (1.0) A	33.8 (0.9) B	69.3	66.5
Gly	20.0 (0.1) A	19.0 (0.6) A	16.1 (0.4) B	16.6	13.3
Pro	17.6 (0.2) A	17.7 (0.5) A	15.4 (0.3) B	18.3	12.8
Ser	17.4 (0.2) A	16.5 (0.4) A	14.4 (0.4) B	20.9	17.1

[a]Steenfeldt and Hammershøj, 2015

[b]Hammershøj and Steenfeldt, 2005

*For each row, mean values with different alphabet letter indicate significantly different values ($p < 0.05$).

Table 5. Overall figures for the green biorefinery.

Plant and development stage		**Protein extractability** g-CP LPC/kg-DM plant	**LPC production** g-dry LPC/kg-DM plant
Chicory	*DS1*	54.6 (11.1) AB*	172.8 (22.0) A
	DS2	56.0 (8.7) AB	170.5 (21.1) A
	DS3	40.6 (1.5) A	142.5 (10.3) A
Red clover	*DS1*	97.7 (9.4) C	235.0 (17.0) B
	DS2	91.5 (6.5) C	225.8 (14.4) B
	DS3	65.1 (10.8) BD	185.8 (5.9) A
Timothy	*DS1*	32.1 (2.1) DE	166.6 (16.4) A
	DS3	15.9 (2.6) E	68.9 (11.6) C

*For each column, mean values with different alphabet letter indicate significantly different values ($p < 0.05$).

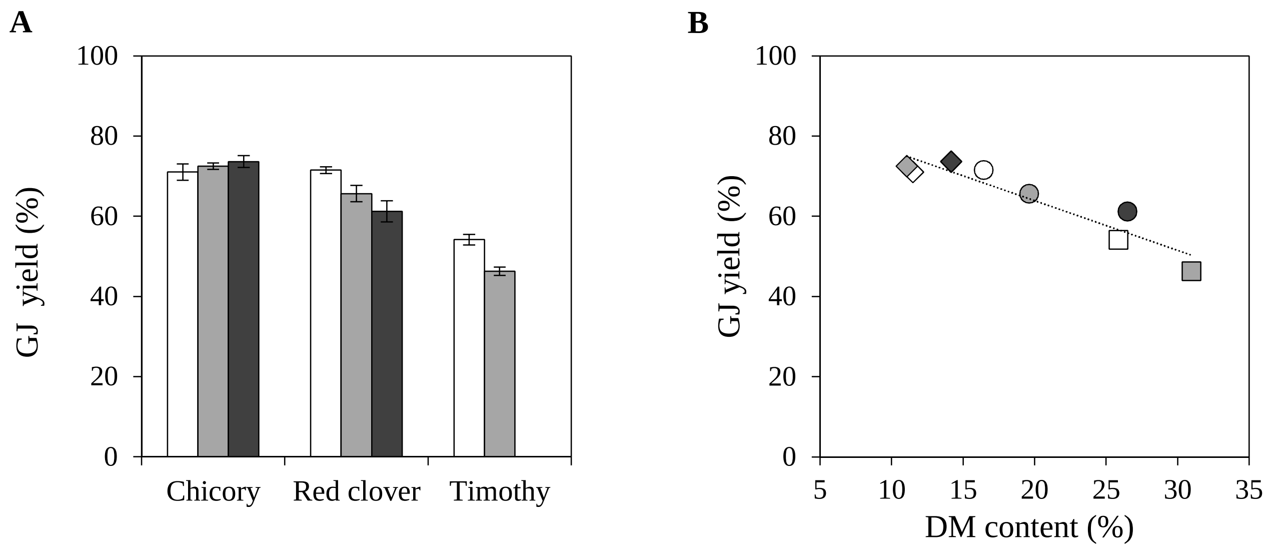
A
100
80
60
40
20
0
GJ yield (%)
Chicory
Red clover
Timothy
B
100
80
60
40
20
0
GJ yield (%)
5
10
15
20
25
30
35
DM content (%)

Figure 1.

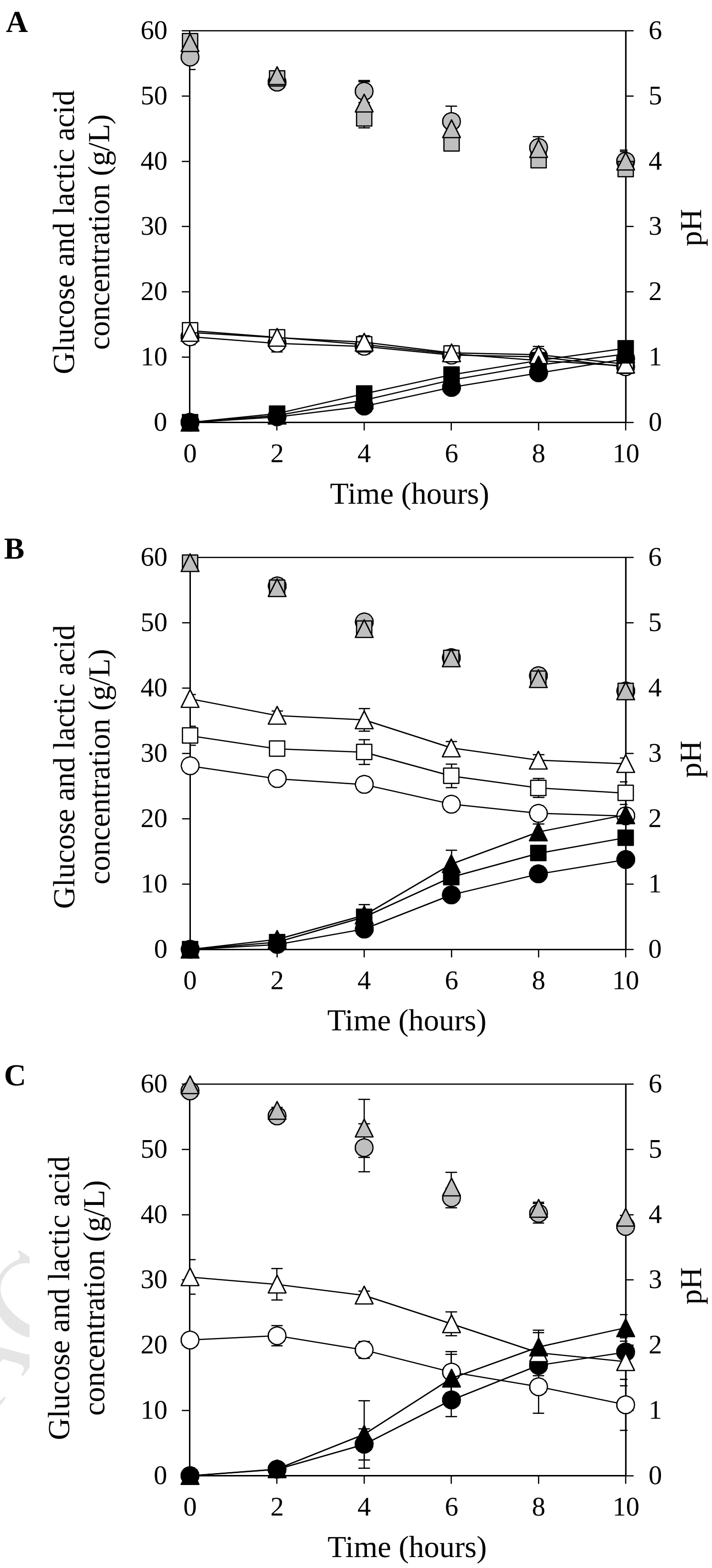
A
B
C
Glucose and lactic acid concentration (g/L)
pH
Time (hours)
0
10
20
30
40
50
60
0
1
2
3
4
5
6
0
2
4
6
8
10

Figure 2.

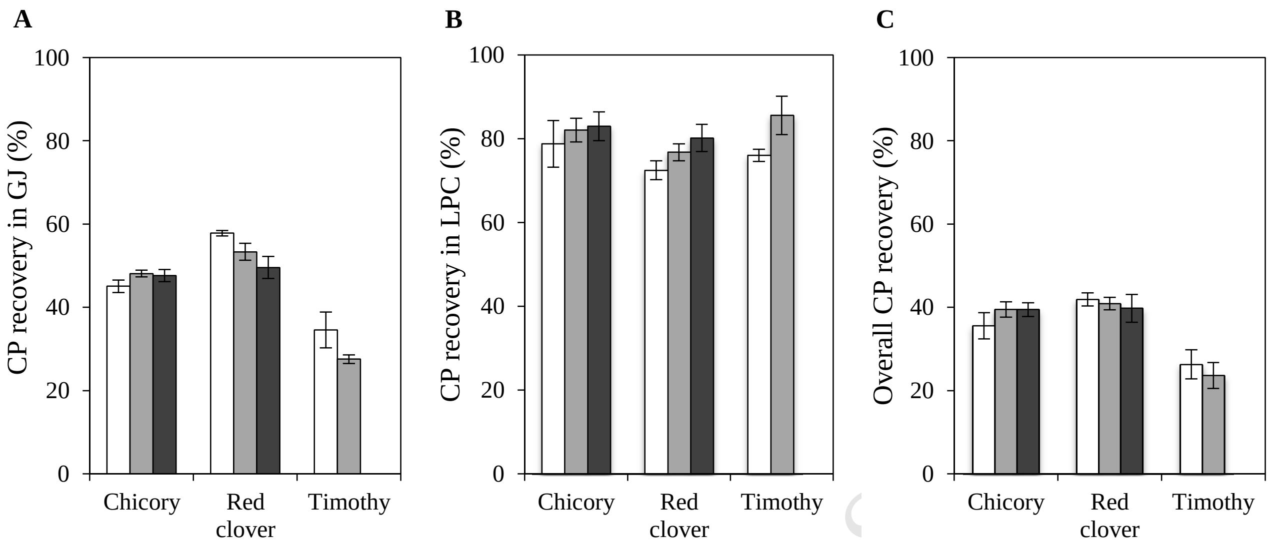

Figure 3.

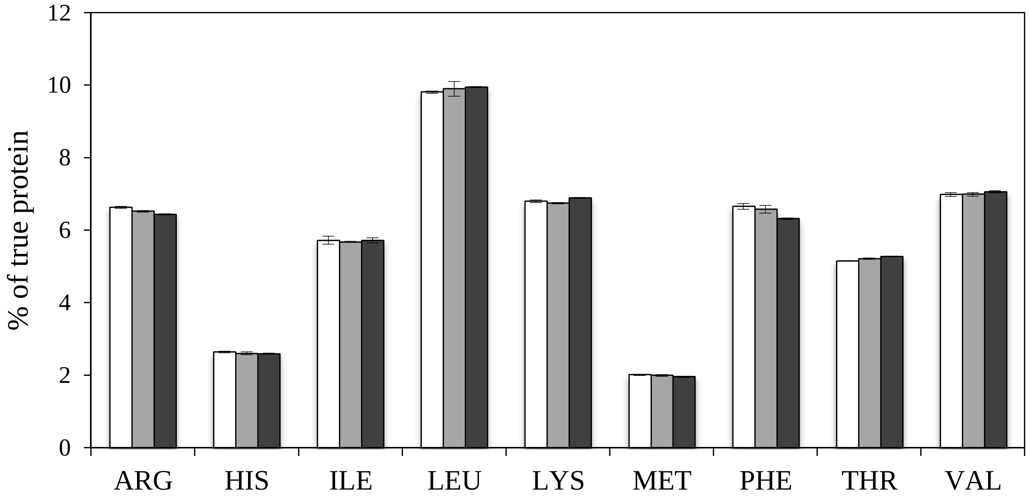

Figure 4.

Highlights

- Crude protein content in the plants decreases with maturity.
- Lactic acid fermentation of green juices efficiently precipitates the proteins.
- Between 24-42% of the plant proteins is recovered in the protein concentrates.
- Protein extractability decreases with plants maturity.
- Red clover results in the highest figures for the green biorefinery process.