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

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

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

1. INTRODUCTION

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

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

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

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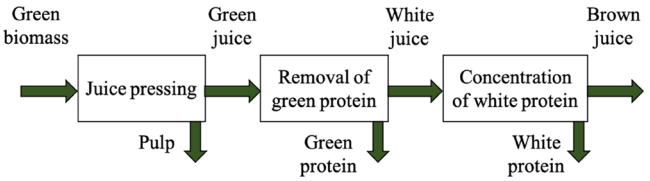

Figure 1. Overview of the protein fractionation process.

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

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

2. MATERIAL AND METHODS

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

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

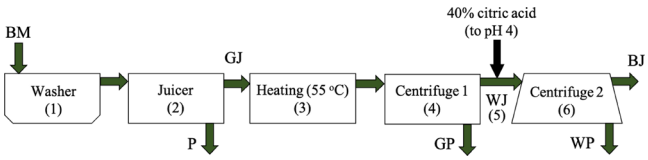

Figure 2. Green biomass fractionation process schematic (BM − unfractionated biomass, *P* − pulp, GJ − green juice, GP − green protein, WJ − white juice, WP − white protein, and BJ − brown juice).

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

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

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

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

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

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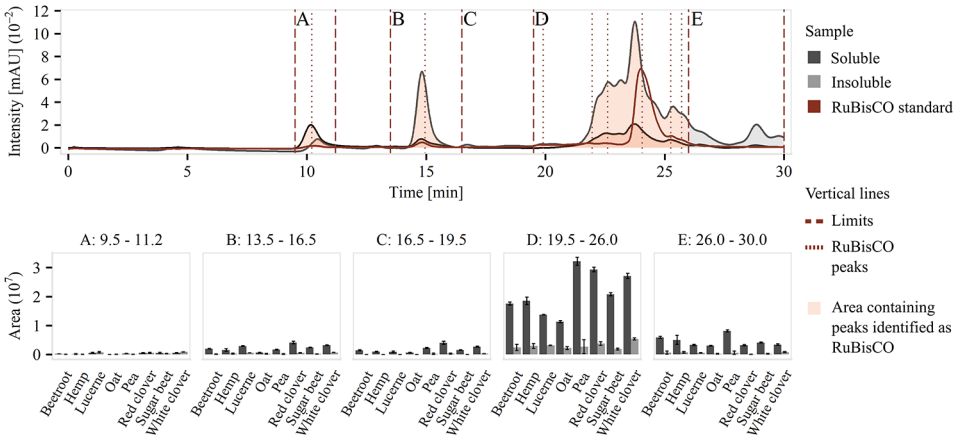

Figure 3. SE-HPLC analysis of protein (absorption at 280 nm) in green biomass from different sources. Top: Chromatogram of the RuBisCO and of "soluble" and "insoluble" protein from unfractionated biomass of oat (as a representative green biomass). The dotted vertical lines represent the approximate elution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

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

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

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

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

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

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

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

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

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

3. RESULTS AND DISCUSSION

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

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

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

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

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

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

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

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

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

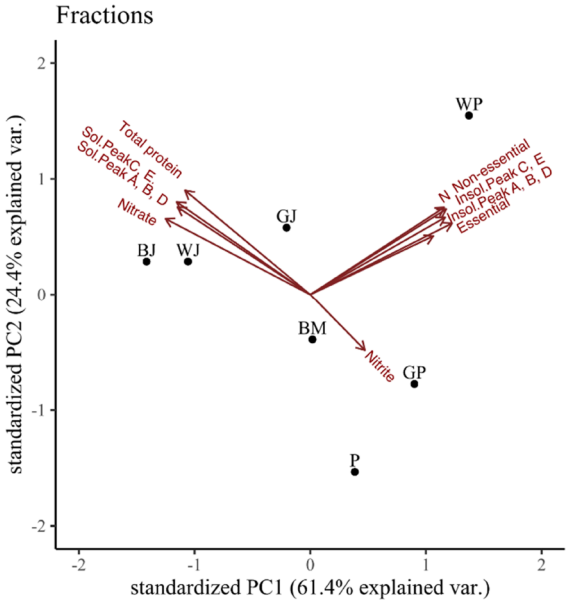

Figure 4. Principal component analysis (PCA) of the content of AAs, nitrogen, nitrate, nitrite, and RuBisCO in the separate fractions in sugar beet, used as one example. Protein components separated by HPLC are denoted Sol. or Insol. Peak A, B, D (RuBisCO containing parts), and Sol. or Insol Peak C,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

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

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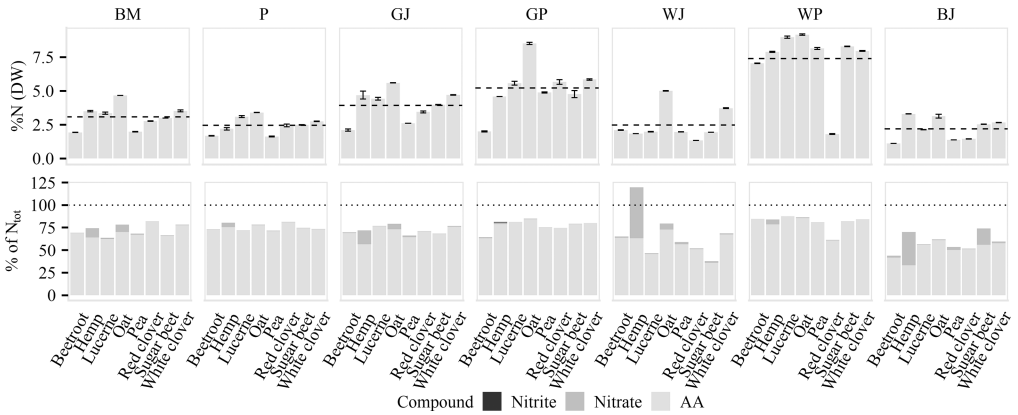

Figure 5. Top: Total *N* content (% dry weight (DW), mean of duplicate measurements) in fractions (unfractionated biomass (BM), pulp (P), green juice (GJ), green protein (GP), white juice (WJ), white protein (WP), and brown juice (BJ) from the different biomass sources. Dashed lines represent the mean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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

■ ASSOCIATED CONTENT

Supporting Information

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

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

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■ ABBREVIATIONS

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

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