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# Production and nutrient composition of forage legume fractions produced by juicing and leaf stripping

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**ABSTRACT**

The large-scale import of soybean products into the EU decreases the self-sufficiency of livestock production. The fractionation of grassland forage crops presents an opportunity to locally produce protein-rich feed for monogastrics. Two promising fractionation methods, *twin-screw press juicing* and *leaf stripping,* were evaluated in parallel in field experiments established in Norway and Sweden to compare the nutrient composition and yield of the resulting *biorefined* and *residual* fractions. The clearest delineation between the methods was in the ash-free neutral detergent fibre (aNDFom) concentration, with juicing producing a biorefined fraction with a lower aNDFom than leaf stripping. Variability in the allocation of crude protein (CP) and biomass to the biorefined fractions occurred in both methods between cuts and locations and is likely due to differing stand characteristics and inconsistency in machine functionality. Additional work is needed to understand how characteristics such as stand density, botanical composition, and plant phenological stage impact each fractionation method's ability to allocate protein, fibre, and biomass into the resulting fractions. Future studies should focus particularly on determining standardised settings for leaf stripping machinery based on a range of stand characteristics to ensure consistency in the yield and nutrient composition of the resulting fractions.

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Biorefinery; clover; crude protein; forages; fractionation; local protein feed; monogastric; neutral detergent fibre; nutrient composition; ruminant

## Introduction

The livestock sector in the EU is heavily reliant on the import of soybean products as a protein feed source. Between 2017 and 2021, an average of over 22 million tons of soybean cake and 16 million tons of soybeans were imported per year to the EU (Food and Agriculture Organization of the United Nations 2023). The dependence on soybeans is particularly apparent for monogastrics due to their high requirements for protein with a balanced amino acid composition (Laudadio et al. 2014). Protein crop production in the EU is estimated to occupy only 3% of arable land, leading to an EU Parliament resolution which proposed solutions to the region's protein feed deficit (Häusling 2011). One solution included in this resolution was to improve the production of animal protein feed based on local and regional crops.

Grasslands make up more than a third of the EU's agricultural area, demonstrating the importance of forage production (Velthof et al., 2014). The temperate grasses and forage legumes included in grassland systems may provide a local alternative to soybean products if processed to maximise their protein yields. Forage-based feed has constrained suitability as a food source for monogastrics due both to the specificity of their protein requirements and their limitation in digesting unprocessed forage fibres (Laudadio et al. 2014). In order to produce forage-based feed suited to the protein requirements of monogastrics, the processing of raw forage becomes essential. Biorefinery can allow for the production of a forage-based protein feed source suitable for monogastrics through the fractionation of fresh forage (Laudadio et al. 2014). The fibre-rich coproduct that remains can serve as a forage source for dairy cows, thereby fully utilising the biomass produced by the cropping system (Damborg et al. 2018).

The call to expand the production of local protein feed has been met by the increased number of studies on forage biorefinery methods. *Twin-screw press juicing*, perhaps the most widely studied biorefinery method in recent years, is a post-harvest fractionation

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method that produces a protein-rich juice and a fibre-rich pulp (Figure 1(a)). Studies have shown that the protein extractability potential is high, with upwards of 60% of the original protein ending up in the juice fraction (Stødkilde et al. 2018; Damborg et al. 2020). The remaining protein is fibre-bound and is thus retained in the pulp fraction, making the nutritive value of the fractionation coproduct potentially suitable for ruminants (Damborg et al. 2018).

An alternative to post-harvest fraction methods involves separating the leaves from the stems during harvest. By utilising the distribution of protein within the plant, the leaves can serve as a protein-rich fraction, while the majority of the fibre remains in the stem fraction. *Leaf stripping*, a harvest-level fractionation method, relies on specially designed harvest machinery that removes the leaves and the soft, upper portion of stem (Figure 1(b)). A previous study on the functionality of leaf stripping machinery showed that upwards of 80% of leaves are successfully harvested by a leaf stripper in pure stands of forage legumes (Liebhardt et al. 2022). The residue left behind is mainly composed of fibrous stem material that can be harvested using conventional machinery. The method shows potential in achieving a protein-rich fraction with an improved feed value for monogastrics compared to conventional harvest methods (Shinners et al. 2007).

Determining the nutrient composition and yield of fractions produced by the juicing and leaf stripping of forage legumes will be essential in determining the potential of these biorefinery methods to create locally produced protein-rich feed products suitable for monogastrics. In the Nordic region, forage legumes are typically grown in mixed stands with grass; however, in this study forage legumes are evaluated in monoculture to remove the influence of grass on their performance. By evaluating these two fractionation methods in parallel, this study aims to compare fractions produced by juicing and leaf stripping in terms of nutrient composition and yield. By comparing the products of both fractionation methods across cuts, locations, and legume cultivars, the potential of each method can be determined under various conditions. The research questions addressed in this paper include: 1. How do the protein- and fibre-rich fractions produced from juicing and leaf stripping differ in terms of crude protein and neutral detergent fibre concentration? 2. How do the two methods differ in yield allocation between the resulting protein- and fibre-rich fractions? 3. How do cut number, cultivar, and location affect the allocation of nutrients and biomass in both fractionation methods?

## Materials and Methods

### Site Description

Two field experiments were established in 2018, one in Norway and one in Sweden. The Norwegian field experiment was established in Tingvoll, Norway (62.92 °N, 8.19 °E). In the experimental fields, the soil is well-drained sandy loam with a high organic matter content (8%). Monocultures of red clover (*Trifolium pratense* L., cv. Gandalf and Lars), alsike clover (*Trifolium hybridum* L., cv. Frida), and lucerne (*Medicago sativa* L., cv. Ludwig) were sown to be harvested conventionally and juiced, or harvested with a leaf stripper. The plots were arranged in a randomised complete block design consisting of four blocks, with each block consisting of one plot of each forage legume cultivar for juicing and one for leaf stripping (32 plots in total). The Swedish field experiment was established in Röbäck, Sweden (63.81°N, 20.24°E). The soil type in the experimental fields is sandy-silt, with good water holding capacity, high capillarity, and high organic matter content (3–6%). The forage legumes sown in Sweden were red clover (*Trifolium pratense* L., cv. Gandalf and Lars),

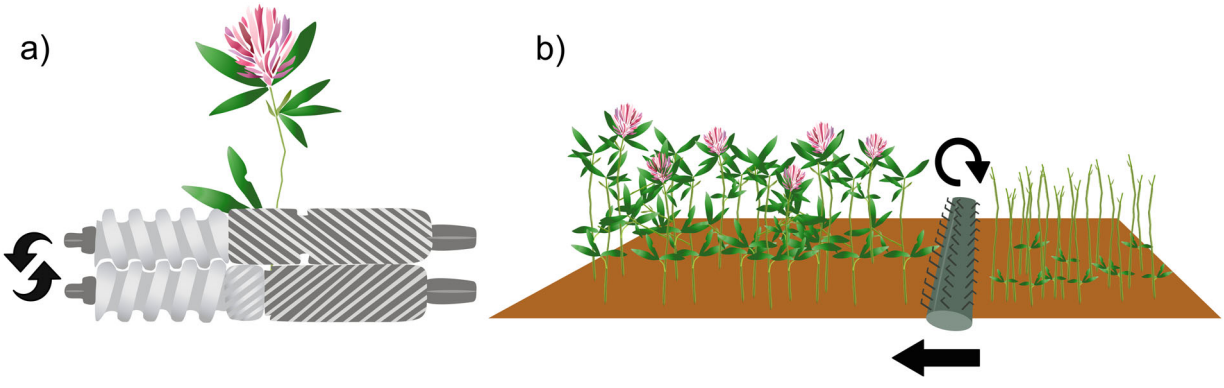


**Figure 1.** Illustrations of (a) A twin-screw press juicer used for fractionation juicing on a red clover plant. (b) The rotating harvest machinery of the leaf-stripper used for fractionation leaf stripping on a stand of red clover.

alsike clover (*Trifolium hybridum* L., cv. Frida), lucerne (*Medicago sativa* L., cv. Ludwig), and yellow lucerne (*Medicago falcata* L. cv. Karlu). The plots were also sown as forage legume monocultures using a randomised complete block design with four blocks, with each block consisting of one plot of each forage legume cultivar for juicing and one for leaf stripping (40 plots in total). For both sites, each plot had an area of 12 $m^2$. Lucerne seeds were inoculated prior to sowing. At both locations, the field experiments were managed organically.

### Data Collection

Pre-harvest data collection was executed the day prior to harvest in Sweden and on the day of harvest in Norway. Stakes were placed roughly 1.5 m from both ends of each plot. Hoops (0.45 $m^2$) were placed around the stakes to serve as two subplots from which to collect pre-harvest measurements for each plot. Height of the tallest plant when stretched, canopy height, and phenology were measured for each subplot considering only the planted forage legume species for each plot. Phenology of red clover and alsike clover was determined according to the scale presented in Nadeem et al. (2019). Lucerne phenology was evaluated according to the scale presented in Kalu and Fick (1981). Hoops were then replaced with 0.25-$m^2$ quadrats to create subplots from which to collect botanical composition samples. All material in the quadrat was hand cut at a stubble height of 8 cm. Samples were then divided into three categories, sown forage legume, grass, and broad leaf weed and dried at 105°C to determine the dry matter concentrations (DM). Botanical composition of the sample was then calculated on a DM basis. Data collection was planned in both countries for the 2019 and 2020 harvest seasons.

### Harvest Methods

The date of harvest in both countries was determined by a combination of growing degree day accumulation, stage of development of clover and nearby timothy (*Phleum pratense* L.), and typical harvest timing of grassland leys in each region. Two harvest treatments were used for each species per block. The first harvest treatment was for plots used for juicing. For these plots, a motor mower (in Norway: Ariens Scandinavia AS, Rygge, Norway) with a harvesting width of 80 cm and a mower harvester (in Sweden: Haldrup F-55, J. Haldrup A/S, Løgstør, Denmark) with a harvesting width of 150 cm cut the plot to an average stubble height of 10 cm. The harvested material was weighed and the yield was calculated on the total area of the plot. A subsample was taken to fractionate through juicing in the lab.

The second harvest treatment was done on plots in which leaf stripping was the fractionation method. Leaf stripper plots were harvested using the PremAlfa Mini electric leaf stripper (Alf'ing – Trust'ing, Nantes, France), a machine designed to fractionate lucerne by harvesting only the leaves and soft upper stems. The leaf stripper harvester consists of rotating tines that separate the leaves from the stem and subsequently collects the leaves in a storage box located within the machine. Rotor height, rotor speed, and ground speed are adjustable to allow the operator to select the ideal settings for the canopy height of the plot, with the objective to maximise leaf collection. The leaf stripper's harvesting width is 80 cm, while the plot width for this experiment was 150 cm. To avoid leaf stripping the same area twice, the harvester was driven through the length of each plot only once. Harvested material was weighed for yield and subsamples were taken for nutrient composition analysis. Yield calculations for the leaves from leaf-stripped plots were then based on the area harvested instead of plot area. The leaf stripper was then driven through the remaining 70 cm of each plot, with a 10 cm overhang into the space between plots to prepare plots for stem harvest. The leaf material collected from this second leaf stripping was discarded. In some cases due to low yield, the leaf-stripped material from the entire plot was utilised to ensure enough material for analysis and yields were based on entire plot area. To harvest the stems, the mower harvesters were driven through each plot in the opposite direction as the leaf stripper to ensure that stems that had been depressed by the leaf stripper would be harvested. The harvested material was weighed and subsamples were taken for nutrient composition analysis. 250 g of the leaf and stem fractions were dried at 105°C for DM determination. An additional 1 kg of each fraction was dried at 55°C for nutrient composition analysis.

### Juicing

Whole plant material harvested from the field experiments was subsampled for juicing. In Norway, the material was juiced fresh directly after harvest, while in Sweden the harvested material was frozen at −20°C and juiced after thawing. A subsample (1 kg) of whole plant material was dried at 55°C and analysed to determine the nutrient composition of the forage before fractionation. Another 250 g of whole plant material was dried at 105°C to determine DM. The remainder of the harvested material was then used for juicing. A twin-

screw press juicer (Angel 7500, Angel CO., LTD., Korea) was used to create juice and pulp fractions from the harvested whole plant material. First, 250 g of plant material was fed into the juicer. The resulting juice and pulp fractions were weighed and dried at 105°C for DM. Then, roughly 1 kg of plant material was juiced and the resulting juice and pulp were weighed. Different subsamples were juiced for DM determination and nutrient composition analysis so that weights of the resulting fractions were obtained from a known weight of whole plant material. Juicing resulted in some loss, mainly in a pulp that remained at the end of the twin-screws. This pulp was added to the pulp fraction. Small amounts of loss also occurred in the form of a foamy substance coating the twin-screws. This foam loss was not collected, as it amounted to a miniscule amount of the total biomass. The pulp fraction was then dried at 55°C for nutrient composition analysis. The juice fraction was frozen at −20°C to preserve the juice for analysis.

### Nutrient composition analysis

In total four fractions were analysed for nutrient composition. The juice and leaf fractions will be referred to as the *biorefined fractions*, as these fractions are the intended product of both fractionation methods. The pulp and the stem fractions will be referred to as the *residual fractions*, as they are considered the coproduct of each fractionation method. Samples of the whole plant and of each fraction were oven dried at 55°C until constant weight in a ventilated oven, with the exception of the juice fraction that was frozen. The dried samples were milled in a Wiley mill (Arthur H. Thomas Co., Philadelphia, PA, US) to pass through a 1-mm sieve and frozen juice samples were thawed in a refrigerator for one night prior to chemical analyses. Samples were analysed according to AOAC official methods (Official Methods of Analysis. Association of Official Analytical Chemists. 15th Edition, 1990) for laboratory DM content (967.03), crude protein (CP) (990.03), and ash (942.05). Amylase treated, ash-free neutral detergent fibre (aNDFom) was determined according to Van Soest, Robertson, and Lewis (1991), adapted to the ANKOM200 Fiber Analyzers Technology (Method 13, Neutral Detergent Fiber in Feeds), omitting sodium sulphite.

### Experimental issues

The planned data collection was hindered by two factors. Due to issues in manufacturing, both sites received the leaf stripper machinery in late summer 2019. This delay resulted in no leaf stripper harvest for all cuts of 2019 in Norway and the first two cuts of 2019 in Sweden. Additionally, harsher than normal conditions during the winter from 2019 to 2020 in northern Sweden caused large amounts of ice build-up on the field, thus killing the majority of plants in the experimental plots. Due to the issues encountered at each experimental site, two datasets were created to maximise the information available from the data collected. As the main objective of these field experiments was to compare two fractionation methods (leaf stripping and juicing), only data from cuts utilising both methods were included. In Norway, both methods were used for all three cuts of 2020. In Sweden, both methods were only used in the third cut of 2019. The first dataset (2020 NO) included all data from the 2020 field season in Norway. Due to issues with lucerne establishment in Norway, only alsike clover and the two red clover cultivars were included in the dataset. In order to include data from Sweden and have the possibility to make comparisons between the two field experiments, a second dataset (3rd Cut SENO) was created that included data from the third cut in Sweden 2019 and the third cut in Norway 2020. This dataset only included the two red clover cultivars, as alsike clover was not harvested in the third cut of 2019 in Sweden due to low yields and lucerne was not harvested in Norway in 2020 due to poor establishment. The third cut in 2019 of the two red clover cultivars was representative of an average year's third cut, yielding similarly to cultivar trials of Gandalf and Lars in northern Sweden between 2018 and 2022 (SLU Fältförsök 2022). In addition to the differing locations of the two field experiments, the harvests included in the 3rd Cut SENO dataset occurred in different years. This signifies that any discrepancy in the results from the two locations must not only be attributed to the location, but also the age of the stand and environmental conditions of the year of harvest.

### Statistical analysis

The pre-harvest measurements and nutrient composition of the unfractionated plant from each dataset were analysed for differences between different cultivars, experimental sites, and cuts using a two-way analysis of variance (ANOVA) (R Studio software version 2023.06.1 + 524, R Core Team 2023). The response variables were plant height, percent legume, total plot yield (kg DM $ha^{-1}$), and DM, CP, and aNDFom of the unfractionated plant. The two-way ANOVA utilised variety, cut, and their interaction as the explanatory variables to analyse the 2020 NO dataset and experimental site, cultivar, and their interaction as the explanatory

variables to analyse the 3rd Cut SENO dataset. Differences among means were tested using Tukey's method ($p < 0.05$).

In order to determine the differences in nutrient composition (CP, aNDFom, DM, and ash), yield (kg DM ha$^{-1}$) and CP yield (kg CP ha$^{-1}$) between the different plant fractions, cultivars, experimental sites, and cuts, several output variables were analysed for both datasets. Linear mixed models were fitted using the SAS procedure MIXED (SAS software version 9.4, SAS Institute Inc., 2008). Output variables were DM, CP concentration, aNDFom concentration, ash concentration, yield, and CP yield. These were analysed separately for the biorefined fraction and the residual fraction. The model for the 2020 NO dataset included cultivar, cut, fractionation method, and all possible interactions of three main effects as fixed-effects factors, and block as a random-effects factor. The REPEATED statement was used with an unstructured covariance structure and with plot as the subject, allowing correlation of residual errors from the same plot. The model for the 3rd Cut SENO dataset included cultivar, location, fractionation method, and all possible interactions of the main effects as fixed-effects factors, and block (within locations) as a random-effects factor. The REPEATED statement was used with location as the group. This specification allowed for heterogeneity of variance between locations. Denominator degrees of freedom were approximated using the Kenward-Roger method. Differences among means were tested using Tukey's test ($p < 0.05$). A normal quantile-quantile plot of residuals and a plot of conditional studentised residuals against fitted values were used to examine for normal distribution and homoscedasticity, respectively. A natural log transformation was used for the output variables residual fraction CP concentration and biorefined fraction aNDFom concentration in the 2020 NO dataset and output variables DM concentration, ash concentration, and aNDFom concentration of the biorefined fraction in the 3rd Cut SENO dataset to achieve homoscedasticity. The mean estimates and 95% confidence interval limits for these outputs variables were subsequently back-transformed.

## Results

### *Field measurements and nutrient composition of the whole legume plant – 2020 NO dataset*

A summary of key field measurements and nutrient composition parameters of the unfractionated whole plant material is presented for each cultivar from the 2020 NO dataset in Table 1. All measurements and nutrient

**Table 1.** Summary statistics of the key field measurements for the stand prior to fractionation including the median value of the plant stage and, mean and standard deviation of plant height, percent legume, and yield.

| | Red clover cv. Gandalf | | | Red clover cv. Lars | | | Alsike clover cv. Frida | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Response variable | 1st Cut | 2nd Cut | 3rd Cut | 1st Cut | 2nd Cut | 3rd Cut | 1st Cut | 2nd Cut | 3rd Cut | *p*-value |
| Plant stage[2] | 2.50 | 3.70 | 3.70 | 2.50 | 3.70 | 3.70 | 2.50 | 3.90 | 3.90 | |
| Plant height (cm) | $36.1^{c} \pm 3.11$ | $65.6^{a} \pm 2.48$ | $42.8^{bc} \pm 3.51$ | $36.7^{c} \pm 2.56$ | $69.3^{a} \pm 4.65$ | $45.6^{b} \pm 4.94$ | $36.5^{c} \pm 3.40$ | $49.6^{b} \pm 7.62$ | $35.6^{c} \pm 2.80$ | <0.001 |
| Legume % | $63.3^{b} \pm 16.8$ | $91.1^{a} \pm 4.53$ | $57.9^{bc} \pm 5.49$ | $70.5^{b} \pm 13.5$ | $89.8^{a} \pm 7.52$ | $68.5^{b} \pm 14.8$ | $42.0^{cd} \pm 10.3$ | $68.3^{b} \pm 11.9$ | $35.8^{d} \pm 9.07$ | 0.623 |
| Yield (kg DM ha$^{-1}$) | $1450^{ab} \pm 279$ | $1390^{ab} \pm 278$ | $519^{c} \pm 129$ | $1620^{a} \pm 198$ | $1550^{a} \pm 307$ | $589^{c} \pm 178$ | $1690^{a} \pm 192$ | $876^{bc} \pm 88.9$ | $438^{c} \pm 124$ | 0.051 |
| DM (g kg$^{-1}$ DM) | $153^{bc} \pm 8.64$ | $196^{a} \pm 9.43$ | $159^{b} \pm 6.66$ | $152^{bc} \pm 3.64$ | $180^{a} \pm 4.14$ | $154^{bc} \pm 3.10$ | $141^{c} \pm 6.11$ | $185^{a} \pm 6.46$ | $153^{bc} \pm 9.07$ | 0.191 |
| CP (g kg$^{-1}$ DM) | $185^{bc} \pm 15.3$ | $160^{d} \pm 5.45$ | $203^{ab} \pm 8.66$ | $193^{b} \pm 6.37$ | $164^{cd} \pm 4.95$ | $216^{a} \pm 5.01$ | $167^{cd} \pm 6.53$ | $159^{d} \pm 6.52$ | $192^{b} \pm 9.37$ | 0.149 |
| aNDFom (g kg$^{-1}$ DM) | $300^{a} \pm 46.9$ | $376^{a} \pm 35.0$ | $318^{a} \pm 36.8$ | $331^{a} \pm 34.5$ | $363^{a} \pm 37.9$ | $330^{a} \pm 41.5$ | $279^{a} \pm 22.2$ | $314^{a} \pm 23.3$ | $302^{a} \pm 47.5$ | 0.758 |

Summary of the nutrient composition parameters of the whole legume plant prior to fractionation including the mean value and standard deviation of dry matter (DM), crude protein (CP), and ash-free neutral detergent fibre (aNDFom). Data included is from the Norwegian experiment in 2020[1]. *P*-values are presented for the interaction of cut and cultivar. Means with common letters within each response variable are not significantly different ($p > 0.05$) according to Tukey's test.

[1]For median of plant stage and mean of plant height, legume %, and yield, n = 8. For mean of DM, CP, and aNDFom, n = 4.

[2]Plant development stage was determined according to the scale from 1.00–4.00 set by Nadeem et al. 2019, where 1.00 signifies first visible leaf and 4.00 signifies seed formation.

composition concentrations are from plant samples taken pre-harvest and thus represent the entire plant prior to fractionation. For all three cultivars, plants were more mature in the second and third cuts than in the first cut, with plants reaching the tallest plant height in the second cut. The botanical composition of plots shifted between cuts, with the highest legume percentage occurring in the second cut for all cultivars. For the red clover cultivars, yields were relatively consistent between the first and second cuts, but decreased drastically in the third cut. The highest yields for alsike clover occurred in the first cut, with yields decreasing by 50% on average between subsequent cuts. The DM concentration was highest in the second cut for all cultivars, while CP concentrations were highest in the third cut. Concentrations of aNDFom were consistent between all cuts and cultivars.

### Fraction nutrient composition and yield results – 2020 NO Dataset

Juicing and leaf stripping produced biorefined and residual fractions with differing nutrient composition and yield in the Norwegian experiment in 2020 (Figure 2). All statements of difference are significant at the threshold of $p < 0.05$. The fixed effects and their subsequent interactions for each output variable are presented in Table 2 with their corresponding *p*-values. The three-way interaction of cultivar, fractionation method, and cut was not significant for any of the output variables analysed. As the focus of this study is on the differences in nutrient composition and yield between the two fractionation methods tested, only the means of two-way interactions of cultivar and fractionation method, and fractionation method and cut are presented. When significant, the means of the highlighted interactions are reported for the output variables yield, CP, and aNDFom. Results for the output variables DM, ash, CP yield, and proportion of the total yield can be found in Supplementary material Figures 1 and 2.

*Results for CP concentration:* There was a significant interaction between cut and fractionation method for the CP concentration of both biorefined and residual fractions (Table 2). For CP concentration of the biorefined fractions, the juice had a higher CP concentration than the leaves in the first cut; however, there was no difference in CP between methods in the remaining cuts (Figure 2(A)). For the juice fraction, the CP concentration was higher in the first and third cut compared to the second cut. An increase in CP concentration of the leaves occurred in the third cut compared to the prior two cuts. A similar trend was present in the CP concentration of the residual fractions, with the stems having higher CP than the pulp in the first cut (Figure 2(B)). There were no differences in CP between fractionation methods for the remaining cuts. Both the stems and the pulp had the highest CP concentration in the third cut.

*Results for aNDFom concentration:* There was a significant interaction between cultivar and fractionation method for the aNDFom concentration of the biorefined fractions (Table 2). In the biorefined fractions, the juice produced from all three cultivars had a lower aNDFom concentration than the leaves (Figure 2(C)). For the juice fraction, a difference in aNDFom concentration was only observed between the two red clover cultivars, in which Gandalf has a higher aNDFom than Lars. A difference in aNDFom concentration between different cultivars for the leaf fraction was only present between Frida, the alsike clover cultivar, and Lars, the tetraploid red clover cultivar, with Lars having a higher aNDFom concentration. The residual fractions had a significant interaction between cut and fractionation method (Table 2). Across all cuts, the pulp fraction had a higher aNDFom concentration than the stems (Figure 2(D)). There was no difference between the cuts in terms aNDFom of the pulp fraction. For the stem fraction, the second cut had a higher aNDFom concentration than the first and third cuts.

*Results for yield:* For both the biorefined and residual fractions, there was a significant interaction between cut and fractionation method for yield (Table 2). Yield of the biorefined fractions was higher for the juice than the leaves in the first and third cut (Figure 2(E)). In the second cut, there was no difference in yield between the two biorefined fractions. For the juice fraction, yield was the highest in the first cut and lowest in the third cut. For the leaf fraction, yield was lowest in the third cut, with no difference in yield between the first and second cuts. For the residual fractions, yield of the stems was higher than the pulp in the first cut; however, no difference in yield occurred between the residual fractions in the second and third cuts (Figure 2(F)). Stem yield was the highest in the first cut and lowest in the third cut. The third cut produced the lowest pulp yields; however, no difference in pulp yield occurred between the first and second cuts.

### Field measurements and nutrient composition of the whole legume plant – 3rd cut SENO dataset

A key measurement and nutrient composition parameter summary for the 3rd cut SENO dataset is presented in Table 3. These values represent the whole plants before harvest and fractionation. Though plants were at the same maturity stage in both locations,

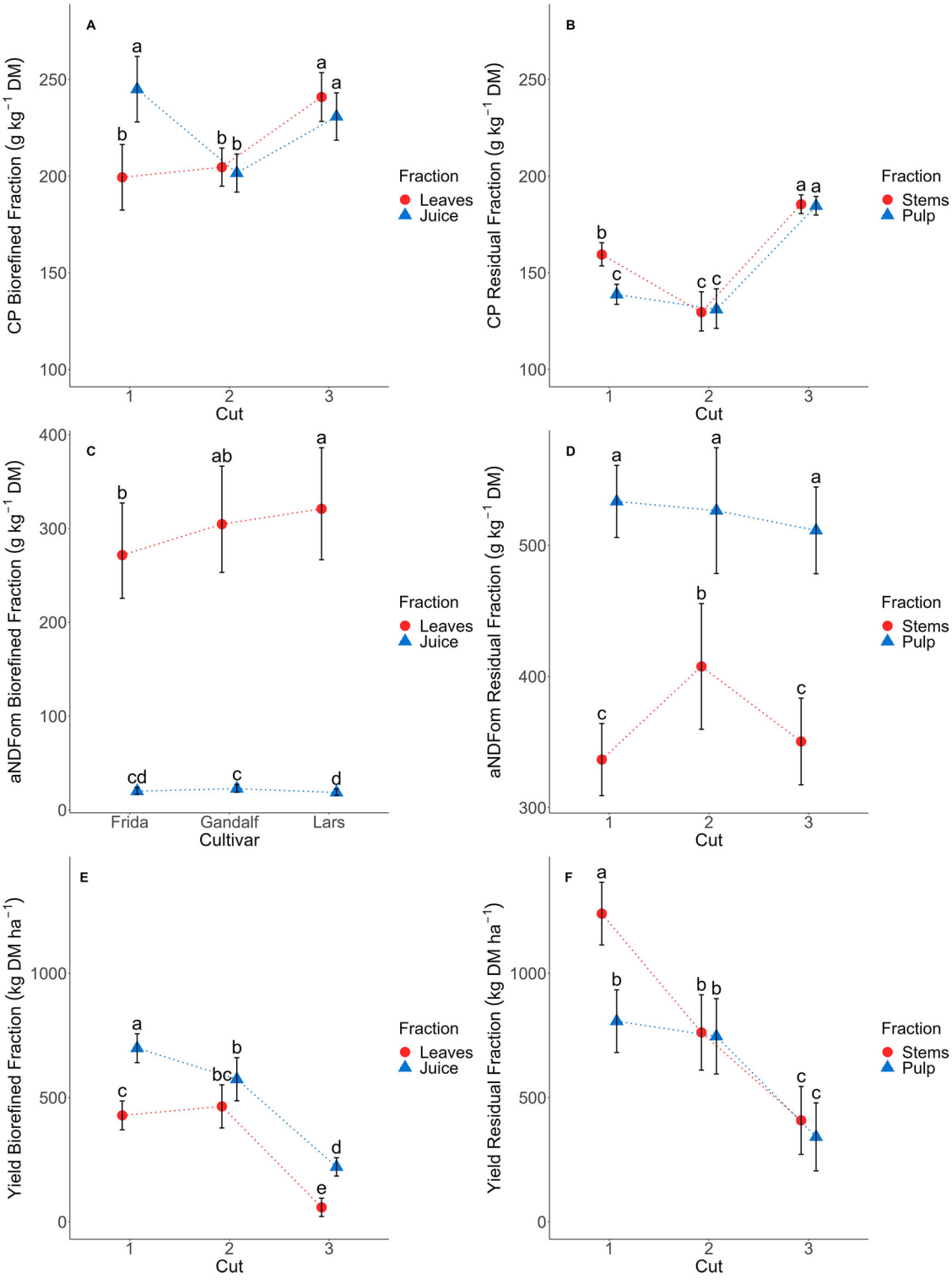


**Figure 2.** Least square means from the linear mixed model for the 2020 NO dataset of (A) crude protein (CP) concentration of the biorefined fraction in response to the interaction of cut and fractionation method; (B) CP concentration of the residual fraction in response to the interaction of cut and fractionation method; (C) ash-free neutral detergent fibre (aNDFom) concentration of the biorefined fraction in response to the interaction of cultivar and fractionation method; (D) aNDFom concentration of the residual fraction in response to the interaction of cut and fractionation method; (E) Total yield of the biorefined fraction in response to the interaction of cut and fractionation method; (F) Total yield of the residual fraction in response to the interaction of cut and fractionation method. These graphs are only for significant interactions. Vertical bars represent 95% confidence intervals. Means with common letters within each graph are not significantly different ($p > 0.05$) according to Tukey's test.

**Table 2.** *P*-values of the fixed-effects factors and their interactions from the linear mixed model for crude protein (CP), ash-free neutral detergent fibre (aNDFom), yield, dry matter (DM), ash, CP yield, and proportion of total yield of the biorefined and residual fractions produced in Norway 2020.

| | Biorefined Fraction | | | | | | | Residual Fraction | | | | | | | Num DF |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Effect | CP | aNDFom | Yield | DM | Ash | CP Yield | Proportion | CP | aNDFom | Yield | DM | Ash | CP Yield | Proportion | |
| Cultivar | 0.109 | **0.047** | **0.001** | **<0.001** | **<0.001** | **0.003** | **0.002** | 0.263 | 0.156 | **0.045** | **0.013** | **0.015** | **0.017** | **0.001** | 2 |
| Method | 0.119 | **<0.001** | **<0.001** | **<0.001** | **<0.001** | **<0.001** | **<0.001** | 0.135 | **<0.001** | **<0.001** | <0.001 | **<0.001** | **<0.001** | **<0.001** | 1 |
| Cut | **<0.001** | **0.018** | **<0.001** | **<0.001** | **<0.001** | **<0.001** | **<0.001** | **<0.001** | 0.135 | **<0.001** | <0.001 | **<0.001** | **<0.001** | **<0.001** | 2 |
| Cultivar*Method | 0.399 | **0.018** | 0.586 | **0.009** | 0.184 | 0.429 | **0.031** | 0.436 | 0.658 | 0.211 | **0.011** | 0.312 | 0.162 | 0.106 | 2 |
| Cultivar*Cut | **0.009** | 0.863 | **<0.001** | **0.045** | **0.020** | **0.006** | **<0.001** | **0.021** | 0.581 | **0.022** | **0.016** | **<0.001** | 0.516 | **<0.001** | 4 |
| Method*Cut | **<0.001** | 0.072 | **0.004** | **<0.001** | **<0.001** | **0.001** | **<0.001** | **<0.001** | **0.028** | **<0.001** | **0.001** | **0.002** | **<0.001** | **<0.001** | 2 |
| Cultivar*Method*Cut | 0.117 | 0.749 | 0.545 | 0.193 | 0.199 | 0.642 | **<0.001** | 0.111 | 0.092 | 0.829 | 0.962 | 0.154 | 0.460 | **<0.001** | 4 |

The fixed-effects factors are the cultivar of forage legume, the fractionation method used, and cut. *P*-values smaller than 0.05 are written in bold. Numerator degrees of freedom for each effect are presented in the final column.

plants were larger in Sweden than in Norway for both cultivars. Additionally, the botanical composition and yield differed between locations, with Swedish plots having a higher legume percentage and higher yields than Norway. There was no difference in CP concentration between cultivars of locations. Plants from the Norwegian experiment had a higher DM, but a lower aNDFom concentration than the plants from the Swedish experiment.

### *Fraction nutrient composition and yield results – 3rd cut SENO dataset*

The 3rd cut SENO dataset was included to determine the repeatability of the results of fractionation with plants grown in different environments. All statements of difference are significant at the threshold of $p < 0.05$. The *p*-values of all fixed effects and interactions are presented in Table 4. None of the output variables had a significant three-way interaction of cultivar, fractionation method, and location. Additionally, the two-way interaction of cultivar and fractionation method was not significant for any output variable. When significant, the means of the interaction of fractionation method and location are reported for the output variables yield and CP in Figure 3. The means of the main effects of aNDFom are presented in Table 5. Results for the remaining output variables are found in Supplementary material Figure 3 and 4.

*Results for CP concentration*: For CP concentration there was a significant interaction of fractionation method and location for both the biorefined and residual fractions. In the biorefined fractions produced in Sweden, the CP concentration of the leaves was higher than the juice (Figure 3(A)). In Norway, there was no difference between the CP of the biorefined fractions. For the residual fractions from Sweden, the pulp had a higher CP concentration than the stems (Figure 3(B)). Similar to the biorefined fractions, there was no difference between CP of stems and pulp from Norway.

*Results for aNDFom concentration*: There was no significant interaction between fractionation method and location for the aNDFom concentration of the biorefined or residual fractions. However, there was a significant main effect of fractionation method for both the biorefined and residual fractions for the output variables aNDFom concentration and yield (Table 4). For the biorefined fractions, the aNDFom concentration of the juice was lower than the leaves, while in the residual fractions, the stems had a lower aNDFom than the pulp (Table 5).

*Results for yield*: The yields of the biorefined and residual fractions were sigificantly impacted by the interaction of fractionation methods and location. For Sweden, there was no difference in yield between the

**Table 3.** Summary statistics of the key field measurements for the stand prior to fractionation including the median value of the plant stage and mean and standard deviation of plant height, percent legume, and yield.

| | Gandalf | | Lars | | |
|---|---|---|---|---|---|
| Response variable | Sweden | Norway | Sweden | Norway | *p*-value |
| Plant stage[2] | 3.70 | 3.70 | 3.70 | 3.70 | |
| Plant height (cm) | $49.4^{b} \pm 5.00$ | $42.8^{c} \pm 3.51$ | $59.0^{a} \pm 2.70$ | $45.6^{bc} \pm 4.94$ | 0.041 |
| Legume % | $99.1^{a} \pm 1.76$ | $57.9^{b} \pm 5.49$ | $99.3^{a} \pm 1.13$ | $68.5^{b} \pm 14.8$ | 0.093 |
| Yield (kg DM $ha^{-1}$) | $1500^{b} \pm 175$ | $519^{c} \pm 129$ | $1830^{a} \pm 269$ | $589^{c} \pm 178$ | 0.103 |
| DM (g $kg^{-1}$ DM) | $138^{b} \pm 6.76$ | $159^{a} \pm 6.66$ | $130^{b} \pm 5.73$ | $154^{a} \pm 3.10$ | 0.665 |
| CP (g $kg^{-1}$ DM) | $247^{a} \pm 45.0$ | $203^{a} \pm 8.67$ | $249^{a} \pm 31.5$ | $216^{a} \pm 5.01$ | 0.748 |
| aNDFom (g $kg^{-1}$ DM) | $477^{a} \pm 58.5$ | $318^{b} \pm 36.8$ | $432^{a} \pm 17.0$ | $330^{b} \pm 41.5$ | 0.254 |

Summary of the nutrient composition parameters of the whole legume plant prior to fractionation including the mean value and standard deviation of dry matter (DM), crude protein (CP), and ash-free neutral detergent fibre (aNDFom). Measurements were taken from the 3rd harvest in 2019 in Sweden and 2020 in Norway[1]. *P*-values are presented for the interaction of location and cultivar. Means with common letters within each response variable are not significantly different ($p > 0.05$) according to Tukey's test.

[1]For median of plant stage and mean of plant height, legume %, and yield, n = 8. For mean of DM, CP, and aNDFom, n = 4.

[2]Plant development stage was determined according to the scale from 1.00–4.00 set by Nadeem et al. 2019, where 1.00 signifies first visible leaf and 4.00 signifies seed formation.

leaves and the juice (Figure 3(C)). In Norway, however, the yield of the juice was higher than the leaves. For each location, there was no difference in yield between the stems and the pulp (Figure 3(D)). The yields of the biorefined and residual fractions in Sweden were higher than fractions produced in Norway.

## Discussion

### *Effect of fractionation method on crude protein*

The CP concentration of the biorefined fractions was relatively consistent between fractionation methods for the 2020 NO dataset. The difference in CP concentration between the two fractionation methods in the first cut (Figure 2(A)) can be explained by the difference in phenology between plants in the first cut versus the second and third (Table 1). Plants in the second and third cut were in the late reproductive stage, with all three cultivars in flower. In the first cut, however, plants were still in the stem elongation stage. Juicing was more successful at allocating protein to biorefined fraction when plants were less mature, as the plants had more soluble protein and less fibre bound protein (Buxton 1996). A study on the protein extractability potential of forage legumes at varying stages of maturity explored the shift in protein distribution, demonstrating that red clover plants in the early vegetative stage had higher concentrations of true protein than those in reproductive stages (Solati et al. 2017). As true protein is the most relevant protein fraction in terms of protein extractability, these results support the higher CP concentration seen in juice produced from plants in the first cut. The leaf stripper allocated less CP to the biorefined fraction in the first cut compared to the subsequent cuts (Figure 2(A) and Table 1), though the cause of this disparity is not explicit. The CP allocation of leaf stripping is susceptible to far more variables than juicing, as there are multiple machine settings that can be modified by the individual user. The creation of standardised machine setting recommendations would be challenging, as great variability can occur in stand characteristics. Results from the 3rd Cut SENO dataset show inconsistencies in the protein allocation of the two fraction methods between locations (Figure 3(A)), suggesting variability in leaf stripper and twin-screw press functionality between the two locations.

The difference in CP concentration between the residual fractions from the 2020 NO dataset, is likely due to lower amounts of fibre-bound protein (Figure 2(B)). The results for residual fractions from the 3rd Cut SENO dataset further demonstrate the difference in protein allocation in Sweden compared to Norway (Figure 3(B)). Additional investigation into the differences in protein allocation between fractionation methods under different environmental conditions is essential to better understand the inconsistencies seen in this study between locations.

Across both datasets, the CP concentrations were relatively similar between the juice and leaves, and the pulp and stems, respectively. Previous studies have investigated the allocation of protein to the residual fraction performed by both fractionation methods included in this study. Pulp produced from red clover in Denmark had a 3.4% lower CP concentration than the whole plant (Damborg et al. 2018). The ratio of CP between the whole plant and the pulp varied between cuts and locations in this study (Figure 2(B) and Figure 3(B)), with the largest decrease in CP from the whole plant to the pulp occurring in the first cut in Norway (24%) and the smallest in Sweden (5.6%). Stem fractions produced from leaf-stripping pure red clover stands in Germany had a 24% lower CP concentration than the whole plant (Liebhardt et al. 2022). There was substantial variability in the ratio of CP between the whole plant and the stems between cuts and locations (9.1 - 29%) (Figure 2(B) and Figure 3(B)). The stems produced in Sweden

**Table 4.** *P*-values of fixed-effects factors and their interactions from the linear mixed model for crude protein (CP), ash-free neutral detergent fibre (aNDFom), yield, dry matter (DM), ash, CP yield, and proportion of total yield of the biorefined and residual fractions produced in the 3rd cut in Sweden 2019 and the 3rd cut in Norway 2020.

| | Biorefined Fraction | | | | | | | Residual Fraction | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Effect | CP | aNDFom | Yield | DM | Ash | CP Yield | Proportion | CP | aNDFom | Yield | DM | Ash | CP Yield | Proportion |
| Cultivar | 0.749 | 0.826 | **<0.001** | 0.440 | **0.013** | **0.003** | **0.014** | 0.074 | **0.017** | 0.176 | 0.490 | **<0.001** | 0.696 | **0.038** |
| Method | **<0.001** | **<0.001** | 0.123 | **<0.001** | **<0.001** | 0.062 | **<0.001** | **<0.001** | **<0.001** | 0.514 | **<0.001** | **<0.001** | **0.007** | **<0.001** |
| Location | 0.056 | 0.219 | **<0.001** | **<0.001** | **<0.001** | **<0.001** | **0.001** | **0.037** | **<0.001** | **<0.001** | **<0.001** | **0.018** | **<0.001** | **0.001** |
| Cultivar*Method | 0.247 | 0.956 | 0.440 | 0.867 | 0.730 | 0.630 | 0.766 | 0.659 | 0.338 | 0.171 | 0.909 | 0.704 | 0.149 | 0.579 |
| Cultivar*Location | 0.133 | 0.716 | **0.025** | 0.766 | **0.019** | 0.137 | 0.884 | 0.195 | 0.454 | 0.258 | 0.388 | 0.358 | 0.737 | 0.291 |
| Method*Location | **<0.001** | 0.897 | **0.001** | **<0.001** | 0.490 | **<0.001** | **<0.001** | **<0.001** | 0.725 | **0.035** | **<0.001** | **<0.001** | **<0.001** | **<0.001** |
| Cultivar*Method*Location | 0.477 | 0.510 | 0.203 | 0.649 | 0.784 | 0.125 | 0.122 | 0.430 | 0.192 | 0.810 | 0.339 | 0.069 | 0.531 | 0.335 |

The fixed effects are the cultivar of forage legume, the fractionation method used, and the location. *P*-values smaller than 0.05 are written in bold. Numerator degrees of freedom for main effects and interactions is 1.

had a 29% lower CP concentration than the whole plants (Figure 3(B)), congruent with the results seen in the German study.

### *Effect of fractionation method on fibre*

The allocation of aNDFom concentration between the fractions was relatively consistent between the two datasets. The difference in aNDFom allocation between the fractionation methods (Figure 2(C, D) and Table 5) can be attributed to the different mechanisms by which they fractionate the plant. Though leaf stripping produced a biorefined fraction containing more of the soluble protein available in the plant, it also contains a significant amount of fibre, particularly from the petioles and upper stems. Juicing, however, is more successful at removing fibre, as the maceration performed by the twin-screw press excludes fibrous plant material from the biorefined juice fraction and instead allocates the majority of the fibre to the residual pulp fraction (Colas et al. 2013). Across cuts, leaf stripping had less consistent aNDFom allocation than juicing. These results suggest that the allocation of aNDFom into the residual fraction performed by leaf stripping is more susceptible to variation based on plant morphology.

Results on the fibre content of raw products from juicing are limited, as most reported values are on the protein paste precipitated from raw juice (Stødkilde et al. 2020). A single previous study on the nutrient composition of fractions produced by leaf-stripping red clover reported crude fibre concentrations, which were considerably lower than the aNDFom values seen in this study (Möller 2014). The difference in fibre concentrations seen between studies is partially influenced by the analysis method, as crude fibre values do not fully represent the hemicellulose and lignin concentrations in the plant. The discrepancy is also influenced by the user settings of the leaf stripper, as fraction composition can vary based on the height and rotor speed of the machine. The lack of reported values on the fibre concentration of fractionated products is the consequence of a greater focus on protein concentration, as the primary goal of the process is to maximise protein allocation to the biorefined fraction. However, the fibre content of the resulting biorefined fractions should be considered due to the limitations in fibre digestion for monogastrics (Laudadio et al. 2014). The fibre content of the residual fractions is also of importance, as the utilisation of biorefinery by-products as ruminant feed ensures a more sustainable and economically viable production system (Mandl 2010). A study on the nutrient composition of the pulp fraction created by twin-screw press

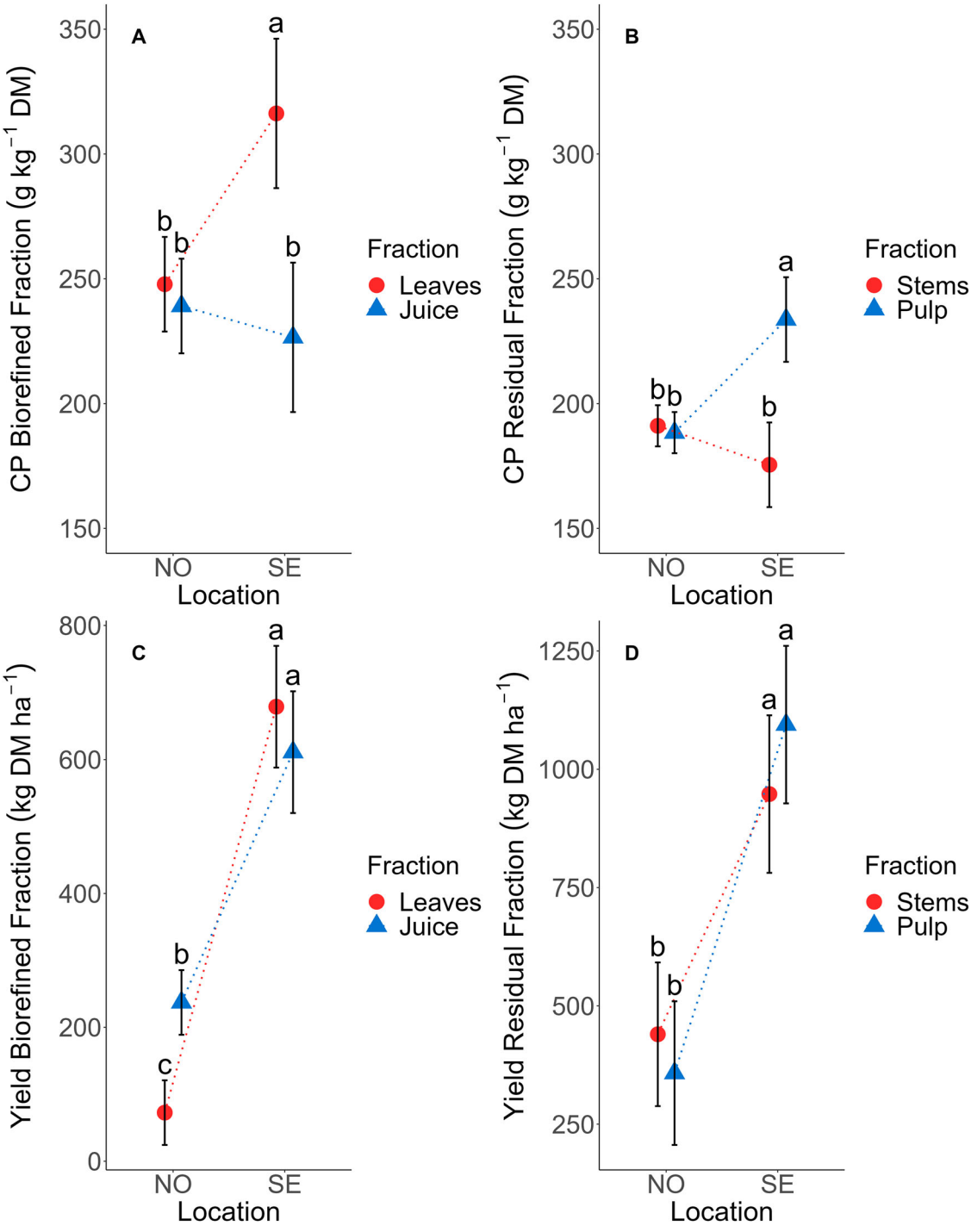


**Figure 3.** Least square means from the linear mixed model for the 3rd Cut SENO dataset of (A) crude protein (CP) concentration of the biorefined fraction in response to the interaction of location and fractionation method; (B) CP concentration of the residual fraction in response to the interaction of location and fractionation method; (C) Yield of the biorefined fraction in response to the interaction of location and fractionation method; (D) Yield of the residual fraction in response to the interaction of location and fractionation method. These graphs are only for significant interactions. Vertical bars represent 95% confidence intervals. Means with common letters within each graph are not significantly different ($p > 0.05$) according to Tukey's test.

juicing reported that pulp produced from red clover in Denmark had a 42% higher aNDF concentration than the original plant, when averaged across three cuts (Damborg et al. 2018). Comparatively, red clover pulp produced in Norway had on average a 62% higher aNDF concentration than the original plant (Figure 2 (D) and Table 1). Results from both studies demonstrate the ability of juicing to concentrate the fibre from the original plant into the pulp, thus reducing the fibre concentration of the juice fraction.

**Table 5.** Least squares means and 95% confidence intervals from the linear mixed model of the effect of fractionation method on ash-free neutral detergent fibre (aNDFom) concentration (g $kg^{-1}$ DM) in the biorefined and residual fractions across both locations for the 3rd Cut SENO dataset.

| | Output Variable | Fraction | Mean Estimate | 95% Confidence Interval |
|---|---|---|---|---|
| Biorefined Fraction | aNDFom | Leaves | 338 | ±13.6 |
| | | Juice | 22.6 | ±13.6 |
| Residual Fraction | aNDFom | Stems | 411 | ±21.3 |
| | | Pulp | 544 | ±21.3 |

### *Effect of fractionation method on yield*

The higher yields of the biorefined fraction from juicing in the 2020 NO dataset (Figure 2(E)) are the result of its ability to utilise the entire plant biomass to create the biorefined fraction, not just leaf material. For the 3rd Cut SENO dataset, the lower biorefined fraction yields from leaf stripping in Norway can be explained by the disparity in legume percent of total botanical composition between locations (Table 1). A study focusing on leaf stripping pure red clover stands reported leaf proportion yields of roughly 46% (Liebhardt et al. 2022). Alternatively, a previous study examining the leaf stripping of mixed stands of red clover and timothy reported leaf proportion yields ranging from 16-57% (Micke et al. 2023). The large variability of leaf proportion reported by Micke et al. (2023) and the low leaf fraction yields seen from Norway in this study (Figure 2(E) and Figure 3(B)) indicate that yields from leaf stripping are more dependent on the composition of the stand compared to juicing, as a result of less leaf biomass available for the biorefined fraction.

The yield fluctuations between cuts and locations when using the leaf stripper are not surprising, considering the larger influence of plant morphology and stand density on the biomass allocation between fractions. In contrast, juicing was far more consistent in its biomass allocation between cuts and locations (Supplementary material Figures 2 and 4). As noted previously, large variability in leaf proportion has been seen in other studies, as a result of differences in stand composition (Liebhardt et al. 2022; Micke et al. 2023). It is thus expected to see large variability in leaf stripper yield allocation between cuts and locations (Figure 2(E, F) and Figure 3(C, D)). In contrast, the proportion of the juice fraction produced in this study was considerably higher than previous red clover juicing studies. Two studies on the mass balance and yield of red clover fractions produced by juicing reported the proportion of juice produced to be 29.8% (Damborg et al. 2020) and 28.2% (Santamaría-Fernández et al. 2017). Plants from the Santamaría-Fernández study were harvested at a pre-flower maturity stage, similar to the first cut in this study; however, the juice proportion was considerably lower than what was achieved in the first cut in Norway. Determining the cause of the higher juice proportion achieved in this study is challenging, as no additional information on fibre content of the plants was reported by Santamaría-Fernández. Results from the Damborg study also demonstrate a lower juice proportion, thought this is likely attributed to the fact that the loss proportion was also calculated into the mass balances presented in the study.

Yield differences between sites were certainly impacted by the age of the ley, as well as lower stand density at the Norwegian site due to harsh winter conditions prior to the year of harvest. Though both sites were managed organically, the Norwegian field experiment was grown on land with a history of organic production, while the land from the Swedish field experiment had a recent history of conventional production. As no herbicides had been used in recent years at the Norwegian site, the lack of weed suppression in previous years allowed for a larger soil weed bank to accumulate and outcompete the clover early in the season (Buhler et al. 1997). Organically managed clover leys have been shown to have high weed density compared to those that are conventionally managed (Kauppila 1990). The results from the 3rd Cut SENO dataset suggest that sites with a higher weed percentage in the stand may be better suited to juicing than leaf stripping to achieve a consistent yield of the biorefined fraction, as the fractions produced from leaf stripping are subjected to greater variability due to fluctuations in botanical composition.

### *Potential for practical implementation of juicing and leaf stripping*

The successful adoption of forage fractionation will require a holistic understanding of both the fractionation mechanisms and their resulting fractions when utilised under variable conditions. This study demonstrated large degrees of variability in the allocation of protein and biomass by both press juicing and leaf stripping. However, the juice fraction's consistently low fibre concentrations under various cuts, cultivars, and locations indicate that fractionation through juicing may be more successful at creating a locally produced protein-feed for monogastrics. Though the high fibre concentrations of the leaf fraction limit its utility as a replacement for soybean in the diet of monogastrics, its relatively high protein concentration demonstrates the potential of leaf stripping to produce a feed product with a superior nutrient composition to conventionally harvested forage. Both methods demonstrate potential to increase the sustainability and self-sufficiency of the production of protein-rich feed.

Both fractionation methods presented in this study have limitations to practicality in adoption on a large scale, as well as their functionality in achieving consistent results in terms of nutrient composition and yield. Though juicing resulted in lower fibre concentration in the biorefined fraction and relatively consistent results in terms of fraction allocation, there are constraints in method adoption due to the multistep nature of the fractionation process. Additionally, the process requires highly specialised machinery and labour to achieve an end product that can serve as animal feed. Previous studies have shown the potential for protein paste precipitated from juice fractions to be a viable protein feed for monogastrics (Stødkilde et al. 2020; Renaudeau et al. 2022). The utilisation of the pulp fraction as an alternative forage source for ruminants shows promise, as the protein concentration of pulp remains consistent with the original plant (Damborg et al. 2018).

Fractions created through leaf stripping are more susceptible to variation based on plant phenology, stand density, and user settings. Significant work is necessary to better understand how this variability can be mitigated by standardised machine settings based on stand characteristics. Additionally, the high fibre concentration in the leaf fraction may hinder its suitability as a protein feed source (Laudadio et al. 2014). Feeding studies have investigated the potential of red clover leaves as a protein source for broilers and pigs and the concluded that the concentration of anti-nutritional factors and fibre present in the leaf fraction hinder its digestibility and utilisation as a protein feed source (Pleger et al. 2021; Renaudeau et al. 2022). Investigation into the feeding potential of the stem fractions for ruminants is still required, as this remains unstudied.

## Conclusions

This study provided an initial look into the nutrient composition and yield of biorefined and residual fractions produced by juicing and leaf stripping. By utilising both fractionation methods within the same field experiments, a direct comparison in fraction nutrient composition and yield was possible. The most consistent difference between the methods was their ability to reduce the fibre concentration of biorefined fractions. Juicing resulted in a biorefined fraction with a lower aNDFom concentration than leaf stripping for all cuts and locations. Results for the CP concentration of the biorefined fractions were less clear, as inconsistencies in protein allocation occurred between cuts and locations. Both fractionation methods, however, achieved higher CP concentrations in the biorefined fractions compared to the residual fractions. Yields of the biorefined and residual fractions were also variable within and between each fractionation method and were likely heavily dependent on stand characteristics and functionality of the fractionation machinery. More work is needed to understand how each fractionation method allocates protein, fibre, and yield, particularly in variable stand conditions.

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No potential conflict of interest was reported by the authors.

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## Data availability statement

Data sets analysed during the current study are available from the corresponding author on reasonable request.

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Conceptualisation, D.P. and S.A.; methodology, B.M., D.P., and S.A.; statistical analysis, B.M., D.P., J.F., and S.A.; data curation, B.M., D.P., J.F., and S.A.; writing – original draft preparation, B.M.; writing – review and editing, B.M., D.P., J.F., and S.A.; visualisation, B.M., D.P., J.F., and S.A.; project administration, D.P. and S.A., funding acquisition, D.P. and S.A. All authors have read and agreed to the published version of the manuscript.

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