Green biorefining: Effect of nitrogen fertilization on protein yield, protein extractability and amino acid composition of tall fescue biomass

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ABSTRACT

Perennial grasses can provide very high biomass yields and a better environmental profile compared to most annual cereal crops. The grass biomass can be processed into a protein-rich fraction for monogastric feed and a fibrous pulp fraction for ruminant feed or energy production. We studied the effect of fertilization of tall fescue grown on two sandy sites in Denmark. Dry matter (DM) and crude protein (CP) yield of first cut was measured at four nitrogen (N) fertilization levels (0, 70, 140 or 210 kg N ha⁻¹). Samples were processed in a screw press, achieving a pulp fraction and a juice fraction from which proteins were precipitated into a protein concentrate fraction. CP allocation between the fractions of protein concentrate and pulp was analysed as well as the amino acid (AA) profile. CP in the brown juice fraction was not analysed.

CP concentration and CP yield of unprocessed tall fescue increased linearly with N fertilization, attaining concentrations up to 126 g kg⁻¹ DM and yields up to 0.76-0.86 Mg ha⁻¹. N fertilization did not affect CP allocation between concentrate and pulp in a consistent way. As a mean of all treatments and both sites, 17% of CP was recovered in the concentrate and 74% remained in the pulp. CP concentration increased linearly with fertilization in both concentrate and pulp with coefficients of 0.50 and 0.21 g kg⁻¹ DM kg⁻¹ N ha⁻¹, i.e. with a higher increase in the concentrate fraction. The AA profile only differed slightly between the two fractions and between the fertilization levels, however, the total sulphur to N ratio in both fractions decreased with increased fertilization, and a similar trend was observed for the relative content of the sulphur-containing amino acids. The AA profile deviated considerably from soybean meal, particularly with higher leucine and lower glutamic acid content. In conclusion, fertilization can greatly increase CP concentration and CP yield. However, the separation process needs improvements to increase the protein in the concentrate fraction.

1. Introduction

Protein is a very important source both for human nutrition and for livestock production. Soybeans constitute an excellent source of protein in monogastric diets with a well-balanced AA profile (Krishnan and Jez, 2018), and although soybean protein may be deficient in sulfur-containing AAs for livestock production (Murphy, 2008), soybean meal serves as the world’s largest source of animal protein feed (Krishnan and Jez, 2018). On a European level, soybean and soybean meal constituted nearly 16 Mt of the annual consumption of 25 Mt protein for feed in 2012, corresponding to 62% (Schreuder and de Visser, 2014). However, there are also concerns about the extensive production of soybean for protein, including potential negative effects related to land occupation and deforestation etc. (Fehlenberg et al., 2017; Lathuilière et al., 2017) as well as loss of nutrients and low profit for soybean farmers (Cavalett and Ortega, 2009). From a European perspective, there is also a desire to be less dependent on imported soybean-based protein. In 2012, 68% of the consumption of protein for feed in EU was based on import and for soybean-based protein, 97% was based on import (Schreuder and de Visser, 2014). Hence, there are various incentives for finding alternative and more locally produced protein sources for livestock feed.

Within Danish conditions, perennial grasses have been shown to have very high yield levels with a potential doubling of the dry matter (DM) yield when compared to traditional crop rotations dominated by annual cereal crops while at the same time reducing the nitrate leaching
70–80% (Manevski et al., 2017, 2018). The grass species festuolium (× Festulolium braunii) and tall fescue (Festuca arundinacea) have been found to be among the highest yielding grass species (Manevski et al., 2017). Thus, a shift towards producing perennial grasses rather than annual cereals may allow a sustainable intensification of the biomass production. However, a prerequisite for such a change is that there is a market and an application of the grass biomass and that the needs for food and feed are still met. Biofinering of the grass biomass may constitute a way of providing products for various purposes. For instance, Dale et al. (2009) proposed a ‘double duty’ of biomass crops by production of both animal protein (‘leaf protein’ from herbaceous or leafy biomass) and feedstock for fuel production. Jørgensen and Larke (2016) suggested that conversion from production of annual cereal crops to high-yielding perennial grasses could contribute to substitution of imported soybean protein. This will require biofinering of the ‘green biomass’ to achieve feedstock with the desired qualities, and the concept of refining protein from green biomass has gained considerable interest in Denmark, as indicated by recent studies (e.g. Santamaría-Fernández et al., 2017a; Solati et al., 2017, 2018).

Technically, grass biomass can be refined by processing in a screw-press to achieve a protein-rich juice and a fibrous pulp fraction. The pulp fraction may be used as feed for ruminants (Sharma et al., 2011), for bioethanol production (Sharma et al., 2011), or for bioethanol production (Sharma et al., 2011). The protein juice can be further processed into a protein-rich concentrate and a residual stream of soluble nutrients (‘brown juice’) by precipitation of the protein by heat coagulation (Sharma et al., 2011) or lactic acid fermentation (Santamaría-Fernández et al., 2017a). Previous studies have shown that the CP concentration in the concentrate fraction could be approximately doubled or tripled compared to unprocessed biomass when extracting protein from orchard grass (Dactylis glomerata) and switchgrass (Panicum virgatum) (Kammes et al., 2011), whereas the increase in CP concentration ranged between doubling and quadrupling in a study on red clover (Trifolium pratense), clover grass, alfalfa (Medicago sativa) and oilseed radish (Raphanus sativus var. Oleiferus) (Santamaría-Fernández et al., 2017a).

The feasibility of biofinering of protein from green biomass may depend on a range of factors including the biomass yield per hectare, the concentration of protein in the biomass as well as the extractability of the protein. Both DM yield and protein concentration in grasses such as tall fescue may vary between cultivars (Butkutė et al., 2014), between sites (Larsen et al., 2016), between harvest times (Butkutė et al., 2014; Kandel et al., 2016, 2017), between harvest frequencies as well as between individual harvests within a site (Kandel et al., 2016, 2017). However, N fertilization is among the most important agronomic factors in forage grass production with a strong response in DM yield (Peyraud and Astigarraga, 1998; Valkama et al., 2016) as well as CP content (Peyraud and Astigarraga, 1998) and, consequently, a very strong response in CP yield per hectare (Peyraud and Astigarraga, 1998). Hence, N fertilization is of great importance for protein production using perennial grasses. For biofinering, however, the extractability of the protein from grass biomass is also an important factor. A recent study has demonstrated that festuolium and tall fescue can provide higher yields of theoretically extractable protein per hectare compared to other grass species (Solati et al., 2018), primarily due to a high CP yield per hectare whereas there appeared to be less difference in extractability. However, little is known about the effect of N fertilization on the extractability of CP from grass biomass.

Soybean meal is considered the standard to which other protein sources are compared (Krishnan and Jez, 2018), and although the AA profile of soybean meal may vary between different sources (Lagos and Stein, 2017), it is still relevant to compare the AA profile of grass protein with soybean meal. Previously, the AA profile has been analysed in unprocessed biomass of various forage species including ryegrass, white clover and alfalfa (Edmunds et al., 2013) and in the unprocessed biomass and the protein concentrate fraction from orchard grass (Kammes et al., 2011), red clover, clover grass, alfa and oilseed radish (Santamaría-Fernández et al., 2017a). To our knowledge, studies of the AA profile of tall fescue have not been published and, moreover, there appears to be very little knowledge on the effect of N fertilization on the AA profile of the pulp and concentrate fractions, respectively, when biofinering grass biomass. Such knowledge is highly relevant when evaluating high-yielding grass species as biomass sources for protein extraction.

The objectives of this study were to examine the following research questions: i) How is CP yield and CP concentration in tall fescue affected by N fertilization level? ii) How is the allocation of CP between the concentrate fraction and the pulp fraction affected by N fertilization level when extracting protein from the biomass? iii) How is the quantity and the profile of AA in the two fractions affected by N fertilization level?

2. Materials and methods

2.1. Study sites and experimental design

Plant material was achieved from selected plots in two field trials which are described in detail in Larsen et al. (2016). The field trials were established in 2011 on two sites near Sunds in Central Jutland, Denmark, with a distance of approx. 2 km. The soil type was coarse sandy soil on both sites with 75.6% coarse sand, 14.8% fine sand, 4.1% silt, 4.7% clay and 6.0% organic matter on site 1 (56°11′29″ N, 009°02′40″ E) and 75.7% coarse sand, 18.1% fine sand, 2.6% silt, 3.1% clay and 5.4% organic matter on site 2 (56°10′16″ N, 009°02′26″ E). The soil had relatively high humidity, especially on site 1.

On both sites, a series of 64 experimental plots were established on 9th of April 2011 by sowing either 35 kg ha⁻¹ of tall fescue cv. Barolex or 20 kg ha⁻¹ of reed canary grass cv. Bamse, both being sown in pure stand and without a cover crop. Net plot size was 1.5 × 12 m = 18 m² and unfertilized plots of the same size were located between net plots. In the establishment year, all plots were treated equally and fertilized with 50, 20 and 105 kg ha⁻¹ of N, phosphorous (P) and potassium (K), respectively, and two cuts were taken at the end of June and in September, respectively.

In the years 2012–2014, the plots were used for a split-split-plot experimental design with four replicate blocks and three experimental factors: 1) The whole-plot factor grass species with either tall fescue or reed canary grass, 2) the sub-plot factor PK fertilization with either no PK fertilization or 25 kg ha⁻¹ of P and 257 kg ha⁻¹ of K, 3) the sub-plot factor N fertilization with annual rates of either 0, 150, 300 or 450 kg ha⁻¹. Three cuts were taken per year, and N fertilization was distributed with 47, 33 and 20% of the N quantity applied prior to first, second and third cut, respectively. The full annual quantity of P was applied prior to first cut, whereas the K fertilization was distributed with 51, 29 and 20% prior to the three cuts, respectively. Sulphur (S) was applied with 45 kg S ha⁻¹ to all plots as spring fertilization and additional 274 kg ha⁻¹ in an application prior to first cut of 45, 56, 65 and 74 kg S ha⁻¹ corresponding to the four N levels.

2.2. Yield measurements and biomass sampling

DM yield and N content was measured for all treatments and all three annual cuts during the years 2012-2014. Within each plot, DM yield of whole plant biomass was calculated by multiplication of fresh matter yield and DM content in the fresh matter. The N content in whole plant biomass was analysed (see Section 2.4) and given on DM basis. For this study, only the plots with tall fescue and with PK fertilization were used and only for the first cut in 2014, i.e. 4 N levels × 4 replicate blocks resulting in 16 plots per field trial. These plots were all fertilized with 25 kg ha⁻¹ P and 130 kg ha⁻¹ K on 28th March 2014 and plots were either fertilized with 0, 70, 140 or 210 kg ha⁻¹ N.
Biomass yield was measured on 4th June 2014 by use of an experimental harvester (by J. Haldrup A/S, Legstor, Denmark). A biomass sample of approx. 1 kg was sampled from each plot and frozen on the same day and until processing and analysis, i.e. a total of 32 samples was collected from the two field trials.

2.3. Processing of biomass

The pressing was conducted using an Angelia 8500S juicer (Angel Co Ltd, Busan, South Korea), a twin-screw press commonly used for extraction of juice from fruits and vegetables. The presser was equipped with a coarse size screen (hole size 1 mm) and the pressing was done at room temperature. For each sample, 200 g wet weight material was mixed with 200 g demineralized water and pressed. The obtained pulp was frozen and later freeze-dried. The protein in the juice was precipitated by heat induced precipitation. The juice was heated in 500 mL centrifugation tubes in a water bath set at 95 °C. The temperature of the juice was monitored and when reaching 80 °C (after approx. 30 min) kept for one more minute in the water bath. Thereafter, the juice was cooled to 10 °C, and the precipitated protein was collected by centrifugation at 6690 g for 20 min. The pellet was freeze dried before further analysis. The supernatant (brown juice) was not analysed. DM content of all fractions including unprocessed material was determined by weighing the samples before and after freeze-drying. Before analysis, the freeze-dried samples were finely ground by use of a ball mill.

2.4. Analysis of crude protein

Total N and S concentration of the freeze-dried samples were analyzed by Dumas combustion using a Vario Macro cube elemental analyzer (Elementar Analyensysteme GmbH, Hanau, Germany). A modified method optimized for plant materials was used. Sulfanilamide and acetonilide were used as calibration standards and matrix matched in-house and certified reference materials were included to evaluate the accuracy and precision of the analysis. A conversion factor of 6.25 was used for conversion of N to CP. The concentrations of N, S and CP were reported on a DM basis of the given material, i.e. for both whole plant material, pulp fraction and concentrate fraction.

2.5. Amino acid analysis

AA analysis was performed on pulp and concentrate samples according to Dahl-Lassen et al. (2018) and reported on a DM basis. In brief, acid hydrolysis of the freeze-dried samples was performed using 6 M HCl with 0.1% w/v phenol at 110 °C for 24 h. After hydrolysis, the samples were neutralized with 6 M sodium hydroxide. For analysis of sulfur-containing AAs, an oxidation with performic acid for 1 h at room temperature was performed prior to hydrolysis. Solid sodium meta-bisulfite was added to quench the reaction. Thereafter, the hydrolysis proceeded as described above.

Derivatization of AAs was done using the analytical grade AccQ-Tag kit (Waters, Millford, MA, USA). Pierce Amino Acid standard H (Waters, Millford, MA, USA) supplemented with cysteic acid, methionine sulfone and hydroxyproline was used as standard. Cell free 13C–15N-labeled AA mixture (Sigma-Aldrich, St. Louis, MO, USA) was added as internal standard.

Sample analysis was performed on a Waters UPLC system with a UPLC Binary Solvent Manager and Sample Manager (Waters, Millford, MA, USA). Derivatized AAs were detected on a Waters QDa single quadrupole mass detector in positive mode. Separation was performed on a Cortecs UPLC C18 (1.6 μm particle size, 2.1 × 150 mm) guard column with a VanGuard Cortecs UPLC C18 (1.6 μm particle size, 2.1 × 5 mm) guard column (Waters, Millford, MA, USA). The column temperature was maintained at 55 °C. Gradient elution was performed using 0.5% formic acid in water as eluent A and 0.5% formic acid in acetonitrile as eluent B. The flow rate was kept constant at 0.500 mL min−1 with the following gradient (expressed as solvent B): Initial conditions: 0.0% B, 0.0–0.54 m in 0.1% B, 0.54–4.00 m in 6.0% B, 4.00–4.50 m in 13.0% B, 4.50–7.50 m in 16.0% B, 7.50–8.04 m in 59.6% B, 8.04–8.05 m in 90.0% B, 8.05–8.64 m in 90.0% B, 8.64–8.73 m in 0.0% B, 8.73–10.00 m in 0.0% B.

2.6. Statistical analysis

Statistical analyses were performed using the Proc Mixed procedures of SAS 9.2 (SAS, 2008). Data on DM yield (Mg ha−1), CP concentration (g g−1 DM) and CP yield (Mg ha−1) in whole plant biomass, CP concentration and CP yield in the pulp fraction and concentrate fraction as well as CP allocation (g g−1) in the pulp and concentrate fractions were all used as response variables in an analysis using the same model. The principle of the analysis is to predict response curves for N fertilization as described in Larsen et al. (2016), with N fertilization level as a numerical explanatory variable and also including second order polynomial effects of N level to account for non-linear relationships. Site was included as a class variable to account for different yield levels on the two sites, and the interaction between site and N level allows for different shapes of the response curves on the two sites. Data were analysed using the following equation:

\[ y = S + B(S) + N × N + S × N + S × N × N \]  

(1)

where \( y \) is one of the response variables above, \( S \) is the effect of site (site 1 or site 2), \( B(S) \) is the effect of block within site (four blocks per site) and \( N \) is the effect of N fertilisation prior to the first cut (0, 70, 140 or 210 kg ha−1 N). \( S \) and \( B(S) \) were included as class variables and \( N \) was included as numerical variable. \( N \) and \( N × N \) accounted for the linear and second order polynomial effects, respectively, of \( N \) fertilization. Interactions between site and \( N \) fertilization were included to account for different linear or polynomial effects of \( N \) fertilization on the two sites.

In each analysis, the model was successively reduced by excluding non-significant variables (at \( P > 0.05 \), type III test), and only significant variables were left in the final model. However, non-significant main effects were kept in the model if they were included in significant interactions. The data sets generally included 32 observations corresponding to two sites by four blocks by four N levels. However, certain observations were lacking due to experimental errors during the laboratory analyses, but data always included at least three replicates per combination of site and \( N \) treatment.

The total AA proportion of the CP content in the pulp and concentrate fractions was analysed by a model using CP concentration as a numerical explanatory variable and with fraction, site and block within site as class variables. Hence, the model can test linear effects of CP concentration on total AA proportion with different levels and slopes for fractions and sites. Data were analysed using the following equation:

\[ y = F + S + B(S) + CP + F × S + F × CP + S × CP + F × S × CP \]  

(2)

where \( y \) is the proportion of AA as percent of the CP content, \( F \) is the effect of fraction (pulp or concentrate), \( CP \) is the effect of CP content in the given fraction (g kg−1 DM), and \( S \) and \( B(S) \) are as for Eq. (1). \( F \), \( S \) and \( B(S) \) were included as class variables and \( CP \) was included as numerical variable. The model was reduced successively as described for analyses using Eq. (1). The data set included 61 observations since three observations were excluded due to experimental errors during the laboratory analyses.

The AA profile in the pulp and concentrate fractions from each \( N \) fertilization level and each of the two sites were evaluated by use of standard deviations.
Fig. 1. Dry matter yield (A), crude protein concentration (B) and crude protein yield (C) in whole plants of tall fescue depending on N fertilization. Dots indicate observed data and lines indicate predicted functions. Each dot represents a sample from a single field plot.
3. Results

3.1. Dry matter and crude protein in whole plant biomass

N fertilization had a significantly positive effect on DM yield, CP concentration and CP yield of whole plant biomass (Fig. 1 and Table 1). DM yield increased non-linearly on both sites with a declining response at higher N levels, reaching 6.3 and 6.0 Mg ha\(^{-1}\) at 210 kg N ha\(^{-1}\) on site 1 and 2, respectively (Fig. 1A). CP concentration increased linearly with N fertilization with the same relationship on both sites and an increase from 68 to 126 g kg\(^{-1}\) DM when increasing the N level from 0 to 210 kg N ha\(^{-1}\) (Fig. 1B). Also, the CP yield increased linearly with N fertilization on both sites, although at different levels (Fig. 1C). At 210 kg N ha\(^{-1}\), CP yield reached 0.86 and 0.76 Mg ha\(^{-1}\) on site 1 and 2, respectively.

3.2. Crude protein in fractions after processing

The proportion of the initial CP allocated to the pulp fraction was not significantly affected by neither N fertilization nor site, with a mean of 0.78 and 0.71 g g\(^{-1}\) for site 1 and 2, respectively (Fig. 2A, Table 1). Thus, 78 and 71% of the initial CP was on average allocated to the pulp fraction on the two sites. For the allocation of CP into the concentrate fraction, there was a significant effect of N fertilization as well as interaction between site and N fertilization (Fig. 2B, Table 1). For site 1, the allocation of CP was only slightly affected by N fertilization whereas for site 2, a relatively higher proportion of CP in the biomass from the 0 kg N ha\(^{-1}\) treatment was allocated to the concentrate compared to the 70–210 kg N ha\(^{-1}\) treatments (Fig. 2B). As a mean of all fertilization levels, the allocation of CP into the concentrate fraction was 0.19 and 0.15 g g\(^{-1}\) for site 1 and 2, respectively. Hence, the total CP recovery in pulp and concentrate was 0.97 and 0.87 g g\(^{-1}\) for site 1 and 2, respectively.

CP concentration increased linearly with increasing N fertilization, both in the pulp fraction and the concentrate fraction (Fig. 3, Table 1). For the pulp fraction, the same pattern was seen on both sites with an increase in CP concentration from 71 to 116 g kg\(^{-1}\) DM when increasing N fertilization from 0 to 210 kg N ha\(^{-1}\) (Fig. 3A). The concentrate fraction contained more CP on site 1 than on site 2, but the response to increasing N fertilization was similar on the two sites (Fig. 3B). The CP concentration in concentrate increased from 193 to 298 g kg\(^{-1}\) DM on site 1 and from 159 to 263 g kg\(^{-1}\) DM on site 2. Hence, the CP concentration was 124–172 % higher in the concentrate fraction than in the pulp fraction. The slope coefficient was 0.21 and 0.50 g CP kg\(^{-1}\) DM kg\(^{-1}\) N ha\(^{-1}\) for the pulp fraction and concentrate fraction, respectively, showing a larger effect of N fertilization on CP concentration in the concentrate fraction than in the pulp fraction.

CP yield per hectare increased significantly and linearly with N fertilization both for the pulp fraction and the concentrate fraction (Fig. 4, Table 1). For the pulp fraction, the same pattern was seen on both sites with an increase in CP concentration from 71 to 116 g kg\(^{-1}\) DM when increasing N fertilization from 0 to 210 kg N ha\(^{-1}\) (Fig. 3A). The concentrate fraction contained more CP on site 1 than on site 2, but the response to increasing N fertilization was similar on the two sites (Fig. 3B). The CP concentration in concentrate increased from 193 to 298 g kg\(^{-1}\) DM on site 1 and from 159 to 263 g kg\(^{-1}\) DM on site 2. Hence, the CP concentration was 124–172 % higher in the concentrate fraction than in the pulp fraction, depending on site and N fertilization. The slope coefficient was 0.21 and 0.50 g CP kg\(^{-1}\) DM kg\(^{-1}\) N ha\(^{-1}\) for the pulp fraction and concentrate fraction, respectively, showing a larger effect of N fertilization on CP concentration in the concentrate fraction than in the pulp fraction.

CP yield per hectare increased significantly and linearly with N fertilization both for the pulp fraction and the concentrate fraction (Fig. 4, Table 1). CP yield was significantly higher on site 1 than on site 2, and for the concentrate fraction, CP yield increased less with N fertilization on site 2 than on site 1. At 210 kg N ha\(^{-1}\), the CP yield was 308% higher in the pulp fraction than in the concentrate fraction on site 1 whereas the difference was 424% for site 2.

3.3. Amino acid concentration and profile in fractions

Overall, there was only a marginal variation in the AA profile between the two grass fractions and between the two sites (Fig. 5). Aspartic acid increased with N fertilization in the pulp fraction and tyrosine increased in both the pulp fraction and the concentrate fraction. In contrast, cysteine and methionine decreased, particularly in the pulp fraction. For the concentrate fraction, N fertilization had a moderate negative effect on particularly lysine, cysteine and methionine. When the fertilization was increased from 0 kg N ha\(^{-1}\) to 70–210 kg N ha\(^{-1}\),
the proportion of lysine as percentage of total AAs in the concentrate fraction was reduced from 5.8% to 4.8–5.5% on site 1 and from 5.9 to 5.2–5.4% on site 2. The proportion of cysteine was reduced from 0.94 to 0.58–0.69% on site 1 and from 1.2 to 0.74–0.94% on site 2, whereas the proportion of methionine was reduced from 2.0 to 1.2–1.4% on site 1 and from 2.4 to 1.5–1.9% on site 2.

Analysis of total S in the pulp and concentrate fractions showed that the S:N-ratio decreased as the N fertilization level increased. The decrease was more pronounced for the pulp fraction where the S/N-ratio decreased by 30% from 0.19 to 0.13 (R² = 0.65) when fertilization was changed from 0 kg N ha⁻¹ to 210 kg N ha⁻¹. For the concentrate fraction, the S:N-ratio decreased by 23% from 0.13 to 0.10 (R² = 0.483).

The AA proportion decreased significantly with increasing CP concentration (P = 0.016) with a common slope of -0.087% g⁻¹ CP kg⁻¹ DM for both sites and for both pulp and concentrate fractions (Fig. 6). The effect of CP concentration did not interact significantly with neither fraction × site (P = 0.176) nor with site (P = 0.689) or fraction (P = 0.595). However, site and fraction interacted significantly on the level of the AA proportion (P = 0.003). The mean AA proportion as percentage of CP was 81.9 and 72.2% in concentrate from site 1 and 2, respectively, whereas the mean AA proportion was 76.4 and 79.6% in pulp from site 1 and 2, respectively, but with a slightly decreasing proportion at higher CP levels within both sites and both fractions (Fig. 6).

In the AA analyses, tryptophan as well as the amide group of glutamine and asparagine were not included. Across sites and fertilization levels, the proportion of essential AAs (Arg, His, Ile, Leu, Lys, Met, Phe, Thr, Val) ranged between 49.9 and 51.4% of total AAs in the concentrate and between 48.8 and 51.1% in the pulp.

4. Discussion

4.1. Dry matter and crude protein in whole plant biomass

This study reports the tall fescue yield of the first of three cuts in the experimental year and, hence, it does not represent the effect of fertilization on potential annual production. However, the pattern in DM yield response to N fertilization seen in Fig. 1A is very similar to the pattern found for the annual yield in 2014 (Larsen et al., 2016). As a mean of the two sites, the annual yield in 2014 at the highest N level was 14.2 Mg DM ha⁻¹ and 1.98 Mg CP ha⁻¹ (Larsen et al., 2016) and, hence, the yields in the first cut correspond to 43% of the annual DM yield and 41% of the annual CP yield. The annual yields of the two experimental sites are lower than the yield range of 16.6–19.7 Mg DM ha⁻¹ and approx. 3 Mg CP ha⁻¹ reported for tall fescue in another...
Danish Study with comparable N fertilization (300–500 kg N ha\(^{-1}\) y\(^{-1}\)) (Manevski et al., 2017; Solati et al., 2018). The primary reason for the lower yield level in the present study is most likely the poorer soil quality with a considerably higher content of coarse sand and lack of irrigation. Nevertheless, the yield level is still relatively high for this type of coarse sandy and semi-marginal land and higher than a traditional crop rotation on a sandy loam soil (Manevski et al., 2017). This emphasizes the considerable potential for biomass production using perennial grass species such as tall fescue.

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\text{N fertilization has a very large effect on DM yield in grasses, although often with a declining response at higher fertilization levels (Morrison et al., 1980; Peyraud and Astigarraga, 1998; Valkama et al., 2016). This response pattern was also found in this study (Fig. 1A). The results are also consistent with those of Pedroso et al. (2014) who found a near-linear response and a tripling of the DM yield in tall fescue when applying up to 200 kg N ha\(^{-1}\) y\(^{-1}\) compared to no fertilization but N fertilization was not applied beyond this level. The difference in yield response between site 1 and 2 is most likely related to differences in the soil N pool and N mineralization from the soil. In general, the effect of N fertilization on CP content in grass is nearly linear up to very high levels of applied N with increases up to 0.5–0.9 g CP kg\(^{-1}\) DM kg N ha\(^{-1}\) (Peyraud and Astigarraga, 1998). Our study also shows a linear increase in CP content (Fig. 1B), although the effect is smaller with a slope of 0.28 g CP kg\(^{-1}\) DM kg N ha\(^{-1}\) (Peyraud and Astigarraga, 1998). Hence, N fertilization is a key factor for optimizing protein production of perennial grasses, both in terms of protein yield per hectare and protein concentration in the biomass. However, the CP content of tall fescue has also been shown to be largely affected by e.g. the growth stage of the grass at harvesting (Butkutė et al., 2014; Kandel et al., 2017), and harvest time and number of cuts per year may constitute another means of optimizing the CP content of tall fescue.

4.2. Crude protein in fractions after processing

Extraction and precipitation of protein from whole plant biomass serves as a way to produce a concentrated protein-rich feed. Compared to the whole plant biomass, the increase in CP concentration in the concentrate ranged from 108 to 183% depending on site and N fertilization and with a numerical increase from 126 to 263–298 g CP kg\(^{-1}\) DM at the highest N fertilization level (Figs. 1B and 3 B). For comparison, the CP concentration was approx. doubled to tripled by extraction of protein from orchard grass and switchgrass as compared to unprocessed grass biomass (Kammes et al., 2011) and increases of 126 and 116% have been found for red clover and alfalfa, respectively, and 325 and 335% for clover grass and oil seed radish, respectively (Santamaría-Fernández et al., 2017a). Hence, there appears to be considerable variation between biomasses in potential effect of extraction on CP concentration, although the specific processing may also affect CP concentration.

The efficiency of extraction of CP into the concentrate fraction was not consistently affected by N fertilization, since there was no clear
effect on site 1 and a declining effect with increasing N fertilization on site 2 (Fig. 2B). For site 2, however, there only appears to be a higher proportion of CP in the concentrate fraction without N fertilization and no clear differences in the range 70 to 210 kg N ha$^{-1}$. This pattern may be related to experimental uncertainty particularly for site 2, reflected in a relatively lower total CP recovery of 83.2–86.8% for 70–210 kg N ha$^{-1}$ compared to 93.2% for 0 kg N ha$^{-1}$. For comparison, the total CP recovery on site 1 ranged between 91.9 and 103.0%. The brown juice after precipitation of protein concentrate was not analysed but is likely to have a certain N content which may, to some extent, reduce the total CP recovery in concentrate and pulp. Consequently, the primary effect of N fertilization seems to be on the DM yield and the CP concentration in the biomass whereas fertilization appears to have a rather limited effect on the extractability of CP from tall fescue biomass. However, the proportion of CP extracted into the concentrate fraction is relatively low with a mean allocation of 19% for site 1 and 15% for site 2 (Fig. 2B). It is likely, that N fertilization could have an impact on CP extractability within conditions with more effective extraction.

On the other hand, studies of CP extraction from green biomass of other plant species have shown similar efficiencies regarding the proportion of protein ending in the concentrate fraction. Kammes et al. (2011) achieved CP extraction efficiencies of 20.6 and 23.5% for immature and mature orchard grass, respectively, and 15.2 and 24.3% for immature and mature switchgrass. Santamaria-Fernández et al. (2017a) obtained CP extraction efficiencies of 23.4, 17.1, 15.1 and 12.1% for red clover, clover grass, alfalfa and oilseed radish, respectively, and with 60.9–72.3% of the CP left in the pulp fraction. These extraction efficiencies are much lower than those reported by Dale et al. (2009), however, these higher values are most likely due to various modification of the extraction process. Increasing the extraction temperature and using alkaline conditions have in several cases been found to improve extraction efficiency dramatically (Dale et al., 2009; Zhang et al., 2015). Washing of the press pulp, i.e. extractions with two or more steps, is also a simple method to improve the extraction efficiency. Besides extraction efficiency, the amount of protein ending in the protein concentrate is also influenced by the efficiency of the precipitation step. In this case, the precipitation was done by heat coagulation of the protein. In other studies, the heat treatment is combined with lowering the pH of the juice (Kammes et al., 2011). Therefore, effective extraction and precipitation of protein from green biomass is clearly a technical challenge, most likely requiring process optimisation for each type of biomass, which was not the aim of this study.

Consequently, the economics of protein extraction from green biomass may not be competitive with other protein sources if the concentrate fraction is the sole product (Dale et al., 2009), and protein extraction should be integrated in a biorefinery concept with various products including the use of the pulp fraction for e.g. ruminant feed or biogas production.

4.3. Amino acid concentration and profile in fractions

The mean AA proportion as percentage of CP was 81.9 and 72.2% in concentrate from site 1 and 2, respectively, and with a slightly decreasing proportion at higher CP levels (Fig. 5). The AA proportion of
CP is considerably lower than found in soybean meal, e.g. with 96.3% found in Brazilian soybean sources (Lagos and Stein, 2017), indicating a lower feeding value of CP from grass concentrate. However, in the AA analysis performed in this study, tryptophan was not measured for technical reasons thereby underestimating AA proportion of CP. Despite this, the AA proportion of CP is similar to the range between 70.4 and 78.6% in unprocessed whole-plant biomass found among forage species and mixtures by Edmunds et al. (2013), whereas Santamaría-Fernández et al. (2017a) found a range between 79 and 100% AA in CP in protein concentrate. Although the AA proportion only decreased moderately but significantly in both the concentrate and the pulp with increasing CP level, this indicates that N fertilization causes an increase in the content of non-protein N in the biomass, e.g. chlorophyll, which may also decrease the value for monogastric feed. Hence, there may be a trade-off between the positive effect of N fertilization on CP concentration and CP yield per hectare and the potentially negative effect on AA proportion of CP. Nevertheless, there is still a marked increase in AA yield per hectare when increasing N fertilization.

The AA profile found in pulp and concentrate of tall fescue has considerable similarities with the profile found in other grass species and legume species used for forage including orchard grass (Kammes et al., 2011), perennial ryegrass and meadow grass as well as legumes such as white clover and alfalfa (Edmunds et al., 2013), as illustrated in Fig. 7 which also shows the AA profile for soybean meal. The most pronounced difference is a higher concentration of leucine in tall fescue than in the other grass species. Compared to soybean meal, tall fescue has considerably lower concentration of glutamic acid and higher concentration of leucine. Besides, there are certain differences in the concentration of certain other AAs between soybean meal and the two grass fractions (Fig. 7). Across sites and fertilization levels, the proportion of essential AAs ranged between 49.9 and 51.4% of total AAs in the concentrate and between 48.8 and 51.1% in the pulp. Hence, the proportion of essential AAs is slightly higher in tall fescue compared to Brazilian soybean meal with 46.1% (Lagos and Stein, 2017) and unprocessed biomass of various forage species with a range between 44.8 and 47.9% essential AAs (Edmunds et al., 2013).

The similarities across the forage species (Fig. 7) indicates a fairly stable AA profile across species used for forage production, probably because Rubisco is the main extracted protein which is rather well conserved among plant species in terms of AA profile (Santamaría-Fernández et al., 2017a). Moreover, Kammes et al. (2011) found remarkably small differences in AA profile between fresh orchard grass and leaf protein concentrate from orchard grass. This is consistent with our results, showing only limited differences in the AA profile between the pulp and the concentrate fractions (Figs. 6 and 7). Hence, extraction of grass juice for protein concentrate production appears to have limited potential for modifying the AA profile of grass protein.

The sulfur-containing AAs cysteine and methionine are often found in inadequate levels in soybean meal when used for feed for monogastric animals (Krishnan and Jez, 2018), and these AAs are limiting e.g. for poultry production (Santamaría-Fernández et al., 2017a). Therefore, alternative protein sources for monogastric feed should ideally have higher proportions of these AAs. Increased N fertilization appears to reduce the proportion of particularly cysteine and methionine in the pulp fraction of tall fescue (Fig. 6A and C). For the concentrate fraction, the proportion of these two AAs appears to be less affected by fertilization, whereas the proportion of leucine was more reduced by fertilization than in the pulp fraction (Fig. 6B and D). For both lysine, cysteine and methionine, the proportion of these AAs in the concentrate were lower at fertilization levels of 70–210 kg N ha⁻¹ compared to 0 kg N ha⁻¹ (Fig. 6B and D), indicating a dilution of these AAs at high N fertilization levels. The decrease in methionine and cysteine could be due to an alteration in the type of proteins that are produced in the plants in response to increased N supply. However, total S analysis of pulp and concentrate fractions revealed that as N fertilization increased from 0 kg ha⁻¹ to 210 kg ha⁻¹, the concentration of total S decreased relative to total N; the S:N ratio decreased linearly from 0.191 to 0.132 (31%) for the concentrate fraction and...
Fig. 6. Total amino acid concentration as proportion of crude protein concentration in pulp and concentrate fractions when extracting protein from tall fescue from site 1 (A) and site 2 (B). Data are analysed according to Eq. (2), and dots indicate observed data and lines indicate predicted functions. Each dot represents a sample from a single field plot.

Fig. 7. Amino acid profile for soybean meal and various grass biomasses. Data for soybean meal is based on the mean of five sources of Brazilian soybean meal from Lagos and Stein (2017). Data for tall fescue pulp and concentrate is for site 1 and fertilization with 210 kg N ha\(^{-1}\) from this study. Data for fresh orchard grass and orchard grass concentrate (‘leaf protein’) is from Kammes et al. (2011). Data for perennial ryegrass (‘First harvest’), white clover (‘First harvest’), meadow grass (‘Meadow grass 1 Fresh’) and alfalfa (‘Fresh’) is from Edmunds et al. (2013) and all based on fresh material (‘original forage’).
from 0.133 to 0.101 (24%) for the pulp fraction (data not shown). Hence, the decrease in S:N ratio was less in the concentrate fraction compared to the pulp fraction which was also the case for the decrease in methionine and cysteine concentration. Although total S is not a direct measure of cysteine and methionine, the fact that both total S concentration and the relative content of the sulphur-containing amino acids decrease could serve as an indicator that S availability or uptake is limiting at conditions with high N application. This could likely be counteracted by fertilizing the grass more intensively with S; although limiting at conditions with high N application. This could likely be acids decrease could serve as an indicator that S availability or uptake is compared to the pulp fraction which was also the case for the decrease from 0.133 to 0.101 (24%) for the pulp fraction (data not shown). This study demonstrates that N fertilization has a strong positive effect on dry matter yield, crude protein content and crude protein yield of unprocessed biomass of tall fescue. When biofering the biomass into a protein concentrate fraction and a fibrous pulp fraction, there was no consistent effect of N fertilization on the protein concentrate allocation between the fractions, i.e. increased fertilization did not increase the proportion of crude protein obtained in the concentrate fraction. However, N fertilization increased the crude protein concentration in both fractions but with a higher effect on the concentrate fraction when calculated per kg applied N. There was only a limited difference in the amino acid profile between the pulp and concentrate fractions. The total sulphur concentration in both fractions decreased with increased fertilization, and a similar trend was observed for the relative content of the sulphur-containing amino acids. Besides this, N fertilization had a limited effect on the amino acid profile. Therefore, the amino acid profile of tall fescue appears to be relatively stable, and the main effect of N fertilization is a larger crude protein yield per hectare and a higher crude protein concentration in both the protein concentrate fraction and the fibrous pulp fraction.

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References


