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The effects of grass biomass preservation methods, organic acid treatment and press type on the separation efficiency in the green biorefinery

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ABSTRACT

Processing green biomass into novel products provides opportunities to improve the sustainability of the bioeconomy. The objective of this study was to evaluate the effects of biomass types (fresh, frozen-and-thawed, dried-and-rehydrated and ensiled grass) as well as formic and propionic acid-based additive on the efficiency of liquid-solid separation and crude protein (CP) yield. Three different pressing methods for liquid-solid separation were used. All preservation methods improved biorefinery efficiency compared to fresh grass, and the effect of additive was more profound on the fresh biomass than other materials. However, due to lower CP concentration in the liquid, presumably caused by lower nitrogen solubility, the amount of CP retained in the liquid was not improved in response to the additive treatment. The type of processing technology plays a key role in the extraction of relevant compounds from biomass. With less efficient separation methods, the effects of pretreatments were more pronounced.

1. Introduction

With an increased demand for food and feed protein due to population growth, novel but sustainable protein sources are required. Grasslands occupy about 69 % of the world's agricultural area and yield high biomass (Dengler et al., 2020) that provides nutritional and ecosystem benefits (Chen et al., 2022; Dengler et al., 2020; Plantureux et al., 2005). This makes grass protein a prospective protein source for animal and human consumption (Chiesa and Gnansounou, 2011). When compared with soybean meal, the amino acid composition of the grass protein was similar (Jørgensen et al., 2022) indicating that it could be successfully used to replace other plant-based proteins.

The use of grass has been mainly limited to the ruminant sector due to the capture of soluble nutrients in the fibre matrix of plant cells. However, green biorefinery provides techniques that liberate these soluble nutrients which can subsequently be used for various added-value purposes (Jørgensen et al., 2022; Mandl, 2010). According to Mandl (2010), the concept of green biorefinery is an innovative approach that utilizes fresh green biomass as raw material to generate valuable industrial products and other side streams that could be further refined. During the first step of the biorefinery process, the two fractions mechanically separated are protein-rich liquid fraction and fibrous solid fraction which can be used as such or serve as raw materials for further products (Kamm et al., 2016; Wilkinson and Rinne, 2018). The efficiency of the biorefinery process is highly variable depending on the extraction technology and biomass quality (Franco et al., 2019) but 30–50 % of biomass dry matter (DM) and 40–60 % protein can be recovered in the liquid fraction (Damborg et al., 2020). The liquid fraction can be fed to monogastrics (Keto et al., 2021), used for food ingredient extraction (Contreras et al., 2019) or provide biomolecules for various purposes, while the pulp with high fibre content can be used, for example as paper and packaging production, animal bedding, biochar, ruminant feed (Savonen et al., 2020), biogas or single cell protein production (Pihlajaniemi et al., 2020).

Fresh grass as well as the solid and liquid fractions produced in the biorefinery process are prone to fast deterioration, and fresh grass biomass accessibility is highly seasonal in most parts of the world. These factors challenge the logistics of the green biorefinery approach. Within livestock production, ensiling (anaerobic lactic acid fermentation) as an efficient method of grass biomass preservation has been established (Wilkinson and Rinne, 2018) and could be used as an alternative to fresh grass. Until now, the major focus in green biorefinery research has been on the use of fresh green crops (Damborg et al., 2020; Thers and Eriksen, 2022) although grass silage has also been used (Franco et al., 2019; Rinne et al., 2020; Schwarz et al., 2016). Ensiling decreases the biomass pH and could promote the release of liquid from cell contents, but direct

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comparisons of the same biomass as fresh or ensiled are lacking. Further, various additives can be used in ensiling, and Rinne et al. (2020) showed that using fibrolytic enzymes as additives in grass silage acted as a pretreatment of the biorefinery process by improving the liquid yield. Ensiling low DM silages may result in high fermentation losses and poor hygienic quality of the silage, but formic acid-based additives have successfully been used to improve the preservation quality (Franco et al., 2022; Jaakkola et al., 2006) and they can also be used to extend the shelf life of fresh biomasses (Rinne et al., 2019).

Ensiling also negatively affects the biomass because some protein is degraded during fermentation (McDonald et al., 1991), and precipitation of the protein from the acidic silage juice is not possible. Other practically feasible potential methods of green biomass preservation include drying and freezing. Drying, e.g., haymaking, is a traditional way of grass preservation (Wilkinson and Rinne, 2018), and weather dependency can be decreased by artificial drying. Freezing is energy intensive and minor losses in the nutritional quality of the material are to be expected while rupturing of the plant cells during the freeze-thaw circle could promote nutrient release (Phalakornkule et al., 2017).

The aim of this study was to use the same grass biomass 1) as fresh, 2) after freezing and thawing, 3) after drying and rehydrating and 4) after ensiling to directly compare the liquid protein yields from these different biomass types. In addition, a formic and propionic acid-based silage additive (FPA) was used for all biomasses except the dried material. We hypothesized that all preservation methods and the use of FPA would increase the liquid protein yield due to their effects on plant cell rupture compared to the use of fresh grass. In addition, three different pressing techniques were used to elucidate the interactions between raw material characteristics and the processing technology.

2. Materials and methods

2.1. Experimental grass biomass and preservation methods

The experiment was conducted at the Natural Resources Institute Finland (Luke) in Jokioinen, Finland (60°48′N, 23°29′E). The grass was harvested on 9 September 2021 from the first regrowth of a ley which was mown and precision-chopped immediately after cutting using farm-scale equipment. The ley was a mixture of 61 % timothy (*Phleum pratense*) and 29 % meadow fescue (*Festuca pratensis*) but also contained 8 % of dead material and 2 % of unsown species, on a fresh matter basis. Fresh grass was representatively sampled before pretreatments to evaluate its chemical composition.

Four biomass types were produced from the fresh grass:

- Fresh grass as such (FR)
- Frozen-and-thawed grass (FZ)
- Dried-and-rehydrated grass (DR)
- Ensiled grass (EN)

Grass material was frozen at −20 °C immediately after harvesting and later thawed at room temperature to produce the FZ biomass. For DR, the fresh grass biomass was dried at 40 °C in a forced air oven for 48 h to reach a moisture content of 67 g kg^{-1}. Before liquid-solid separation, the dried biomass was rehydrated to the original DM content and let soak for ca. 1 h refrigerated. The EN was produced by ensiling fresh grass material in pilot-scale silos (cylinder shape, 12 L effective volume). The silos were sealed air-tight and stored in the dark at an average room temperature of 19.5 ± 0.34 °C for 90 days until opening according to EFSA (2018) recommendations. After ensiling, samples were taken to analyse the fermentation quality and chemical composition of the silages.

All biomass types, except DR, were included as such (Control, without additive) and after application of FPA (5 L/ton fresh matter; AIV Ässä Na, Eastman; Oulu, Finland). The additive was applied to the FR and FZ biomass using a commercial applicator attached to the precision chopper, while for the EN biomass, FPA was manually added to a batch of grass before filling the silos.

2.2. Mechanical liquid-solid separation

Liquid-solid separation of the four biomass types was performed using two laboratory scale methods: pneumatic press (LPP; Luke in-house built equipment, Jokioinen, Finland) and twin-screw press (LTS; Angel Juicer Ltd., Busan, South Korea); and a custom-made pilot-scale single screw press (CSS; Pellon Group Ltd., Ylihärmä, Finland). The capacity and processing procedure of the mechanical separation methods were adopted from Franco et al. (2019) and Damborg et al. (2020).

Three replicates per sample of the biomass materials were processed using each separation method as follows:

- LPP: A 100 g biomass sample was placed in a mesh bag and pressed for 2 min between two piston plates at a pressure of six bars (×100 kPa). Before processing the actual samples, the mesh bags were wetted and pressed to exclude the effect of absorbed moisture into the mesh bags.
- LTS: A 300 g biomass sample was used in the press that has a gear grinding force of 3 hp and screw rotation speed of 82 rpm. To achieve a steady state and optimal performance, the press was first fed with about 150 g of biomass before the actual processing of experimental biomass sample batches.
- CSS: A batch of 50 kg was used, and the press was previously filled with biomass to achieve a steady state. Only FR was processed with this press.

From each processing round, all extracted liquid was quantitatively collected, weighed, and frozen at −20 °C until further analysis.

2.3. Analytical procedures and calculations

The FR and EN were dried for 16 h at 105 °C to determine DM content and at 60 °C until dry and subsequently, ash, nitrogen (N), soluble N and neutral detergent fibre (NDF) were analysed. Silage DM concentration was corrected with equations provided by Huida et al. (1986) for the loss of volatile compounds. The ash concentration was determined by igniting the samples at 600 °C for 2 h according to AOAC (2019, method 942.05). Soluble N and total N were analysed according to Kjeldahl procedure based on standard methods of AOAC (2019, method 984.13) using Cu as a digestion catalyst and Foss Kjeltec 2400 nitrogen analyzer unit. The crude protein (CP) concentration was calculated by multiplying N content by 6.25. According to Van Soest et al. (1991), NDF was analysed using ANKOM 220 Fiber analyzer (ANKOM Technology, Macedon, NY, USA) with sodium sulphite and expressed without residual ash. The buffering capacity (BC) of FR was determined by using the lactic acid method according to Weissbach et al. (1974).

The silage samples were analysed for fermentation quality. Ammonia N was determined from water extract of samples according to McCullough (1967). Ammonia N and soluble N proportion in total N were calculated using total N content of individual samples. Soluble non ammonia N was calculated by subtracting the concentration of ammonia N from soluble N. The pH of FR and EN (Control and FPA-treated) was measured from homogenised samples using a Mettler Toledo 345 pH meter while water soluble carbohydrates (WSC) were determined according to Somogyi (1945) using the Schimadzu double-beam UV-VIS spectrophotometer UV-1800 (Schimadzu Co., Kyoto, Japan). Ethanol determination was done through a spectrophotometric method using a commercial kit (Cat. No. 10 176 290 035, Boehringer Mannheim GmbH, Mannheim, Germany) according to the manufacturer's instructions. Determination of volatile fatty acids was done according to Huhtanen et al. (1998) by using an external standardization and HP 6890 gas chromatograph with an automatic injector HP 7683, FID detector and

GC Chemstation Rev.C.01.04, while lactic acid was determined according to Haacker et al. (1983). The *in vitro* organic matter digestibility based on cellulase solubility was calculated with the correction equation provided by Huhtanen et al. (2006). All liquid samples were analysed for DM, ash and N as described above. Fermentation coefficient (FC) was calculated according to the equation provided by Pahlow and Weissbach (1999):

$$FC = \frac{DM\left(g\,kg^{-1}\right) + \left[8 \times \left(WSC\left(g\,kg^{-1}\,DM\right)/BC\left(g\,lactic\,acid\,100\,g^{-1}\,DM\right)\right]}{10}$$

Liquid yield was calculated using the following formula:

$$\text{Liquid yield}\left(g\,g^{-1}\right) = \text{Fresh matter in liquid}\,(g)/\text{Fresh matter in original biomass}\,(g).$$

Retained compounds in liquid were calculated as follows:

$$\text{Retained compound in liquid}\left(g\,g^{-1}\right) = \frac{\text{Liquid yield}\,(g\,g^{-1}) \times \text{Liquid DM}\left(g\,kg^{-1}\right) \times \text{Nutrient concentration in liquid}\left(g\,kg^{-1}\,DM\right)}{\text{DM of original biomass}\left(g\,kg^{-1}\right) \times \text{Nutrient concentration in original biomass}\left(g\,kg^{-1}\,DM\right)}$$

The same chemical composition of the original biomass was used for FR, FZ and DR.

2.4. Statistical analysis

Data were analysed using the SAS MIXED procedure (version 9.4; SAS Inst. Inc., Cary, NC, USA). The normality of analysed variables was checked using box plot and scatter plot of residuals and fitted values generated using the UNIVARIATE procedure of SAS. Separately for both laboratory scale presses, the model contained fixed effects of biomass type, additive and their interaction, while replicate was used as a random effect. In a separate analysis, the main and interaction effects of the three separation methods and additive treatment on FR biomass were analysed and Tukey test was used to establish the differences in pairwise comparisons of treatment means. Differences were declared significant at $P < 0.05$, while a trend was considered at $P < 0.10$.

3. Results and discussion

3.1. Fresh herbage and silage characteristics

The chemical composition of the FR and EN biomasses is shown in Table 1. The DM content of FR and subsequent EN was low as the biomass was collected immediately after mowing. In practical silage making, it has become routine to wilt grass in the field prior to picking it up (Wilkinson and Rinne, 2018). This results in farm silages having a higher DM content, for example, 321 g kg^{-1} in a Finnish data set containing over 110,000 farm silage samples analysed in a commercial laboratory (Salo et al., 2014). Silage DM content is a key parameter affecting the liquid yield (Franco et al., 2019). Therefore, biomass with relatively low DM content (218 g kg^{-1}; FR) was intentionally used in this study in order to facilitate high liquid yield.

The CP concentration of 123 g kg^{-1} DM in FR biomass can be considered low, as compared to the farm silage data set where it was 147 g kg^{-1} DM (Salo et al., 2014). The grass biomass CP concentration is highly variable and can be increased by manipulating factors such as harvest time (Rinne et al., 1997), N fertilization (Salo et al., 2014), or by using forage legumes (Huhtanen et al., 2007). Strategic manipulation of the management factors could thus be used to achieve higher CP yields in the biorefinery process.

A low DM content of grass biomass for ensiling purpose poses a risk to the hygienic quality of silage and is likely to increase losses due to effluent run-off and extensive fermentation (McDonald et al., 1991). The ease of grass biomass ensiling can be evaluated using the FC, where a value above 45 indicates a material easy to ensile (Pahlow et al., 2002). The FC in FR was 55 even though its DM content was low. Factors elevating the FC were the low BC and high WSC concentration of the grass. Despite the high FC, the FPA application improved the fermentation quality of EN compared to Control as indicated by the lower proportion of ammonia N (Huhtanen et al., 2013) and soluble N in total

Table 1
Chemical composition and fermentation quality of fresh and ensiled grass.

Item	Fresh	Ensiled	
		Without additive	With FPA[a]
Dry matter (DM), g kg^{-1}	218	208	208
pH	6.14	3.87	3.96
Buffering capacity, g lactic acid 100 g^{-1} DM	2.97		
Fermentation coefficient	55		
In total N[b], g kg^{-1}			
Ammonia N		66.2	31.8
Soluble N	232	588	449
Soluble non-ammonia N		522	417
In DM, g kg^{-1}			
Ash	84	94	94
Crude protein	123	122	123
Water soluble carbohydrates	125	38	106
Ethanol		9.7	5.7
Neutral detergent fibre	509	458	451
Lactic acid		96	57
Acetic acid		26.2	17.5
Propionic acid		0.33	0.05
Butyric acid		0.05	0.05
Total volatile fatty acids[c]		26.6	22.7
Total fermentation acids[d]		122	79
Cellulase solubility, g kg^{-1} OM[e]	839	826	835
IVOMD[f], g g^{-1} OM	0.786	0.771	0.781

[a] FPA = Formic and propionic acid-based additive.
[b] N = Nitrogen.
[c] Acetic acid + propionic acid + butyric acid + minor volatile fatty acids (data not shown).
[d] Total volatile fatty acids + lactic acid.
[e] OM = Organic matter.
[f] IVOMD = *In vitro* organic matter digestibility based on cellulase solubility.

Table 2
The effects of biomass types (Fresh; Frozen; Dried and rehydrated (Dry); Ensiled) and additive treatment on the biorefinery outputs using lab-scale pneumatic press and twin screw press.

Biomass type (BT)	Fresh		Frozen		Dry	Ensiled		SEM[b]	*P*-value		
Additive (Add)	Control	FPA[a]	Control	FPA	Control	Control	FPA		BT	Add	BT × Add
Lab-scale pneumatic press											
Liquid yield, g g^{-1}	0.125	0.346	0.408	0.418	0.420	0.519	0.534	0.0091	<0.001	0.005	<0.001
Liquid dry matter (DM), g kg^{-1}	22.7	61.3	51.6	56.4	59.2	72.5	80.8	1.47	<0.001	<0.001	<0.001
Liquid CP[c], g kg^{-1} DM	79.8	66.3	78.7	67.6	123.5	241.4	175.6	4.05	<0.001	<0.001	<0.001
Liquid ash, g kg^{-1} DM	244	224	210	233	213	178	189	5.7	<0.001	0.303	<0.001
In liquid, g g^{-1} original material											
DM	0.013	0.097	0.096	0.108	0.114	0.181	0.208	0.0032	<0.001	<0.001	<0.001
CP	0.008	0.052	0.061	0.059	0.114	0.356	0.299	0.0051	<0.001	0.646	<0.001
Ash	0.038	0.260	0.241	0.300	0.291	0.342	0.418	0.0065	<0.001	<0.001	<0.001
Lab-scale twin screw press											
Liquid yield, g g^{-1}	0.644	0.663	0.658	0.673	0.620	0.703	0.698	0.0047	<0.001	0.042	<0.001
Liquid DM, g kg^{-1}	97.6	95.9	93.6	94.5	92.7	89.6	86.7	2.61	0.022	0.556	0.126
Liquid CP, g kg^{-1} DM	172.8	105.5	129.8	108.9	151.0	225.1	207.4	6.73	<0.001	<0.001	<0.001
Liquid ash, g kg^{-1} DM	136	146	150	146	131	147	154	4.9	0.055	0.367	0.070
In liquid, g g^{-1} original material											
DM	0.288	0.291	0.282	0.291	0.263	0.302	0.292	0.0087	0.055	0.933	0.141
CP	0.403	0.249	0.297	0.257	0.322	0.554	0.492	0.0043	<0.001	<0.001	<0.001
Ash	0.468	0.507	0.506	0.507	0.412	0.472	0.478	0.0217	0.039	0.389	0.080

[a] FPA = Formic and propionic acid-based additive.
[b] SEM = Standard error of the mean.
[c] CP = Crude protein.

N. Control EN could also be regarded as well fermented due to the ammonium N proportion in total N which was within 50–100 g kg^{-1} DM range indicating good quality (McDonald et al., 1991; Wilkinson, 1990). FPA restricted fermentation and resulted in a clearly higher residual WSC and lower lactic acid concentration than Control as typically observed in response to formic acid application (Franco et al., 2022; Jaakkola et al., 2006). Relative to Control, also acetic acid production was hindered by FPA treatment, and lower total fermentation acid concentration was observed consistent with Seppälä et al. (2016).

During ensiling, some protein hydrolysis occurs which degrades protein into peptides, free amino acids and non-protein N such as ammonia due to microbial activities as well as enzymatic processes due to the presence of plant proteinases and peptidases (McDonald et al., 1991). Formic acid decreases proteolysis, ammonia N as well as free amino acid production (Franco et al., 2022), and the extent of effects depends on the level of formic acid used (Jaakkola et al., 2006). The mode of action of formic acid includes a direct drop in acidity, which reduces the activity of amino and carboxypeptidases with optimum pH of 5–7 (Heron et al., 1989).

3.2. Biomass type and additive effects on biorefinery output

The effects of biomass type and additive treatment on liquid-solid separation efficiency by the two laboratory scale methods, LPP and LTS, are presented in Table 2. Interactions between biomass type and additive treatment were detected in all constituents studied for LPP ($P < 0.001$) while for LTS, interactions were found for liquid yield, liquid CP concentration and CP retained in liquid ($P < 0.001$).

The choice of biomass type is very important regarding the costs and efficiency of logistics of a prospective biorefinery plant. All the biomass types used in the current study can be considered practically feasible, but the choice depends on the particular case. The liquid yields for FR, FZ, DR and EN were 0.236, 0.413, 0.420 and 0.527 for LPP, and 0.654, 0.666, 0.620 and 0.700 for LTS, respectively. All processing methods of grass had a beneficial effect on liquid yield, particularly when the mechanically less efficient LPP was used. The freezing and thawing cycle involves the expansion of large ice crystals causing a disruption in the plant cell wall, which stimulates the release of intracellular molecules, while thawing promotes starch gelatinization and inhibits enzymatic activity and protein denaturation (Jan et al., 2008) contributing to the higher liquid yield of FZ compared to FR. As far as we know, this is the first time when fresh and ensiled grass were compared directly regarding the separation efficiency in a biorefinery concept, and it seems that the ensiling process can be considered an efficient pretreatment to improve liquid yield. This could be due to cell wall hydrolysis and fibre degradation which occurs during the ensiling period.

The ensiling process can be further refined by the use of additives. De La Rosa et al. (1994) and Rinne et al. (2020) studied the application of fibrolytic enzymes on biorefinery efficiency with positive outcomes. The utilization of enzymes such as proteinases (Contreras et al., 2019) and carbohydrases (Sari et al., 2015) could also improve protein extraction in the liquid. In Dotsenko and Lange (2017), 80 % protein recovery from the press cake of white clover and perennial ryegrass was observed when proteases were used. In the current experiment, FPA with a different mode of action, i.e., direct acidification and antimicrobial effect (McDonald et al., 1991), was used. There was a three-fold and seven-fold increase in liquid yield and DM retained in liquid, respectively, with FPA treatment for FR, but only minor benefits in FZ and EN when LPP was used. For LTS, the liquid yield was slightly improved by FPA in FR and FZ, but not in EN.

The decrease in liquid CP concentration for all biomass types in both LPP and LTS in response to FPA treatment was remarkable. This resulted in a decrease in CP recovered in liquid fraction as a proportion of the original biomass for both LPP and LTS for all biomass types treated with FPA. The only exception was FR processed with LPP, where the great increase in liquid yield overcame the reduction in liquid CP concentration. Reduction of liquid CP concentration in response to formic acid application was also observed in the data set of Rinne et al. (2018) although such an effect did not reach significance in the meta-analysis of Franco et al. (2019).

The efficacy of formic acid in reducing the extent of proteolysis during silage fermentation is generally accepted (Contreras-Govea et al., 2013). Therefore, the decrease in liquid CP concentration could be associated with the reduced soluble N concentration in the FPA-treated EN relative to the Control (449 vs 588 g kg^{-1} N in the current material). It is likely that FPA had the same proteolytic-inhibiting effect in FR and FZ to preserve true protein in the biomass (Jaakkola et al., 2006) making less soluble N available in the liquid fraction. Generally, about half of

grass protein is accounted for in the soluble fraction predominated by RuBisCo (ribulose 1,5-bisphosphate carboxylase-oxygenase; Wang et al., 2008) while the insoluble part is enclosed in the cell wall attached to the polysaccharides. These hard cell walls could restrict protein extraction, but during pretreatment such as ensiling, cell walls are partly hydrolysed (Rinne et al., 1997; Jaakkola et al., 2006).

Even with limited or no benefits of FPA on liquid-solid separation and liquid composition in EN, silage fermentation quality improvement is still beneficial in the biorefinery process, because it results in lower fermentation losses and higher hygienic quality of the biomass. This may be particularly relevant for low DM silages suitable for liquid-solid separation, which are otherwise prone to poor fermentation quality (McDonald et al., 1991). Further, the protein in liquid is susceptible to enzymic and microbiological degradation and needs to be further processed or preserved quickly to conserve the nutrients. Using ensiled material, or a preservative (such as FPA in this study) inhibits the spoiling processes and extends the shelf-life of both the liquid and solid fractions.

The protein content of the liquid fraction is critical because the increase in its composition will enhance the supply of nutrients to animals (Stødkilde et al., 2019). There may also be a dual effect of using additives such as organic acids (lactic, acetic and formic acid) in the feedstock, because they may provide prophylactic and growth-promoting effects on pigs (Luise et al., 2020) if liquid fraction is used as a feed component for them. It is also noteworthy that even though proteins are degraded during the ensiling process, only a small proportion in the form of ammonia N is not useful for the metabolism of monogastric animals. The ammonia N was as low as 66 and 32 g kg^{-1} total N in the current Control and FPA-treated silages, respectively.

Majority of the minerals in plant cell are present in the cell solubles so that the extraction rate of all components studied was highest for ash in line with the meta-analysis of Franco et al. (2019). Liquid ash and ash retained in liquid of LPP increased with FPA treatment for FZ and EN but the opposite effect was found for FR. When LTS was used as a separation technique, ash retained in liquid ($P = 0.039$) was lowest in DR and liquid DM concentration ($P = 0.022$) was highest in FR but no effect of additive was observed. Also, for LTS, liquid ash concentration ($P = 0.055$) tended to be lowest in DR and DM retained in liquid (P = 0.055) tended to be

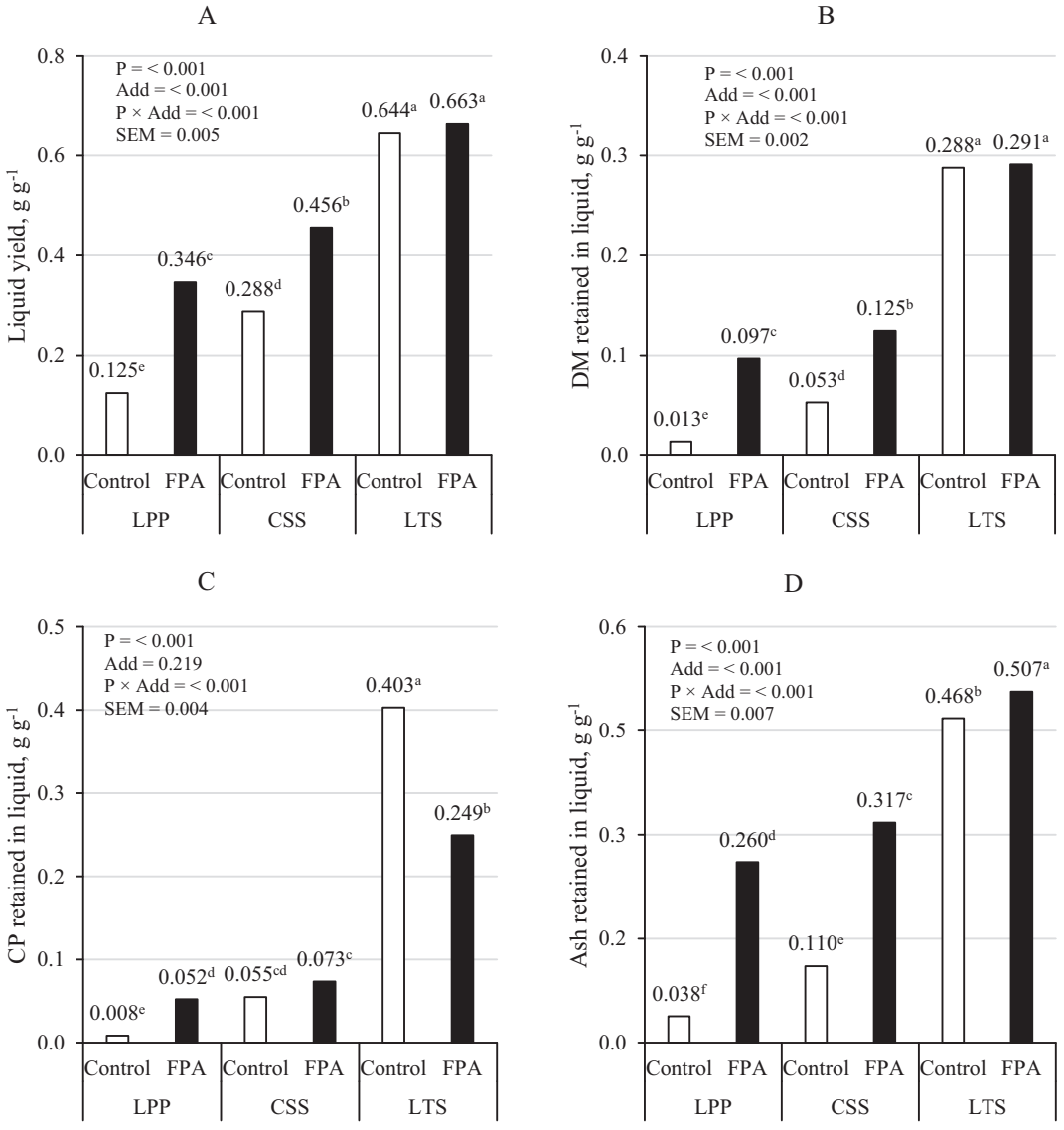

Fig. 1. Effects of separation methods (P) and additive treatment (Add) on A) liquid yield, B) dry matter (DM), C) crude protein (CP) and D) ash retained in liquid of original fresh biomass. Main and interaction effects of P and Add are presented within each graph. LPP: Laboratory scale pneumatic press; CSS: Custom-made pilot scale single screw press; LTS: Laboratory scale twin screw press. $^{a\text{-}f}$values with different lower-case letters within a graph in the bars are significantly different at 5 % probability ($P < 0.05$), SEM = standard error of the mean.

highest in EN but additive did not have any effect.

3.3. Comparison of pressing methods for Control and FPA-treated fresh grass

The choice of the liquid-solid separation technology in a biorefinery process is crucial for liquid yield and constituent recovery (Franco et al., 2019; Rinne et al., 2018). Only FR was processed using all three press types utilized in this study. There was a clear difference in the liquid-solid separation efficiency between the presses (Fig. 1) with the average liquid yields being 0.236, 0.372 and 0.654 for LPP, CSS and LTS, respectively. Interaction effects ($P < 0.001$) between press type and additive treatment were observed for liquid yield and recovery of constituents (DM, CP and ash) in the liquid fraction of FR. Liquid yield and DM retained in liquid increased with FPA treatment more profoundly when processed with LPP than CSS and not in LTS. FPA treatment showed an increasing CP retained in liquid for LPP while the reverse effect was seen in LTS and no effect in CSS. FPA treatment increased ash retained in liquid 7 times more with LPP and 3 times more with CSS while only a slight increase was observed when LTS was used. The meta-analysis of Franco et al. (2019) pointed out that variation in silage quality plays a more important role when a low-efficiency press is used compared to more efficient separation methods, which was confirmed in the current study.

An efficient mechanical liquid-solid separation process is required to break the fibrous cell walls of grass material and solubilize the proteins through the splitting of protein-polysaccharide, protein-pigment and protein-polyphenol complexes. Optimization of the separation technology has resulted in an increased grass protein yield from 30 to 50 % (Kamm et al., 2016; Mandl, 2010) to 40–60 % (Damborg et al., 2020) and it can still be further enhanced by improving the management of raw materials (Franco et al., 2019).

The current pilot scale setting did not allow the possibility of data collection to conduct an economic assessment of the various factors affecting the green biorefinery process. The economic performance of a green biorefinery plant depends on the prices of raw materials, products produced, equipment, labour, and energy etc. of each particular case therefore, general conclusions are difficult to draw and should rather be assessed case by case.

4. Conclusions

The characteristics of the green biomass and type of processing technology play a key role in the extraction efficiency of relevant compounds. The laboratory scale twin screw press proved to be the most efficient among all presses used however the effects of pretreatments were greater with the less efficient laboratory scale pneumatic press. The preservation methods: freezing, drying, and ensiling increased protein solubility and protein extraction from the cell wall compared to the use of fresh grass, but the techno-economic feasibility and environmental impact need to be explored further. The use of a formic and propionic acid-based additive increased liquid yield but decreased protein solubility so that benefits in crude protein yield in the liquid fraction were not always achieved. In the current study, only crude protein yield was considered, but both quality and quantity of the protein extracts as well as other compounds should be studied in more detail.

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CRediT authorship contribution statement

Nisola Ayanfe: Investigation, Data curation, Formal analysis, Writing – original draft. **Marcia Franco:** Methodology, Investigation, Data curation, Formal analysis, Supervision, Writing – review & editing. **Tomasz Stefański:** Methodology, Investigation, Writing – review & editing. **Nora Pap:** Writing – review & editing, Project administration. **Marketta Rinne:** Conceptualization, Methodology, Investigation, Resources, Writing – review & editing, Supervision.

Declaration of competing interest

The authors declare no competing interests.

Data availability

Data will be made available on request.

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