Research article

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Optimisation of alkaline extraction of protein from brewer's spent grain

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Extraction of proteins from industrial residues is a potential source for animal feed. Alkaline extraction combined with isoelectric precipitation may be a useful method for isolating protein from brewer's spent grain (BSG), an abundant by-product from the brewing industry. The objective of this study was to examine the effect of temperature (30, 45 and 60°C), time (30, 60, 120 and 180 minutes), agitation (shaking and stirring), precipitation salinity (0.5 M sodium chloride), isolation mode (freeze drying and precipitation), pre-treatment (defatting and delignification) and repeated extraction on the protein yield and protein content from BSG. Generally, the protein content decreased while protein yield increased with increasing extraction temperatures. Yield and content were maximised after 30 minutes reaching the highest protein content at 30°C (54.8%) and the highest protein yield at 60°C (10.5%). Precipitation with 0.5 M sodium chloride reduced the protein content to 36.4% and the yield to 4.2%. Freeze drying combined with triple extraction yielded a protein content of 20-25%, together with a relatively high protein yield (ca. 45-50%). Delignification increased the protein content from 27% to 32% whereas defatting reduced the overall protein yield from 45% to 38%. Combining isoelectric precipitation and single extraction was associated with a higher protein content (40-55%) but poor protein yields (5-10%). In the light of these findings, further research is required to establish conditions that maximise both the protein content and yield.

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Keywords: Brewer's spent grain; protein; amino acids; alkaline extraction; extraction yield

Introduction

Protein is the costliest ingredient in formulated diets for farmed animals. Currently, fishmeal and plant derived meals are the most widespread protein sources in the production of animal feeds, particularly aquafeed (Tacon et al, 2011). These protein sources have a high nutritional value; however, sourcing them is associated with economic and environmental concerns (Olsen and Hasan, 2012). Therefore, there is a need for more sustainable protein sources in animal feeds.

Brewer's spent grain (BSG) is the most abundant by-product from the brewing industry with an annual production of more than 40 million tonnes of wet BSG (Petit et al, 2020). At present, the main uses of BSG include supplementation of feed for livestock animals, composting and energy production through incineration (Petit et al, 2020). In recent years, increasing attention has been directed at alternative uses of BSG in food and non-food applications as BSG represents a source of different bioactive compounds and is a favourable substrate for microbial cultivation. As a result, several approaches to valorisation have been investigated for BSG including chemical extraction, enzymatic conversion and microbial production of value added compounds and enzymes (Crowe et al, 1985; Forssell et al, 2008; Pejin et al, 2017; Tišma et al, 2018). BSG has a relatively high protein content of dry matter (20-25% DM) and its availability from the brewing industry is stable (Lynch et al, 2016). As such, BSG constitutes a potential protein source for animal and fish feeds. However, BSG contains high levels of lignin and non-starch polysaccharides (NSP), mainly cellulose and hemicellulose, which has prevented the direct use of unprocessed BSG as a protein source in feed applications (Sousa et al, 2018). To facilitate implementation in animal feeds, BSG must undergo refinement where lignin and NSP are reduced, and the protein content is increased. This may be achieved by either chemical fractionation or conversion of BSG. During chemical fractionation, the protein fraction is isolated by extraction whereas the conversion strategy is based on a chemical transformation of lignin and NSP into digestible compounds. Furthermore, the digestibility of lignin and NSP differs between different animal groups. While ruminants are capable of digesting large amounts of cellulose, a high cellulose content will reduce digestion in most fish species. As a result, the levels of lignin and NSP vary depending on their digestibility by the target animal which must be considered when designing a refinement strategy for BSG.

Protein extraction may offer an efficient and convenient method for obtaining protein rich material while eliminating indigestible components. Extraction of protein from BSG has been explored in previous studies to assess different methods including sequential extraction, alkaline and acidic extraction, salt and detergent mediated extraction and ultrasound assisted extraction (Crowe et al, 1985; Diptee et al, 1989; Tang et al, 2010; Qin et al, 2018; Connolly et al, 2019; Wen et al, 2019). In addition, several procedures for precipitation of solubilised protein have been evaluated. For instance, Ervin et al (1989) reported that ethanol precipitation was more effective than isoelectric precipitation when recovering protein from BSG. Earlier research has focused on optimising the extraction of crude proteins from BSG and characterising their

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amino acid profile and physicochemical properties such as water solubility and proteolytic digestibility. For successful protein extraction, two criteria must be satisfied: 1) high recovery of proteins from the original material and 2) high protein content and purity. To be considered a suitable protein source for feed applications, particularly aquafeeds, the protein content should be similar to that of fishmeal (70-85%) and plant based protein meals (50-80%) (Gorissen et al, 2018).

This study was undertaken with the objective of evaluating the impact of duration, temperature, precipitation salinity, protein isolation mode, agitation method and chemical pre-treatment on the recovery of crude protein and the true protein and amino acid content after alkaline extraction from BSG. Furthermore, the effectiveness of isoelectric precipitation in terms of protein content and recovery was assessed. In this study, all extractions were performed with micronised BSG, which reduces the particle size and leads to increased surface area and exposure of protein to solvent molecules. Accordingly, micronisation would be expected to increase the extraction efficiency of protein from BSG.

Materials and methods

Spent grain

Brewer's spent grain was provided by Carlsberg Denmark two different batches - batch 1 (B1) and batch 2 (B2). The BSG was autoclaved at 121°C for 15 min and dried at 60°C until the dry matter content (DM) was above 90%. Subsequently, BSG was mechanically processed at the Danish Technological Institute and was autoclaved to prevent any microbial colonisation.

BSG - mechanical processing

Before protein extraction, BSG from B1 and B2 was processed by micronisation to increase the surface area. This process combines milling and sifting to fractionate the raw material into portions with a range of different particle sizes. The micronisation of BSG was performed using a Rotor FITZ®MILL size-reduction instrument and a SCAN-Vibro VÅV vibrational sifter. During the micronisation process, different size fractions were generated where the most abundant fraction accounted for 50-70% of the micronised material and had a particle size of 125-250μm. This fraction was used for protein extraction with the other fractions discarded.

BSG - defatting

BSG samples were defatted using a modification of the method described by Bligh and Dyer (1959). Methanol and chloroform at a ratio of 1:2 (v/v) was added to BSG at a ratio of 15:1 (w/v). The suspension was stirred for 1 hour at room temperature. The extracted lipids were removed by vacuum filtration and the BSG residue was oven dried at 60°C. This procedure resulted in the removal of 90-95% of the lipids in BSG. Defatted BSG was split into triplicate for protein extraction.

BSG - delignification

Delignification of BSG was performed according to the method of Prado et al. (2013). BSG was mixed with a 60% aqueous ethanol solution at a ratio of 1:4 (w/v) and the suspension heated under reflux at 180°C for 90 min. The delignified BSG fraction was recovered by vacuum filtration and the solid residue was washed three times with 60% ethanol and oven dried at 60°C. The delignified BSG was divided into triplicate and used for protein extraction.

Alkaline protein extraction of BSG

Protein from BSG was solubilised and recovered as protein rich isolates using alkaline extraction followed by isoelectric precipitation (Lam et al. 2018). Sodium hydroxide (pH 12.4, 0.01 M) was used for extraction, and the solubilised proteins were precipitated at pH 4.5. To separate the liquid and solid fraction, the extract was centrifuged for 15 min at 3871 x *g* at 25°C (3-18 KS, Sigma, Germany).

The experimental work was divided into two parts to optimise the extraction conditions and to determine the influence of several factors on protein yield and content. The first part investigated the following parameters on the protein content and yield: temperature and time, agitation mode during extraction, precipitation salinity and isolation mode of extracted proteins (Figure 1A). The second part considered how the protein content and yield were affected by repeated extraction and different pre-treatments including delignification, defatting and a combination of these (Figure 1B). All protein extractions were performed in triplicate.

Optimisation - temperature and time

A two factorial experiment was designed with three temperatures (30, 45 and 60°C) and four extraction times (30, 60, 120 and 180 min). For each temperature, the effect of the different extraction times was evaluated.

Optimisation - salinity and precipitation

The influence of salinity on isoelectric precipitation was evaluated by comparing protein isolates obtained from precipitation of proteins from a sodium chloride (0.5 M) enriched supernatant against a control (water). Prior to precipitation, the salinity of the supernatant was adjusted by addition of NaCl.

Protein isolation

Two methods for obtaining protein isolates from the liquid extracts were evaluated: freeze drying and isoelectric precipitation. Besides quantifying the protein content and yield, the recovery of non-protein nitrogen (NPN) and amino acids was determined for both approaches.

Agitation

Protein extractions were carried out using orbital shaking or magnetic stirring. Orbital shaking was in a SW22 shaking water bath (Julabo, Germany) at a speed of 150 rpm. Magnetic stirring was performed on an AREX Digital PRO Hot Plate Stirrer (VELP Scientifica, Italy) at a speed of 1000 rpm.

Repeated extraction and pre-treatment

Before protein extraction, BSG was subject to three chemical pre-treatments including delignification, defatting and a combination of the two. Additionally, extraction of the pretreated material

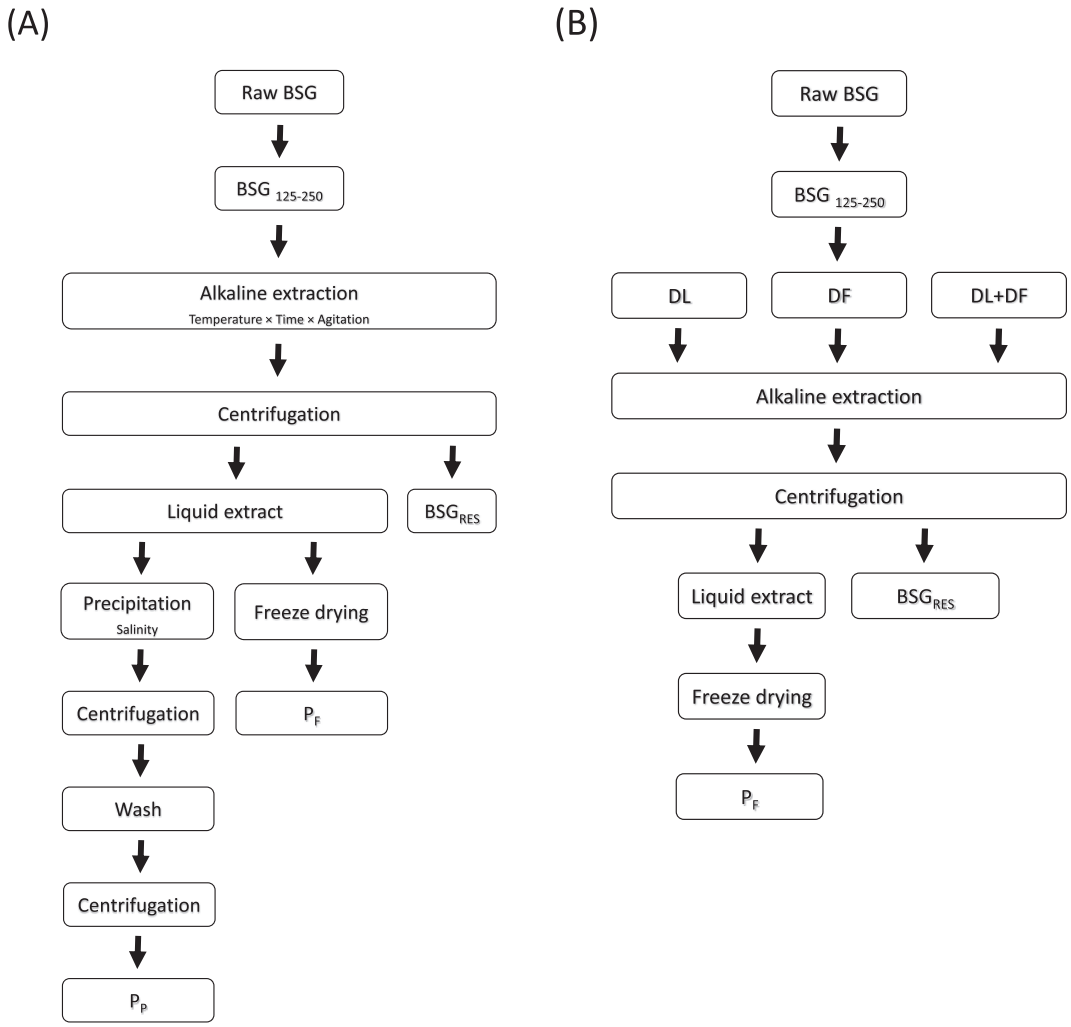

Figure 1. Experimental design of part 1 (panel A) and part 2 (panel B). BSG_{RES}: solid residue of extracted brewer's spent grain (BSG) obtained after alkaline extraction, centrifugation and drying. P_P: protein isolate obtained by isoelectric precipitation. P_F: protein isolate obtained by freeze-drying of the liquid extract. DL: delignification of BSG. DF: defatting of BSG. $BSG_{125\text{-}250}$: micronised BSG fraction used in protein extraction where the numbers in lowercase refer to the particle size of BSG.

and control (untreated BSG) was performed three times to assess how repeated extraction affects the protein yield and content.

Protein content

The concentration of protein produced by extraction of BSG is expressed as a percentage of the dry matter. Crude protein content (C_P) was determined from the nitrogen content measured with the Kjeldahl method while total amino acid protein (AA_P) - also referred to as the true protein content - was based on the sum of the amino acids. The protein content was calculated by multiplying the Kjeldahl nitrogen by a conversion factor of 6.25.

Amino acid content

Amino acids were quantified using the standard procedure described by Otter (2012). Amino acids were liberated from protein by acid hydrolysis with 1.8 mL 6M HCl with 0.2% phenol, for 24 hours at 110°C. Following hydrolysis, 100 µL 30 mM norvalin (internal standard) was added and HCl was removed by evaporation in a RVC 2-18 vacuum centrifuge from CHRIST (Germany) at 210 x *g*. The concentrated sample were resuspended in 3 mL 0.01 M HCl solution and 0.7 mL centrifuged for 10 min at 3000 x *g*. The supernatant was diluted 10-fold and the amino acids were derivatised (Cohen, 1994) with 6-aminoquinolyl-N-hydroxy-succinimidyl carbamate (AQC) to yield short term stable, UV-active amino acid-AQC adducts. Amino acid derivatisation was performed using the AccQ•Tag Ultra Derivatization Kit (Waters, Milford, Massachusetts). The amino acid derivatives were separated and quantified using reverse-phase liquid chromatography with UV detection at 260 nm (Waters ACQUITY UltraPerformance liquid LC (UPLC)). Separation was performed on an ACCQ-TAG ULTRA C18, 2.1x100 mm, 1.7 µm column using water and acetonitrile as eluent systems. A gradient of 0-60% acetonitrile over 10 min and a flow rate of 0.7 mL/s was applied for separation of amino acids.

Protein yield

Protein yield (Y_P) measures the proportion of protein recovered from the original material after extraction. In this study, two Y_P types have been integrated for assessment of protein extraction under different conditions: a Kjeldahl based yield ($Y_{P,\ Kjeldahl}$) and an amino acid-based yield ($Y_{P,\ AA}$). The $Y_{P,\ Kjeldahl}$, which includes

protein derived nitrogen and non-protein nitrogen (NPN) is calculated by formula (1):

$$Y_{P,\ Kjeldahl} = \frac{m_{Kjeldahl\ protein,\ isolate\ (g)}}{m_{Kjeldahl\ protein,\ BSG\ (g)}} \cdot 100\%$$

$m_{Kjeldahlprotein}$ is the mass of Kjeldahl protein in isolates or the micronised BSG sample before extraction.

The $Y_{P,\ AA}$ is the sum of amino acids present in the isolate and excludes the NPN component. In this work, the two different amino acid-based yields are designated $Y_{P,\ AA1}$ and $Y_{P,\ AA2}$. The $Y_{P,\ AA1}$ estimates the amount of extracted protein nitrogen relative to the total amount of extractable nitrogen in micronised BSG according to formula (2):

$$Y_{P,\ AA1} = \frac{m_{AA\ protein,\ isolate\ (g)}}{m_{Kjeldahl\ protein,\ BSG\ (g)}} \cdot 100\%$$

The $Y_{P,\ AA2}$ is used to quantify the proportion of extractable protein nitrogen recovered from micronised BSG during extraction and is calculated from formula (3):

$$Y_{P,\ AA2} = \frac{m_{AA\ protein,\ isolate\ (g)}}{m_{AA\ protein,\ BSG\ (g)}} \cdot 100\%$$

Amino acid yield

The yield of individual amino acids (Y_{AA}) was calculated using the formula (4) as the amount of extracted amino acid in the isolate relative to the amount that can be extracted from micronised BSG:

$$Y_{AA} = \frac{m_{AA,\ isolate\ (g)}}{m_{AA,\ BSG\ (g)}} \cdot 100\%$$

Statistics

All statistical analyses were performed using the open source software R version 3.5.1 (R Core Team, 2018) while graphical illustrations were constructed using the platform SigmaPlot 14.0. A significance level of 0.05 was applied to all statistical tests in this study. To evaluate the impact of time, temperature and their interactions, the data was subjected to a two-way analysis of variance while the different treatment combinations were compared by a pairwise t-test. The influence of salinity on isoelectric precipitation was evaluated using a t-test. Statistical evaluation of the impact of isolation method on protein content and yield was performed using a t-test whereas the effect on amino acid yields was evaluated by a two-way analysis of variance with amino acid type and isolation mode as the explanatory variables. The effect of pre-treatment was evaluated by a one-way analysis of variance. In addition, the amino acid recovery was examined for protein isolates produced by extraction of defatted BSG and a control to determine the effect of defatting on amino acid extractability. A two-way analysis of variance was performed using amino acid type and pre-treatment (control and defatting) as independent variables.

Results and discussion

Chemical composition of BSG

The amino acid composition was determined for non-micronised and micronised BSG (125-250 μm) to validate the compositional similarities between these BSG fractions. This analysis revealed that micronised and non-micronised BSG from the same batch (B1) were similar in protein and lipid content (Table 1). Although micronisation was employed to increase the extraction efficiency of proteins from BSG, separation of the different size fractions led to losses of micronised material (30-50%). Although this could be overcome by extracting micronised BSG containing all size fractions from the micronisation process, this was not progressed any further. A comparison of the three size fractions suggested that the protein content decreased with increasing particle size. For the smallest fraction (<125 μm), a protein content of about 40% was measured whereas the largest fraction (>250 μm) had a protein content of about 15%. Because of the high dust of the small size fraction and low protein content of the large size fraction, these fractions were less suitable for protein extraction. Accordingly, the medium size fraction (125-250 μm) was use for extractions in this study.

The amino acid composition of BSG reflects its nutritional quality as a protein source for feed applications, and it may prove useful in predicting the amino acid content of BSG derived protein isolates. As shown in Table 1, the micronised BSG contains high levels of glutamic acid (5.31 ± 0.33 g/100 g DM), proline (2.46 ± 0.13 g/100 g DM) and aspartic acid (1.75 ± 0.11 g/100 g DM) while it is deficient in methionine (0.31 ± 0.04 g/100 g DM). Consequently, alkaline extraction of micronised BSG may generate protein isolates rich in glutamate, proline and aspartate but may not be an ideal source of methionine. Moreover, the amino acid composition reported here is consistent with the previous studies presented in Table 1 (Gmoser et al, 2020; Gutiérrez-Barrutia et al, 2022; Oluseyi et al, 2011; Waters et al, 2012; Yu et al, 2020). Due to its thermal instability, tryptophan is susceptible to breakdown when exposed to high temperatures (Cuq et al, 1983) and therefore lost in the hydrolysis conditions used for amino acid analysis. As a result, the true protein content (AA_P) will be underestimated while the NPN content will be overestimated. Waters et al (2012) found that BSG had a relatively low content of tryptophan and therefore the underestimation of AA_P may be of minor concern. Proteins and amino acids may also degrade with autoclaving of the raw material owing to the high temperature. To determine whether these components deteriorate during autoclaving, the composition of autoclaved BSG were compared with that without autoclaving. The results showed that autoclaved and non-autoclaved BSG had a similar content of protein and amino acids indicating that autoclaving did not result in any compositional changes in the BSG (data not shown).

Optimisation of protein extraction

Temperature and time. A nested design was employed to explore the single and combined effects of time and temperature on Kjeldahl based protein yield ($Y_{P,\ Kjeldahl}$) and content (C_P). The two-way analysis of variance indicated that time and temperature significantly affected both protein yield and content ($p<0.001$) while the combined effect of these variables appeared to be insignificant ($p_{yield} = 0.256$, $p_{content} = 0.729$). Protein content decreased with increasing temperature for all extraction times (Figure 2A).

Table 1. Chemical composition of micronised and non-micronised brewer's spent grain (BSG) and comparison of amino acid composition from this study with that from previous publications. All measurements were in duplicate, and results reported as mean ± SD.

	Micronised BSG*	Non-micronised BSG*	Previous studies‡
Composition			
Dry matter (DM) (%)	93.5 ± 0.00	95.9 ± 0.00	-
Ash (%)	3.67 ± 0.03	3.66 ± 0.00	-
Lipids (%)	10.5 ± 0.08	9.90 ± 0.06	-
C_P (%)[a]	25.2 ± 0.11	24.1 ± 0.31	-
AA_P (%)[b]	22.2 ± 1.03	20.9 ± 0.61	-
NPN (%)[c]	3.03 ± 0.92	3.10 ± 0.91	-
Essential amino acids (g/100 g DM)			
Phenylalanine (Phe)	1.24 ± 0.07	1.23 ± 0.04	1.03 - 1.59
Histidine (His)	0.48 ± 0.03	0.45 ± 0.01	0.50 - 5.81
Valine (Val)	1.22 ± 0.06	1.13 ± 0.03	1.02 - 1.45
Isoleucine (Ile)	0.96 ± 0.07	0.90 ± 0.03	0.73 - 1.11
Leucine (Leu)	1.76 ± 0.08	1.64 ± 0.04	1.35 - 3.19
Methionine (Met)	0.31 ± 0.04	0.26 ± 0.03	0.40 - 0.59
Threonine (Thr)	0.87 ± 0.04	0.79 ± 0.01	0.16 - 1.11
Lysine (Lys)	0.93 ± 0.04	0.75 ± 0.00	0.67 - 3.17
Non-essential amino acids (g/100 g DM)			
Arginine (Arg)	1.12 ± 0.01	1.01 ± 0.01	0.50 - 1.22
Alanine (Ala)	1.10 ± 0.49	0.97 ± 0.01	0.80 - 2.05
Aspartate (Asp)	1.75 ± 0.11	1.54 ± 0.01	1.06 - 1.95
Glutamate (Glu)	5.31 ± 0.33	5.28 ± 0.25	3.67 - 5.36
Glycine (Gly)	0.89 ± 0.04	0.80 ± 0.00	0.39 - 1.08
Serine (Ser)	1.04 ± 0.06	0.98 ± 0.03	0.83 - 1.46
Tyrosine (Tyr)	0.70 ± 0.00	0.67 ± 0.03	0.57 - 0.94
Cysteine (Cys)	0.00 ± 0.00	0.00 ± 0.00	0.00 - 0.49
Proline (Pro)	2.46 ± 0.13	2.48 ± 0.11	1.40 - 2.99
Cystine (Csn)	0.10 ± 0.00	0.10 ± 0.01	-

* Values are based on micronised or non-micronised brewer's spent grain (BSG) from batch 1. The micronised BSG had a particle size of 125-250 μm.

‡ Amino acid composition of BSG from previous studies, presented as a range of the values reported by Gmoser et al (2020), Gutiérrez-Barrutia et al (2022), Oluseyi et al (2011), Waters et al (2012) and Yu et al (2020).

[a] Kjeldahl protein calculated by multiplying the Kjeldahl nitrogen by 6.25.

[b] True protein content calculated as the sum of all measured amino acids and excludes any non-protein nitrogen (NPN) found in the Kjeldahl protein.

[c] NPN is the non-protein nitrogen calculated as the difference between Kjeldahl protein and true protein.

Conversely, a positive correlation was observed between protein yield and temperature at 30 minutes, although not statistically significant, while no general trend was found at 60, 120 or 180 minutes (Figure 2B). The inferred relationship between temperature and protein yield could suggest that protein solubilisation increased as the temperature was raised. Within all temperature groups, a declining tendency was observed in protein content and yield when extraction times were prolonged from 30 to 120 and 180 minutes. For protein yield, this trend may reflect a higher degree of complete protein hydrolysis resulting in the loss of protein derived amino acids during isoelectric precipitation. In contrast, the tendency of reduced protein content may stem from the enhanced solubilisation of lignin at higher temperatures and longer extraction times as lignin is partly soluble in dilute alkaline solutions (Melro et al, 2020). This was supported by the visual appearance of the protein isolates as increased extraction time and temperature yielded darker material. These impurities may originate from co-isolated lignin and/or lignin-hemicellulose adducts.

The impact of time and temperature on protein yield are in agreement with Qiaoyun et al (2017) and Surasani et al (2017), where protein yield was positively correlated with time and temperature within a specific range, above which the yield would decrease or remain constant. Further, much of the variability was attributable to factors that were not accounted for in the statistical model. One possible explanation is that protein isolates are produced in a multiple step process which may increase the degree of uncertainty. Moreover, the high residual variation can also be explained by minor fluctuations in pH during isoelectric precipitation which may influence the amount of precipitated protein. This is supported by Chavan et al (2001) who reported that small pH deviations from the isoelectric point affect the protein solubility and the efficiency of isolation. With respect to the protein yield, a combination of 60°C and 30 minutes was the most efficient while the

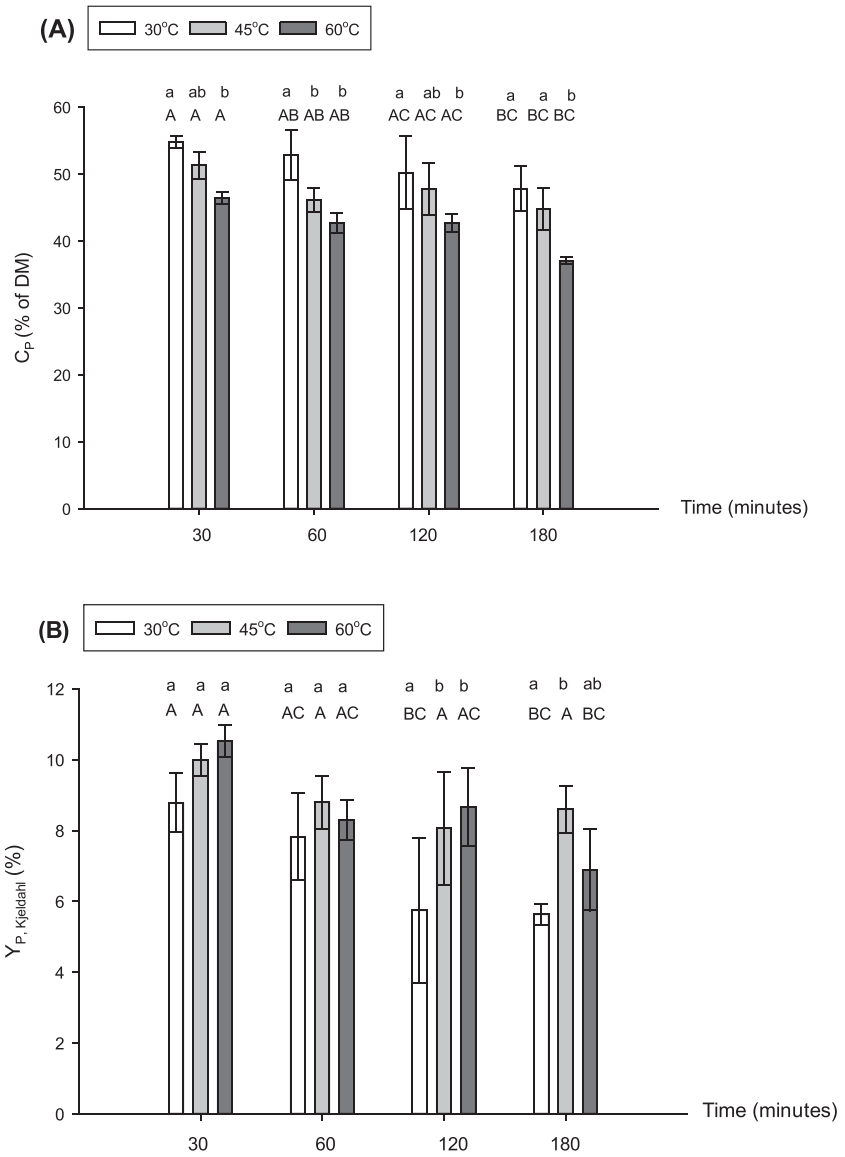

Figure 2. Effect of temperature and time on Kjeldahl-based protein content of isolates (C_P) (A) and Kjeldahl-based protein yield ($Y_{P, Kjeldahl}$) (B) of brewer's spent grain (BSG). Within each time group, three different temperatures were evaluated. Uppercase and lowercase letters are used to designate significant differences between and within different time groups, respectively. C_P = Kjeldahl N (%)·6.25 and $Y_{P, Kjeldahl} = m_{Kjeldahl\ protein,\ isolate\ (g)}/m_{Kjeldahl\ protein,\ BSG\ (g)} \cdot 100\%$. All treatment combinations were tested in triplicate and the results reported as mean±SD.

protein content in the extract was maximised using a temperature of 30°C and an extraction time of 30 minutes. Interestingly, all combinations of temperature and time led to low protein yields (6-10%), although the protein content was relatively high in all isolates (37-55%). There are several explanations for this including: 1) loss of water soluble proteins during washing of the precipitate, 2) complete hydrolysis of proteins to amino acids resulting in a loss of protein derived amino acids during isoelectric precipitation or 3) poor protein extractability caused by interactions with other macromolecules in the BSG matrix. To optimise the protein recovery and identify possible causes of the low protein yield, five optimisation experiments were carried out at a common temperature of 60°C and an extraction time of 30 minutes.

Agitation and salinity. To examine the effect of agitation on protein yield and content, extractions were performed with stirring or orbital shaking. Initially, it was thought that stirring would be more effective than orbital shaking, as stirring may ensure constant exposure of proteins to the extraction solvent. However, the results show that both protein yield and content were unaffected by the type of agitation (p_{yield}=0.629 *and* $p_{content}$=0.847) (Supporting Information, Figure S1). This is in agreement with the findings from Crowe et al (1985) who reported that equal amounts of nitrogen were solubilised from BSG when shaking and stirring were employed. Also, Surasani et al (2017) reported that the velocity and time of stirring did not adversely affect the protein yield and content; although protein solubilisation was maximised with continuous stirring. Based on the principles of the salting-out technique, which takes advantage of the reduced solubility of proteins at higher salt concentrations, it was thought that protein precipitation could be enhanced by increasing the salinity of the liquid extract. This was tested by precipitating out proteins from the liquid extract by addition of 0.5 M sodium chloride. Contrary to the initial hypothesis, this approach showed a marked

reduction in protein yield and content when isoelectric precipitation was performed with a saline extract as compared to the control (Figure 3). It is possible that protein solubility is influenced by an interplay of external factors including medium pH and salinity, protein structure and the amino acid composition of the proteins. This was demonstrated by Sousa et al (2007) who reported that the protein solubility of egg derived proteins was largely dependent on salinity, pH and the type of salt. Similarly, Chavan et al (2001) reported that the solubility of proteins extracted from peas increased with increasing NaCl concentrations near the isoelectric point. This may explain the reduced protein yield observed when proteins were precipitated from a saline extract.

Protein isolation. The low protein yield observed could also be due to a loss of water soluble proteins during the washing step or to the generation of free amino acids by protein hydrolysis. Owing to differences in the isoelectric points, amino acids and proteins may have different solubilities at the pH value (4.5-5) used for precipitation. As a result, the released amino acids may be retained in the liquid extract during precipitation. To establish whether the poor recovery was due to protein loss or protein hydrolysis, proteins were concentrated from the liquid extract by freeze drying (Figure 1B). The protein isolates generated by precipitation and freeze drying were compared with respect to their protein content, protein yields and recovery of NPN (Figure 4). The crude protein content reported in Figure 4A, showed that freeze drying led to a significantly lower protein content compared to precipitation

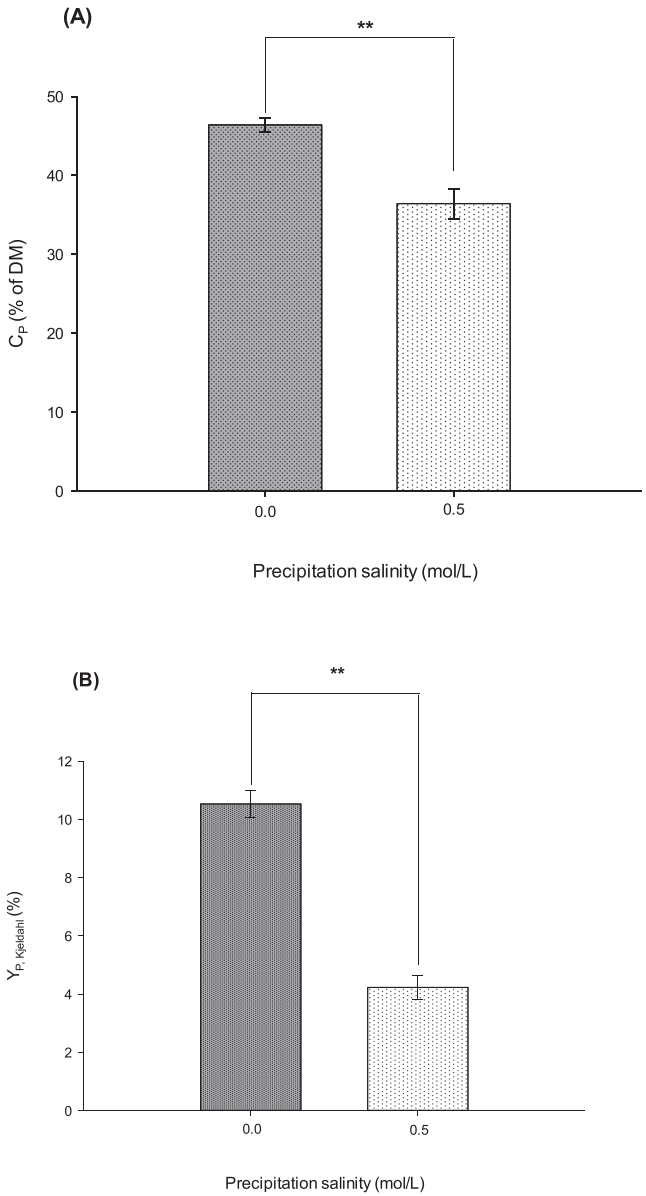

Figure 3. Effect of precipitation salinity on Kjeldahl-based protein content of isolates (C_P) (A) and yield ($Y_{P, Kjeldahl}$) (B). Double asterisks (**) indicate that groups are significantly different. Results are presented as mean±SD.

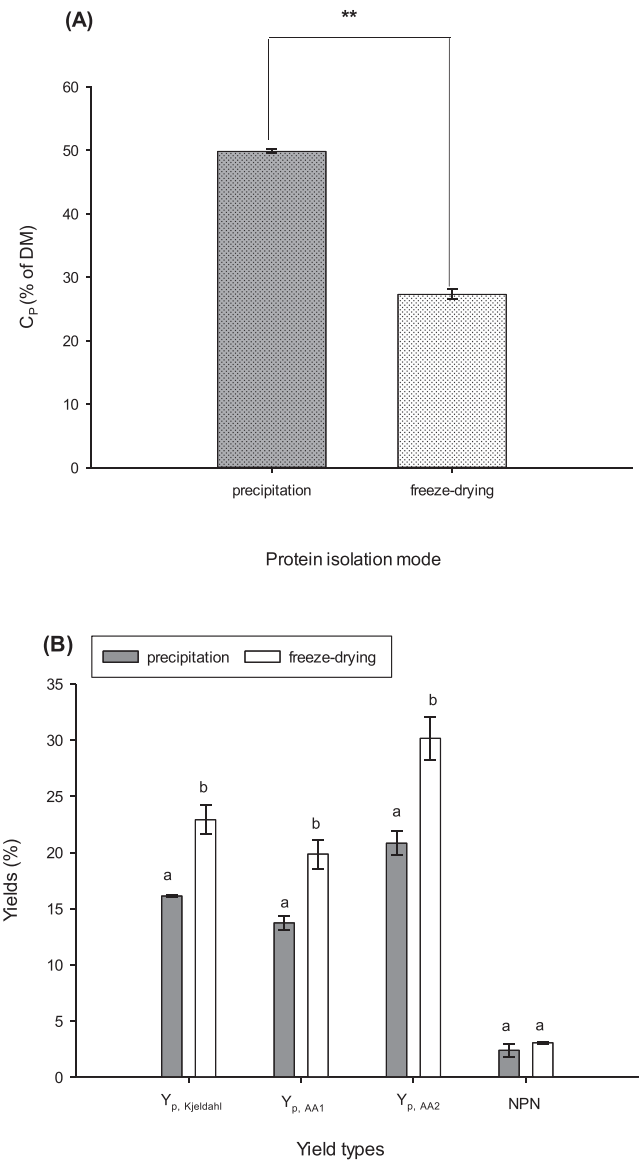

Figure 4. Effect of protein isolation mode on protein content of isolates and extraction yield from brewer's spent grain (BSG). A: Kjeldahl-based protein content (C_P) of isolates obtained by isoelectric precipitation and freeze-drying. B: Three different protein yields and recovery of non-protein nitrogen (NPN) for isolates generated by isoelectric precipitation and freeze-drying. Protein yields and NPN were calculated by the following formulas: $Y_{P, Kjeldahl} = m_{Kjeldahl\ protein,\ isolate\ (g)}/m_{Kjeldahl\ protein,\ BSG\ (g)} \cdot 100\%$, $Y_{P, AA1} = m_{AA\ protein,\ isolate\ (g)}/m_{Kjeldahl\ protein,\ BSG\ (g)} \cdot 100\%$, $Y_{P, AA2} = m_{AA\ protein,\ isolate\ (g)}/m_{AA\ protein,\ BSG\ (g)} \cdot 100\%$, $NPN = Y_{P, Kjeldahl} - Y_{P, AA1}$. Lowercase letters indicate significant differences in protein and NPN recovery obtained by the two different methods. Data is from triplicates and presented as mean± SD.

($p<0.001$). A likely cause of this is the occurrence of co-extracted impurities in the liquid extract which remain in the isolate after freeze drying.

To evaluate the effect of isolation method on protein and NPN recovery, two different yield types were determined (Figure 4B: 1) with the crude protein yield ($Y_{P,\ Kjeldahl}$) and 2) the true protein yield ($Y_{P,\ AA}$). Further, the true protein yield was measured as the extracted amount of protein relative to the total nitrogen ($Y_{P,\ AA1}$) and the total protein ($Y_{P,\ AA2}$) in BSG. The results presented in Figure 4B indicated that the crude protein yield was significantly higher for isolates produced by freeze drying compared with precipitation ($p_{Y_{P,\ Kjeldahl}} = 0.0113$).

To establish whether this observation was due to the enhanced extraction of proteins, NPN or both, the true protein yield ($Y_{P,\ AA1}$ and $Y_{P,\ AA2}$) and NPN recovery were determined and compared for isolates obtained from both isolation methods. With the crude protein yield, the true protein yield increased significantly when freeze drying was used instead of precipitation ($p_{Y_{P,\ AA1}} = 0.00548$ and $p_{Y_{P,\ AA2}} = 0.00453$). Another interesting finding was that the NPN recovery did not differ significantly between the two isolation modes ($p_{NPN} = 0.193$) which implies that the isolation method only influences the recovery of protein derived nitrogen.

The amino acid yield (Y_{AA}) was used for assessing the degree of protein hydrolysis under the extraction conditions (Figure 5). Here, a substantial increase in Y_{AA} was detected for amino acids when freeze drying was used for protein isolation. This suggests that a greater proportion of proteins are hydrolysed to amino acids when extraction was at 60°C for 30 minutes. Increasing the temperature above 60°C is thought to enhance the hydrolysis of proteins, leading to a further reduction in protein recovery. Hence, increased protein hydrolysis may be an underlying cause of the decreasing protein yield observed at prolonged extraction times (Figure 2B). In the light of this, future research should focus on the effect of time and temperature on protein depolymerisation during extraction. Although freeze drying is associated with higher protein and amino acid recoveries, this method is not suitable for producing protein isolates for feed applications due to the low protein content of the resulting isolates. However, for the purpose of optimising protein recovery, this approach was used in subsequent experiments in this study.

Repeat extraction and pre-treatment. Extraction of proteins from a heterogeneous biomass such as BSG may be restricted by intermolecular interactions between proteins and lipids, carbohydrates and lignin. These associations can lead to the formation of clusters which may impair protein extraction as the proteins are less accessible to solvent molecules. Previous studies have focused on describing the effect of carbohydrate-protein interactions on the extractability of BSG proteins. For instance, Crowe et al (1985) found that cellulase aided degradation of cellulose led to a seven-fold increase in the protein yield from alkaline extraction suggesting that proteins form clusters with the structural carbohydrates of BSG.

In the current study, possible interactions of protein with lipid and lignin were examined to determine whether the low protein recovery resulted from macromolecular interactions. Accordingly, BSG was subjected to three pre-treatments before protein extraction: 1) defatting, 2) delignification and 3) defatting and delignification. In addition, the effect of repeat extraction was evaluated for pretreated and untreated BSG by exposing the same biomass to three consecutive extractions. When repeated extraction was applied, the total protein yields were increased by about

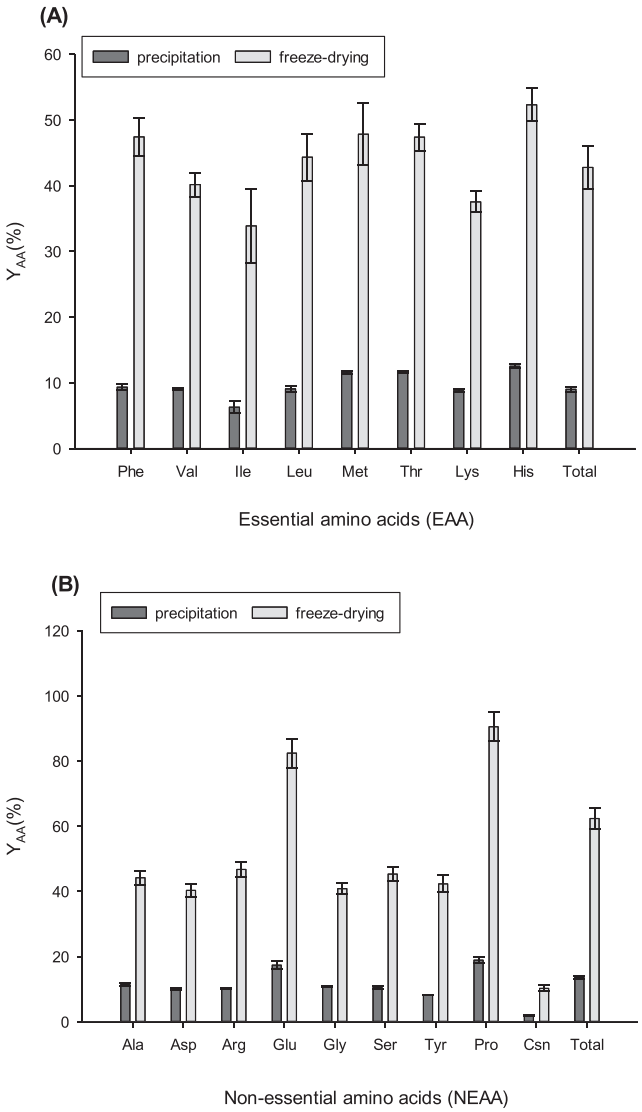

Figure 5. Yields of essential (A) and non-essential (B) amino acids recovered by isoelectric precipitation and freeze-drying of liquid extracts from brewer's spent grain (BSG). The yield of all amino acids differed significantly between the two isolation methods ($p<0.05$) (indicators of significance were omitted for clarity). The total amino acid recovery, which reflects the recovery of the true protein, is calculated by the formula: $Y_{AA,\ total} = \Sigma m_{AA,\ isolate\ (g)}/\Sigma m_{AA,\ BSG\ (g)} \cdot 100\%$. Extractions were carried out in triplicate and the results are presented as mean±SD.

50% compared to a single extraction (Figure 6B). Moreover, similar amounts of biomass were extracted in all three treatments although the yield of crude protein, true protein and NPN decreased with each extraction (Figure S2). It is likely that greater amounts of lignin and carbohydrate are co-extracted by the second and third extraction as most of the protein was recovered during the first extraction. This work was qualitative, and more studies are needed to determine the magnitude of co-extraction.

The one-way analysis of variance indicated that pre-treatment had significant impact on the crude protein yield and content ($p<0.05$). Furthermore, a pairwise analysis revealed that the three pre-treatments had different effects on protein yield and content (Figure 6). The effect of delignification on the total protein yield was insignificant while the protein content was significantly higher in the extract from delignified BSG compared to the control. That protein yields are unaffected by delignification, suggests that

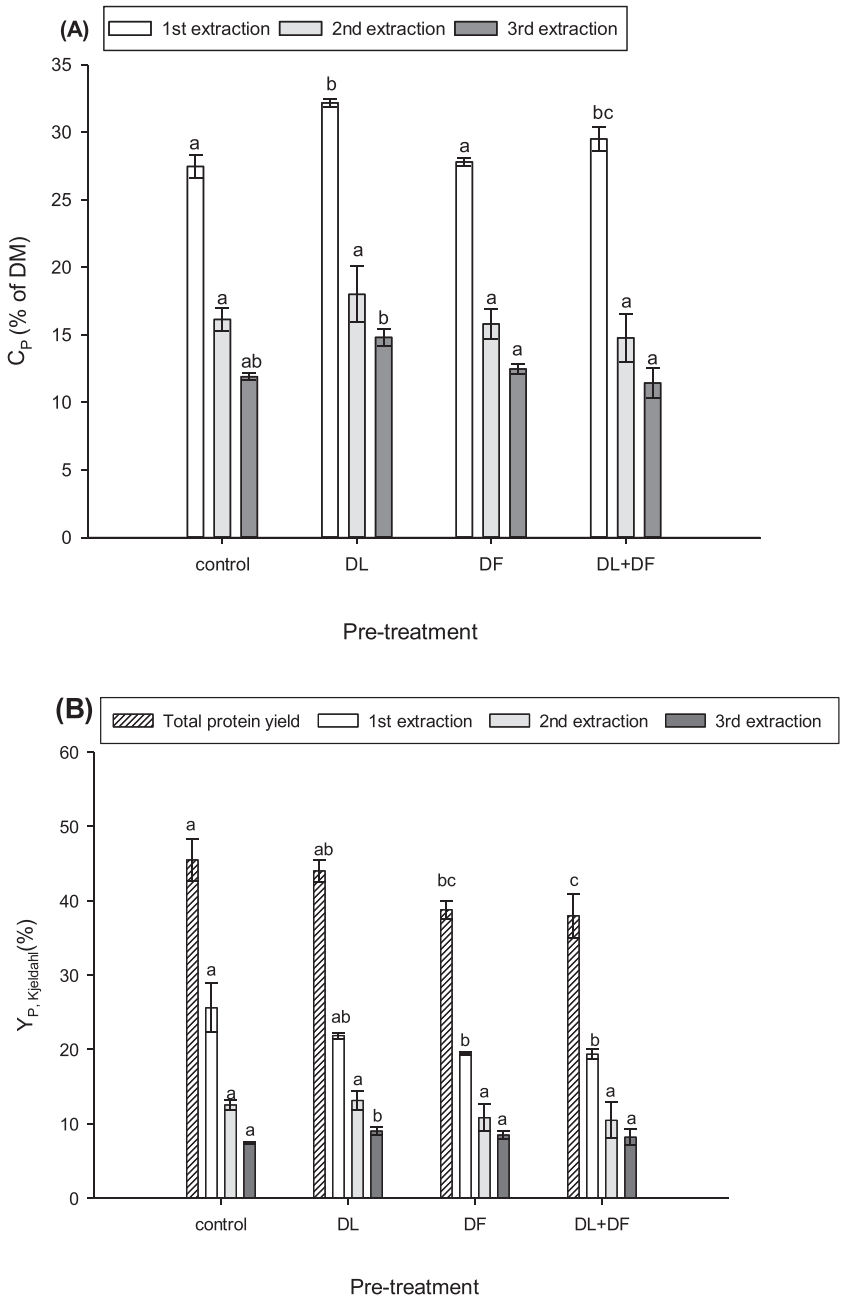

Figure 6. Effect of pre-treatment and repeated extraction on Kjeldahl-based protein content of isolates (C_P) (A) and Kjeldahl-based protein yield ($Y_{P,\ Kjeldahl}$) (B). For each pre-treatment group, extractions were performed three times and compared to a control experiment in which untreated brewer's spent grain (BSG) was extracted three times. Lowercase letters are used to denote significant differences between the pre-treatment groups within the same extraction. Abbreviations: DL = delignification, DF = defatting, DL+DF = defatting and delignification. Results are presented as mean±SD.

lignin and proteins do not interact through intermolecular forces. The lack of protein-lignin interactions is probably a result of protein denaturation during alkaline extraction; a theory supported by Salas et al. (2013) who demonstrated that interactions between lignin and proteins are weakened when proteins are denatured. In contrast, the higher protein content may result from reduced co-extraction of lignin and lignin-hemicellulose adducts, as most of these compounds have been removed during the delignification process. The protein content appeared to be unaffected by defatting suggesting that BSG-bound lipids are not solubilised during alkaline extraction. In addition, a significant reduction was observed in the total protein yield when proteins were extracted from defatted BSG. An explanation for this is that water soluble proteins in BSG are dissolved by the extraction solvent due to their moderate solubility in methanol. Alternatively, the chloroform-methanol solvent for defatting may induce structural changes in the protein which may reduce protein extraction in alkaline solution.

To establish whether the reduced protein yield was due to impaired protein extraction or protein loss during defatting, the amino acid yields (Y_{AA}) were quantified for each of the three extractions (Table 2). Data from the first and second extraction showed that the total amino acid yield, as well as those of individual amino acids, were reduced by defatting. Following the third

Table 2. Comparison of amino acid yields (Y_{AA}) by extraction of defatted and untreated (control) brewer's spent grain (BSG). All extractions were carried out in triplicate and yields are reported as mean ± SD*.

Amino acid **	First extraction		Second extraction		Third extraction	
	$Y_{AA(control)}$ (%)	$Y_{AA(defatted)}$ (%)	$Y_{AA(control)}$ (%)	$Y_{AA(defatted)}$ (%)	$Y_{AA(control)}$ (%)	$Y_{AA(defatted)}$ (%)
Phe	21.9 ± 3.0^a	15.8 ± 2.6^a	9.5 ± 0.5^a	9.0 ± 1.2 ^{a}	5.9 ± 0.2^a	7.0 ± 0.3^b
Val	26.9 ± 4.7^a	20.3 ± 1.0^a	14.1 ± 0.9^a	11.7 ± 2.6^a	9.3 ± 0.2^a	11.0 ± 0.5^b
Ile	20.5 ± 1.0^a	17.5 ± 0.5^b	7.6 ± 0.5^a	8.5 ± 0.9^a	4.8 ± 0.2^a	5.8 ± 0.2^b
Leu	25.6 ± 4.5^a	17.7 ± 2.6^a	12.3 ± 0.7^a	10.9 ± 2.1^a	8.1 ± 0.2^a	9.5 ± 0.5^b
Met	33.5 ± 3.1^a	36.8 ± 10.7^a	13.7 ± 0.7^a	11.5 ± 2.4^a	8.6 ± 0.5^a	10.4 ± 1.2^a
Thr	31.6 ± 7.3^a	20.0 ± 4.5^a	15.3 ± 1.4^a	13.0 ± 2.4^a	9.5 ± 0.2^a	10.8 ± 0.5^b
Lys	26.2 ± 4.2^a	16.8 ± 2.3^b	11.7 ± 1.0^a	9.7 ± 1.9^a	7.1 ± 0.2^a	8.1 ± 0.3^b
Arg	27.6 ± 6.4^a	16.7 ± 3.8^a	12.4 ± 0.9^a	10.4 ± 1.6^a	8.4 ± 0.5^a	9.0 ± 0.7^a
His	33.6 ± 7.8^a	22.0 ± 5.4^a	17.5 ± 2.6^a	15.0 ± 3.1^a	10.9 ± 0.3^a	12.8 ± 0.5^b
Ala	32.9 ± 7.6^a	20.4 ± 4.8^a	17.2 ± 1.4^a	14.1 ± 1.7^a	10.8 ± 0.2^a	12.6 ± 0.5^b
Asp	29.8 ± 7.1^a	18.2 ± 4.1^a	14.9 ± 1.0^a	12.3 ± 2.6^a	9.4 ± 0.2^a	10.8 ± 0.5^b
Glu	44.4 ± 10.4^a	27.7 ± 5.7^a	21.1 ± 0.9^a	17.2 ± 2.8^a	11.7 ± 0.5^a	12.8 ± 0.7^a
Gly	29.6 ± 6.8^a	19.8 ± 5.0^a	15.5 ± 1.4^a	13.6 ± 2.8^a	10.0 ± 0.3^a	12.0 ± 0.5^b
Ser	29.0 ± 6.8^a	18.1 ± 3.6^a	14.8 ± 1.2^a	12.6 ± 2.6^a	9.7 ± 0.2^a	11.0 ± 0.5^b
Tyr	21.8 ± 2.6^a	16.5 ± 0.7^b	9.5 ± 0.7^a	8.3 ± 1.6^a	6.2 ± 0.2^a	7.1 ± 0.3^b
Pro	44.4 ± 9.5^a	28.6 ± 6.1^a	20.5 ± 1.2^a	17.1 ± 2.3^a	10.7 ± 0.5^a	11.8 ± 0.7^a
Csn	6.9 ± 0.2^a	6.9 ± 2.8^a	2.0 ± 0.4^a	1.8 ± 0.5^a	1.0 ± 0.5^a	1.0 ± 0.5^a
Total***	**31.0^a**	**20.8^a**	**14.6^a**	**12.5^a**	**8.7^a**	**10.0 ^{b}**

* Yields of individual amino acids (Y_{AA}) are calculated by the equation: $Y_{AA} = m_{AA,\ isolate\ (g)}/m_{AA,\ BSG}\cdot 100\%$.

** Lowercase letters indicate significant differences between the two pre-treatment groups (defatted and control) within the same extraction.

*** The total amino acid yield from each extraction is calculated using the formula: $Y_{AA,\ total} = \Sigma m_{AA,\ isolate\ (g)}/\Sigma m_{AA,\ BSG\ (g)}\cdot 100\%$.

extraction, the total and individual amino acid yields appeared higher for isolates originating from defatted BSG. Overall, these results imply that defatting had a negative impact on the total amino acid yield from all three extractions, supported by the corresponding reduction in the crude protein yield. Comparing the amino acid composition of defatted and untreated BSG showed a lower amino acid content of defatted BSG compared to untreated BSG (Supporting Information, Table S1). This suggests that protein may be solubilised and lost during defatting with chloroform-methanol. When defatting and delignification were combined, a significant decrease was observed in the total protein yield which may be explained by defatting. On the other hand, the combined treatment had a positive impact on the crude protein content, probably due to delignification.

Conclusions

In the present study, solubilised proteins were either recovered by isoelectric precipitation or freeze drying. Each method had both shortcomings and benefits that need to be addressed in future work. When using isoelectric precipitation, the protein yield was generally low (5-10%) while the protein content in the isolates were high enough to be suitable for feed applications (40-55% DM) (Figure 2). Freeze drying resulted in a higher protein yield (25-30%) with the protein content similar to that of BSG before extraction (20-25%). Furthermore, when the same biomass was extracted three times, the total protein yield increased to 45% compared to 25% from the single extraction; though, the protein content from the second and third extraction were significantly lower compared to the first extraction (Figure 6A). Consequently, the isolates produced by freeze drying are likely to be of low nutritional quality and unsuitable for feed applications.

Previously, protein yields higher than 70% have been reported for alkaline extraction of BSG (Qin et al, 2018). Although the protein yield increased with freeze drying instead of precipitation, the maximum protein yield obtained in this study was significantly lower than the yields obtained in previous study. This suggests that the extraction efficiency is low and needs to be optimised. A likely cause of the low extraction efficiency is that the extraction pH (ca. 12.4) was not optimal for the solubilisation of protein from the BSG batches used here. According to previous work, extraction pH affects protein solubility, and therefore the extraction efficiency could be improved by raising the pH of the alkaline solution (Ruiz et al, 2016; Gao et al, 2020). Initially, a pH range of 8.3-12.4 was evaluated to determine the optimum extraction pH (data not shown). When the pH of the extraction solution was below 12.4, a rapid decline was observed in pH of the BSG suspension which led to low protein solubilisation and a low yield. This decrease may have been caused by lactic acid produced by native lactic acid bacteria in the BSG before autoclaving and suggests that better pH control is required to maintain pH at the desired level during protein extraction (Asurmendi et al, 2014). Furthermore, more alkaline conditions during extraction are associated with reduced purity of protein isolates which may have adverse implications for their use in feed (Ruiz et al, 2016). Therefore, an extraction pH $>$ 12.4 may reduce the nutritional quality of BSG protein.

The solid to liquid ratio (S/L) is also known to influence protein solubilisation during alkaline extraction. Previous studies have employed S/L ratios of 1:5 to 1:60 in the alkaline extraction of protein from different sources of biomass and have shown the optimum

S/L varies with the type of biomass (Vareltzis and Undeland, 2012; Surasani et al, 2017; Vilg and Undeland, 2017). In this study, a S/L ratio of 1:15 was utilised, however the protein yield could possibly be improved by increasing the extraction volume. The low protein yield obtained by isoelectric precipitation could be a result of inadequate precipitation at pH 4.5, as a slight shift in pH during precipitation may cause a significant loss of protein in the liquid fraction (Vilg and Undeland, 2017). Based on the findings of this study, future research should focus on identifying a combination of extraction pH, precipitation pH and S/L ratio that maximises the efficiency of extraction without reducing the quality of protein from BSG.

Author contributions

Freja Karlsen: experimental work, writing (original draft), visualisation and conceptualisation.
Peter Vilhelm Skov: writing (review and editing), funding acquisition and conceptualisation.
Ivar Lund: writing (review and editing).

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Conflict of interest

The authors declare there are no conflicts of interest.

References

Asurmendi P, Pascual L, Dalcero A, Barberis L. 2014. Incidence of lactic acid bacteria and *Aspergillus flavus* in brewer's grains and evaluation of potential antifungal activity of these bacteria. *J Stored Prod Res* 56:33-37. https://doi.org/10.1016/j.jspr.2013.11.002

Bligh EG, Dyer WJ. 1959. A rapid method of total lipid extraction and purification. *Can J Biochem Physiol* 37:911-917.

Chavan UD, McKenzie DB, Shahidi F. 2001. Functional properties of protein isolates from beach pea (*Lathyrus maritimus* L.). *Food Chem* 74:177-187. https://doi.org/10.1016/S0308-8146(01)00123-6

Cohen SA. 1994. Applications of amino acid derivatization with 6-carbamate: analysis of feed grains, intravenous solutions and glycoproteins. *J Chromatogr A* 661:25–34. https://doi.org/10.1016/0021-9673(93)E0821-B

Connolly A, Cermeño M, Crowley D, O'Callaghan Y, O'Brien NM, FitzGerald RJ. 2019. Characterisation of the in vitro bioactive properties of alkaline and enzyme extracted brewers' spent grain protein hydrolysates. *Food Res Int* 121:524-532. https://doi.org/10.1016/j.foodres.2018.12.008

Crowe NL, Baker BE, Inteaz A. 1985. Solubilisation of nitrogenous constituents of brewer's spent grain. *J Inst Brew* 91:148–150. https://doi.org/10.1002/j.2050-0416.1985.tb04320.x

Cuq JC, Vié M, Cheftel JC. 1983. Tryptophan degradation during heat treatments: part 2-degradation of protein-bound tryptophan. *Food Chem* 12:73–88. https://doi.org/10.1016/0308-8146(83)90019-5

Diptee R, Smith JP, Alli I, Khanizadeh S. 1989. Application of response surface methodology in protein extraction studies from brewer's spent grain. *J Food Preserv* 13:457-474.

Ervin V, Alli I, Smith JP, Li Z. 1989. Extraction and precipitation of proteins from brewer's spent grain. *Can Inst Food Sci Technol J* 22:216-221. https://doi.org/10.1016/S0315-5463(89)70386-2

Forssell P, Kontkanen H, Schols HA, Hinz S, Eijsink VGH, Treimo J, Robertson JA, Waldron KW, Faulds CB, Buchert J. 2008. Hydrolysis of brewers' spent grain by carbohydrate degrading enzymes. *J Inst Brew* 114:306-314. https://doi.org/10.1002/j.2050-0416.2008.tb00774.x

Gao Z, Shen P, Lan Y, Cui L, Ohm JB, Chen B, Rao J. 2020. Effect of alkaline extraction pH on structure properties, solubility, and beany flavor of yellow pea protein isolate. *Food Res Int* 131:1-9. https://doi.org/10.1016/j.foodres.2020.109045

Gmoser R, Fristedt R, Larsson K, Undeland I, Taherzadeh MJ, Lennartsson PR. 2020. From stale bread and brewers spent grain to a new food source using edible filamentous fungi. *Bioengineered* 11:582-598. https://doi.org/10.1080/21655979.2020.1768694

Gorissen SHM, Crombag JJR, Senden JMG, Waterval WAH, Bierau J, Verdijk LB, Loon LJC. 2018. Protein content and amino acid composition of commercially available plant-based protein isolates. *Amino Acids* 50:1685-1695. https://doi.org/10.1007/s00726-018-2640-5

Gutiérrez-Barrutia MB, Castillo MD, Arcia P, Cozzano S. 2022. Feasibility of extruded brewer's spent grain as a food ingredient for a healthy, safe, and sustainable human diet. *Foods* 11:1-20. https://doi.org/10.3390/foods11101403

Lam ACY, Karaca AC, Tyler RT, Nickerson MT. 2018. Pea protein isolates: structure, extraction, and functionality. *Food Rev Int* 34:126-147. https://doi.org/10.1080/87559129.2016.1242135

Lynch KM, Steffen EJ, Arendt EK. 2016. Brewers' spent grain: a review with an emphasis on food and health. *J Inst Brew* 122: 553-568. https://doi.org/10.1002/jib.363

Melro E, Filipe A, Sousa D, Valente AJM, Romano A, Antunes FE, Medronho B. 2020. Dissolution of kraft lignin in alkaline solutions. *Int J Biol Macromol* 148:688-695. https://doi.org/10.1016/j.ijbiomac.2020.01.153

Olsen RL, Hasan MR. 2012. A limited supply of fishmeal: impact on future increases in global aquaculture production. *Trends Food Sci Technol* 27:120-128. https://doi.org/10.1016/j.tifs.2012.06.003

Oluseyi AK, Dawodu FA, Ajanaku CO, Nwinyi OC. 2011. Functional and nutritional properties of spent grain enhanced cookies. *Am J Food Technol* 6:763-771. https://doi.org/10.3923/ajft.2011.763.771

Otter DE. 2012. Standardised methods for amino acid analysis of food. *Br J Nutr* 108:230–237. https://doi.org/10.1017/S0007114512002486

Pejin J, Radosavljević M, Kocić-Tanackov S, Mladenović D, Djukić-Vuković A, Mojović L. 2017. Fed-batch L-(+)-lactic acid fermentation of brewer's spent grain hydrolysate. *J Inst Brew* 123:537-543. https://doi.org/10.1002/jib.452

Petit G, Korbel E, Jury V, Aider M, Rousselière S, Audebrand L, Turgeon SL, Mikhaylin S. 2020. Environmental evaluation of new brewer's spent grain preservation pathways for further valorization in human nutrition. *ACS Sustain Chem Eng* 8:17335-17344. https://doi.org/10.1021/acssuschemeng.0c04236

Prado R, Erdocia X, Labidi J. 2013. Lignin extraction and purification with ionic liquids. *J Chem Technol Biotechnol* 88:1248–1257. https://doi.org/10.1002/jctb.3965

Qiaoyun C, Xinghong N, Liang Z, Zheng T, Jin L, Kang S, Xuan C, Xinghui L. 2017. Optimization of protein extraction and decoloration conditions for tea residues. *Hortic Plant J* 3:172–176. https://doi.org/10.1016/j.hpj.2017.06.003

Qin F, Johansen AZ, Mussatto SI. 2018. Evaluation of different pretreatment strategies for protein extraction from brewer's spent grains. *Ind Crops Prod* 125:443-453. https://doi.org/10.1016/j.indcrop.2018.09.017

R Core Team, 2018: a language environment for statistical computing. Vienna, Austria. https://www.r-project.org/

Ruiz GA, Xiao W, Boekel M, Minor M, Stieger M. 2016. Effect of extraction pH on heat-induced aggregation, gelation and microstructure of protein isolate from quinoa (*Chenopodium quinoa* Willd). *Food Chem* 209:203–210. https://doi.org/10.1016/j.foodchem.2016.04.052

Salas C, Rojas OJ, Lucia LA, Hubbe MA, Genzer J. 2013. On the surface interactions of proteins with lignin. *ACS Appl Mater Interfaces* 5:199-206. https://doi.org/10.1021/am3024788

Sousa D, Venâncio A, Belo I, Salgado JM. 2018. Mediterranean agro-industrial wastes as valuable substrates for lignocellulolytic enzymes and protein production by solid-state fermentation. *J Sci Food Agric* 98:5248-5256. https://doi.org/10.1002/jsfa.9063

Sousa R, Coimbra JSR, Rojas EEG, Minim LA, Oliveira FC, Minim VPR. 2007. Effect of pH and salt concentration on the solubility and density of egg yolk and plasma egg yolk. *LWT - Food Sci Technol* 40:1253-1258. https://doi.org/10.1016/j.lwt.2006.08.001

Surasani VKR, Khatkar SK, Singh S. 2017. Effect of process variables on solubility and recovery yields of proteins from pangas (*Pangasius pangasius*) frames obtained by alkaline solubilization method: Characteristics of

isolates. *Food Bioprod Process* 106:137-146. https://doi.org/10.1016/j.fbp.2017.09.008
Tacon AGJ, Hasan MR, Metian M. 2011. Demand and supply of feed ingredients for farmed fish and crustaceans: Trends and prospects. *FAO Fisheries and Aquaculture Technical Paper* 564:1-87. http://www.fao.org/docrep/015/ba0002e/ba0002e.pdf
Tang D, Tian Y, He Y, Li L, Hu S, Li B. 2010. Optimisation of ultrasonic-assisted protein extraction from brewer's spent grain. *Czech J Food Sci* 28:9-17. https://doi.org/10.17221/178/2009-CJFS
Tišma M, Jurić A, Bucić-Kojić A, Panjičko M, Planinić M. 2018. Biovalorization of brewers' spent grain for the production of laccase and polyphenols. *J Inst Brew* 124:182-186. https://doi.org/10.1002/jib.479
Vareltzis PK, Undeland I. 2012. Protein isolation from blue mussels (*Mytilus edulis*) using an acid and alkaline solubilisation technique-process characteristics and functionality of the isolates. *J Sci Food Agric* 92:3055–3064. https://doi.org/10.1002/jsfa.5723
Vilg JV, Undeland I. 2017. pH-driven solubilization and isoelectric precipitation of proteins from the brown seaweed *Saccharina latissima*—effects of osmotic shock, water volume and temperature. *J Appl Phycol* 29:585-593. https://doi.org/10.1007/s10811-016-0957-6
Waters DM, Jacob F, Titze J, Arendt EK, Zannini E. 2012. Fibre, protein and mineral fortification of wheat bread through milled and fermented brewer's spent grain enrichment. *Eur Food Res Technol* 235:767-778. https://doi.org/10.1007/s00217-012-1805-9
Wen C, Zhang J, Duan Y, Zhang H, Ma H. 2019. A mini-review on brewer's spent grain protein: isolation, physicochemical properties, application of protein, and functional properties of hydrolysates. *J Food Sci* 84:3330-3340. https://doi.org/10.1111/1750-3841.14906
Yu D, Sun Y, Wang W, O'Keefe SF, Neilson AP, Feng H, Wang Z, Huang H. 2020. Recovery of protein hydrolysates from brewer's spent grain using enzyme and ultrasonication. *Int J Food Sci Technol* 55:357-368. https://doi.org/10.1111/ijfs.14314

Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.