Extractability and characteristics of proteins deriving from wheat DDGS

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Abstract

Wheat Distillers' Dried Grains with Solubles (DDGS) and in-process samples were used for protein extraction. Prolamins were the predominant protein components in the samples. The absence of extractable α- and γ-gliadins in DDGS indicated protein aggregation during the drum drying processing stage. Prolamin extraction was performed using 70% (v/v) ethanol or alkaline-ethanol solution in the presence of reducing agent. DDGS extracts had relatively low protein contents (14-44.9%, w/w), regardless of the condition applied. The wet solids were the most suitable raw material for protein extraction, with recovery yields of ~ 55% (w/w) and protein content of ~58% (w/w) in 70% (v/v) ethanol. Protein extracts from wet solids were significantly rich in glutamic acid and proline. Mass balance calculations demonstrated the high carbohydrate content (~ 50%, w/w) of solid residues. Overall, the feasibility of utilising in-process samples of DDGS for protein extraction with commercial potential was demonstrated.

Keywords: DDGS, protein, wheat, distillery, extraction, in-process samples, amino acids

1. Introduction

Distillers' Dried Grains with Solubles (DDGS) is the principal by-product of the dry-grind distillation process, generated mainly from beverage alcohol plants (e.g. whisky and neutral spirits distilleries) or from grain-based fuel-ethanol plants. In the case of distilleries, single or blended grains including wheat, barley, maize and rye can be utilised as feedstock, whereas fuel-ethanol plants use either corn (maize) (US) or wheat (Europe) as starting materials.

During the dry-grind process, in the case of bioethanol production, whole grains are milled and liquefied, followed by the addition of amylolytic enzymes for starch conversion into fermentable glucose. In distillery plants, saccharification of the milled grain is carried out using malted barley instead of external enzymes and a food-grade process is followed, as the end-product (potable ethanol) is intended for human consumption. For both bioethanol and potable ethanol production, yeast is added to ferment the sugars into ethanol and carbon dioxide. At the end of the fermentation, the whole stillage undergoes distillation by direct steam injection. Ethanol is further purified via dehydration, whereas the non-volatile components (spent solids) are centrifuged to produce a liquid fraction (thin stillage) and a solid fraction (wet solids). Around 15% or more of the thin stillage is recycled to the liquefaction process of the ground grain, whereas the remaining is concentrated in a series of steam driven evaporators, mixed with wet solids and drum dried to produce the final DDGS (Kim et al. 2008; Liu, 2011). The drying process applied at the last stage is intensive, as the air temperature can be over 500 °C at the dryer inlet and over 100 °C at the dryer outlet. Partial recycling of DDGS to the drum dryer can also occur in order to increase the drying efficiency of the equipment and improve the consistency of the produced

DDGS (Kingsly et al. 2010). Overall, for 100 kg of grain approximately 40 litres of ethanol, 32 kg of DDGS and 32 kg of CO_2 are generated (Schingoethe, 2006).

Because it is enriched in protein, as well as in water-soluble vitamins and minerals, DDGS has been long marketed as feed for livestock (including poultry) (Klopfenstein, Erickson & Bremer, 2008; Schingoethe, Kalscheur, Hippen & Garcia, 2009). DDGS derived from wheat contains around 28-38% (w/w) of protein, whereas for maize DDGS the protein levels range within 28-31% (w/w) (Kim et al. 2010). The major parameters influencing the cost-effectiveness of bioethanol production from cereal grains include the cost of raw materials, as well as the revenue derived from DDGS. In Europe, bioethanol production is currently driven by the EU mandates on biofuel framework (Directive 2009/28/EC), thus the increased bioethanol demand is likely to result in increased DDGS availability. As a result, current research is focused on identifying alternative uses of DDGS, other than animal feed. To this end, existing bioethanol or distillery companies could implement a biorefinery approach, where DDGS is fractionated into several added value compounds including proteins, carbohydrates and phytochemicals.

In contrast to the literature on maize DDGS, a limited number of studies have investigated the extraction of protein from wheat-based DDGS (Bandara, Che & Wu, 2011; Hong, Avramenko, Stone, Abbott, Classen & Nickerson, 2012; Xu, Reddy &Yang, 2007). Wheat grains contain gluten proteins that account for 80% of the total wheat protein, with the remaining 20% corresponding to a heterogeneous group of structural and metabolic proteins, including a major group of water soluble components with molecular weight (MW) lower than 25 kDa (Veraverbeke & Delcour, 2002). By contrast, gluten proteins are largely insoluble in water due to their

high non-polar amino acids content (in particular proline and glutamine) and serve as storage reserves in the wheat grain (prolamins) (Shewry, 1999). Prolamins comprise both alcohol-soluble monomers (gliadins) and alcohol-insoluble polymers (glutenins) with the individual glutenin subunits being alcohol-soluble in their reduced state. Prolamin monomers and subunits show considerable diversity in MW, ranging from 10 to 100 kDa (Shewry & Halford, 2002). The extraction of proteins from DDGS at high yield and purity remains a challenge; DDGS proteins often show low extractability possibly due to the intensive heating applied at the final stage of the production process. Looking towards potential applications, DDGS proteins can be exploited for the production of biodegradable films, coatings and biodegradable plastics, which can be used for food, agricultural and industrial applications (Day, Augustin, Batey & Wrigley, 2006). Wheat protein (gluten) has therefore been extensively studied as a natural starting material for the development of biodegradable films, due to its remarkable cohesive and elastic properties, as well as its susceptibility to chemical modifications (Irissin-Mangata, Bauduin, Boutevin & Gontard, 2001; Kuktaine et al. 2011). Further applications of gluten include in aquaculture feed and in pet food, as an adhesive material in tapes and medical bandages, or as a biodegradable polymer material for the slow release of pesticides or fertilising agents (Day et al. 2006; Majeed, Ramli, Mansor & Man, 2015).

The aim of this study was to investigate the extractability of proteins from various samples originating from a distillery plant, i.e. wheat DDGS, wet and spent solids (the latter also known as whole stillage). The composition of the extracted proteins and their amino acid content were determined and are discussed in order to evaluate the

effect of the multi-step DDGS production process on the properties of the proteins at each stage of production.

2. Materials and methods

2.1 Raw materials

Distillers' Dried Grains with Solubles (DDGS) and in-process samples of wet solids and spent solids were kindly provided by a distillery plant in UK. The distillery plant uses a mixture of 95% (w/w) wheat and 5% (w/w) barley as starting material for potable ethanol manufacture. After being received, samples were frozen at -80 °C. After determination of their moisture content (Section 2.2), samples were lyophilised in a VirTis Bench Top (USA) freeze-drier, initially set at -55 °C for 48 h, packed in polyethylene bags and subsequently stored at -20 °C, until further analysis.

2.2 Compositional analysis of samples

All samples were milled using a conventional coffee grinder in order to reduce their particle size to less than 0.5 mm. The moisture content was determined by drying at 105 °C until a constant weight was reached (at least 18 h of drying needed). Ash was determined after drying the samples in a muffle furnace at 550±10 °C for at least 6 h until a constant weight was reached. Kjeldahl analysis was used to determine total protein using N×5.7 as the conversion factor. Starch content was measured using the Megazyme total starch assay kit (Megazyme International, Ireland). The lipid content was measured gravimetrically after extraction with a Soxhlet apparatus using petroleum ether (Merck, Germany) as solvent.

The composition of the carbohydrates in the samples was determined after a two-step acid hydrolysis procedure according to the National Renewable Energy Laboratory

protocol (NREL/TP-510-42618). The material (300 mg) was first hydrolysed with 72% v/v of sulphuric acid at 30 °C for 1 h and then in diluted acid (4%, v/v) at 121 °C for 30 min. During hydrolysis the polysaccharides are hydrolysed into monosaccharides (glucose derived from cellulose and β-glucan, and xylose and arabinose derived from hemicellulose) which were quantified by HPLC (Agilent, 1100 series) with an Aminex HPX-87H column (300 mm × 7.8 mm, Bio-Rad, California, USA) and a refractive index detector. The operating conditions were: sample volume 20 μL; mobile phase 0.005 M H_2SO_4; flow rate 0.6 mL/min; column temperature 65 °C. According to the NREL protocol, during acid hydrolysis lignin is fractionated into acid soluble and acid insoluble material. Acid-soluble lignin was measured with a UV-Vis spectrometer at 320 nm and acid-insoluble lignin gravimetrically after subtracting the ash and protein contents of the samples. The lignin content of samples is presented as the sum of acid soluble lignin and acid insoluble residue.

2.3 Osborne fractionation of DDGS and in-process samples

DDGS, wet and spent solid samples were subjected to Osborne fractionation according to the method of Lookhart and Bean (1995). Briefly, 100 mg of sample were sequentially extracted with deionised water, 0.5 M NaCl (Sigma, UK), 70% (v/v) aqueous ethanol (Sigma, UK) and 50% (v/v) 1-propanol (Merck, Germany) with 1% (w/v) dithiothreitol (DTT) (Sigma, UK), in order to extract the water-soluble albumins, salt-soluble globulins, ethanol-soluble prolamins and ethanol-insoluble prolamins (as reduced subunits), respectively. A 1:10 (w/v) solids-to-liquid ratio was used for the extractions, which were performed in a thermomixer (Eppendorf, UK) with constant mixing (1400 rpm), at 60 °C for 30 min. Extractions for each sample

were done in duplicate and the supernatants were collected by centrifugation (8,000×*g* for 5 min). In the case of sodium chloride, an additional wash with deionised water was performed in order to remove the residual salt. The protein contents of the Osborne fractionated supernatants were determined using the Bradford reagent assay (Sigma, UK) (Bradford, 1976).

2.4 Protein extraction

2.4.1 Aqueous-ethanol extraction of proteins

Lyophilised and milled samples were subjected to protein extraction using different extraction conditions. Initially, all samples were treated with hexane at a 1:10 (w/v) solid-to-hexane ratio at room temperature for 8 h in order to remove the oil content. Hexane was removed by filtration though a Whatman No 1 paper and the solids were placed in an oven at 45 °C overnight to remove any residual hexane. A two-stage process was subsequently applied to the de-fatted samples to extract the water insoluble proteins. Specifically, 10 g of each sample were mixed with 70% (v/v) aqueous ethanol in a 1:10 (v/w) ratio and incubated under constant shaking for 30 min at different temperatures (50, 70 and 90 °C). Supernatants were removed by centrifugation (8,000×*g*, 15 min) and the residues mixed with 70% (v/v) of aqueous ethanol in a 1:10 (v/w) ratio containing varying concentrations of sodium metabisulfite (Fluka, UK) (0.5, 1.0 or 1.5% w/v) as reducing agent. After mixing the samples for 30 min at different temperatures, again at 50, 70 and 90 °C, they were centrifuged (10,000×*g*, for 10 min at 25 °C), and the second step of the extraction was repeated. Deionised water was added to the collected supernatants in order to dilute the ethanol concentration to below 20% (v/v) and the samples were placed at -20 °C for 4 h to precipitate the proteins. The precipitated proteins were collected by

centrifugation (15,000×g, for 20 min at 2 °C), washed with distilled water, lyophilized in a VirTis Bench Top (USA) freeze-drier for 48 h, and stored at -20°C.

2.4.2 Alkaline-ethanol extraction of proteins

Alkaline conditions were also investigated for the extraction of the proteins in DDGS, wet and spent solid samples. These were incorporated in the second stage of the 2-step extraction process described in 2.4.1, in which aqueous ethanol (45 or 70%, v/v) was mixed with 0.05 or 0.1 M of NaOH (Fluka, UK) and 1.0% (w/v) sodium metabisulfite, in a solid-to-liquid ratio of 1:10. This extraction step was carried out twice at 70 °C for 30 min and the supernatants were collected following centrifugation (10,000×g, for 10 min at 25°C). Extracted proteins were then precipitated with 2 M HCl at pH 5.5 and collected by centrifugation (15,000×g, for 20 min at 10°C), washed with distilled water, lyophilized (VirTis Bench Top, USA) and stored at -20 °C. For both aqueous-ethanol and alkaline-ethanol extractions, the protein contents of the dried extracted samples were determined by Kjeldahl analysis. The protein content and protein yield of dried extracts were calculated as follows:

$$Protein\ content\ of\ extract\ (\%) = \frac{Total\ Kjeldalh\ Nitrogen\ \times 5.7}{Dry\ weight\ of\ extract} \times 100$$

$$Protein\ yield\ (\%) = \frac{Protein\ content\ of\ extract}{Protein\ concentration\ in\ original\ sample} \times 100$$

2.5 SDS-PAGE of samples and protein isolates

To identify the sub-units of water-insoluble proteins present in the original samples, they were extracted sequentially according to Singh, Shepherd and Cornish (1991). Briefly, gliadins were extracted three times from 20 mg samples with 0.1 mL 50% (v/v) 1-propanol for 30 min at 65 °C and the supernatants from the three extractions containing the gliadin fraction were collected by centrifugation (3,000×g for 2 min)

and pooled together. The solid residues, free of gliadins, were incubated with 50% (v/v) 1-propanol in 0.08M Tris-HCl (pH 8.0) with 1% (v/v) p-mercaptoethanol (Sigma, UK) as reducing agent and 1.4% (v/v) 4-vinylpyridine (Sigma, UK) as alkylating agent of sulfhydryl groups, in order to extract glutenin subunits; the supernatant containing the glutenin fraction was collected by centrifugation (3,000×*g* for 2 min). The supernatants containing the gliadin and glutenin fractions, respectively, were diluted in sample buffer [2% (v/v) SDS, 40% (w/v) glycerol, 0.02% (w/v) bromoethyl-blue in 0.08M Tris-HCl (pH=8.0)] and loaded onto a 1.0 mm 4-12% Bis-Tris pre-casted gel (NuPAGE Novex, UK). Proteins were separated in an XCell SurelockTM unit (Invitrogen, UK) at constant voltage (200 V) for 35 min. Gels were washed three times with purified water, stained with SimplyBlue SafeStain buffer (Life Technologies, UK) for 1 h at room temperature and washed with distilled water to obtain a clear background. The molecular weights of the visualised bands were estimated using Novex Sharp pre-stained protein standards (Invitrogen, UK).

The protein fractions extracted after aqueous-ethanol and alkaline-ethanol treatments of the samples were also separated based on their molecular weights using an XCell SurelockTM unit (Invitrogen, UK) according to the protocol provided by the supplier. Specifically, protein samples were reduced by treatment with NuPAGE LDS buffer and reducing agent (dithiothreitol) at 70 °C for 10 min. Electrophoresis was performed as described above.

2.6 Amino acid analysis

The original solid samples as well as lyophilized protein extracts (10 mg) were hydrolysed using 6M HCl (Fluka, UK) and 1% (w/v) phenol (Sigma, UK) at 110 °C for 24 h, in oxygen-free pressure tubes. After hydrolysis, aliquots (100 µL) were

neutralised and derivatised using the EZ-Faast amino acid derivatisation kit (Phenomenex, UK). The kit is based on a solid-phase extraction that binds amino acids and enables the derivatisation in aqueous solution of both the amine and carboxylic groups of amino acids at room temperature. Amino acid profiles were determined using a Gas Chromatography-Mass Spectrometry instrument (Agilent 6890/5975) as described by Elmore, Koutsidis, Dodson, Mottram & Wedzicha (2005). Norvaline was used as internal standard and detected amino acids were quantified according to standard solutions supplied by the manufacturer. Methionine, cysteine and tryptophan were not detected as they were degraded by acid hydrolysis.

2.7 Thermogravimetric analysis (TGA)

TGA analysis was carried out on the protein extracts using a Thermogravimetric Analyzer (TA-Q600SDT TGA). 10 mg samples were heated in an aluminium open pan (Perkin-Elmer) from 30 to 800 °C, with a heating rate of 20 °C/min under nitrogen flow (20 mL/min).

2.8 Statistical analysis

Data are presented as mean values and their respective standard deviations from three replicates. One-way ANOVA was used to calculate the significance between the means of the samples treated under different extraction conditions at $p < 0.05$.

3. Results and discussion

3.1 Composition of DDGS, wet solids and spent solids

The compositions of DDGS, wet solids and spent solids are shown in Table 1 as percentage concentration per dry weight basis (db). As expected, the dry matter was

241 higher in the case of DDGS, due to the thermal drying process carried out at the final
242 stage of DDGS production. By contrast, wet and spent solids contained significant
243 amounts of moisture (66.8% and 77.9%, respectively). In terms of protein, DDGS
244 contained around 30% (db) of protein, whereas lower concentrations were present in
245 wet (20%, db) and spent solids (25%, db). Similar values for wheat DDGS have been
246 previously reported (Pedersen et al. 2014; Cozannet et al. 2010; Ortín & Yu, 2009),
247 with the small differences probably resulting from differences in the processes used
248 between different plants, seasonal variation in the harvested wheat, and a different N-
249 to-protein conversion factor (6.25 over 5.7). The lipid content was similar in DDGS
250 (3.4% db) and wet solids (2.9% db) but significantly higher ($P<0.05$) in spent solids
251 (5.4%, db). Low concentrations of starch were detected in all samples (1.4-2.6%, db).
252 In terms of the non-starch carbohydrate content, the values for cellulose and
253 hemicellulose (Table 1) did not vary significantly between DDGS and the in-process
254 samples. The ash content was slightly higher in spent solids (4.4%, db) than in DDGS
255 (3.9%, db). Thin stillage, and consequently spent solids, as also shown in this study,
256 typically contain the highest contents of ash among the different in-process samples
257 (Liu, 2011; Hong et al. 2012). Blending of wet solids with condensed thin stillage and
258 subsequent drying to give DDGS resulted in a lower ash content of DDGS compared
259 to spent solids. Finally, DDGS had a higher lignin content (5.3%, db) compared to
260 wet and spent solids. Pedersen et al. (2014) determined the composition of DDGS of
261 various origins, including wheat, maize and mixed cereals, and found differences in
262 the Klason lignin content among the DDGS samples. These were attributed to an
263 extent to the presence of non-lignin sources in the Klason lignin fraction, such as
264 Maillard-reaction products. The latter are formed during the mixing and drying of wet

265 solids as a result of the reaction between reducing sugars and lysine residues, and are
266 condensed in thin stillage (Pahm, Pedersen & Stein, 2009).

267

268 **3.2 Osborne fractionation of DDGS and in-process samples**

269 A modified Osborne protocol was carried out for DDGS and in-process samples in
270 order to identify the nature and solubility in different solvents of the various protein
271 fractions present in the samples (Fig 1). Salt-soluble globulins were the least abundant
272 group in all samples, accounting for 14% (w/w) of the total extracted protein in spent
273 solids and about 10% (w/w) or less in wet solids and DDGS, respectively. The
274 albumin content varied significantly among samples; it was the major protein fraction
275 of spent solids accounting for ~ 41% (w/w), followed by ~18% (w/w) in DDGS and
276 ~10% (w/w) in wet solids. Taking into account the fact that spent solids are a mixture
277 of fermentation liquid and grain residues, it is expected that a substantial amount of
278 the protein content in spent solids could be attributed to non-gluten proteins. These
279 are mainly water soluble, metabolic or structural proteins and also include the
280 amylolytic enzymes used in the fermentation process. Moreover, from a process point
281 of view, the mixing of wet solids and the concentration of thin stillage taking place
282 during the production of DDGS, contributed considerably to the presence of about
283 20% of albumins in DDGS. Alcohol soluble gliadins were the second most abundant
284 protein fraction in all samples. Small differences in their concentration occurred
285 between spent and wet solids (Fig. 1), whereas in DDGS they accounted for 33%
286 (w/w) of the total extracted protein. Despite the fact that gliadins are readily soluble in
287 aqueous alcohol, it is unlikely that they are solubilised during the fermentation
288 process, as the ethanol concentration is only around 18% (v/v) at the end point of the
289 fermentation. Glutenins were the major fraction in wet solids and DDGS, accounting

290 for 55% (w/w) and 42% (w/w) of the total extracted protein, respectively. Glutenins
291 and gliadins contain high levels of proline and glutamine and serve as storage proteins
292 in the starchy endosperm cells of the wheat grain (Shewry et al., 2002). Glutenins
293 comprise a heterogeneous mixture of high and low molecular weight subunits
294 assembled into polymers stabilised by inter-chain disulphide bonds (Veraverbeke &
295 Delcour, 2002). However, they are only extractable in aqueous alcohol as reduced
296 subunits in the presence of a reducing agent. It should be noted that the yield of total
297 extracted proteins according to the Osborne fractionation method (measured by
298 Bradford and compared to the initial protein content of the samples), was 20.3% for
299 DDGS, 27.9% for wet solids and 28.4% for spent solids, respectively. Although the
300 Osborne method has been widely used to extract proteins based on solubility,
301 quantification can be problematic due to the fact that the different protein groups can
302 overlap in their solubility in the different solvents, leading to partial cross-
303 contamination of the fractions (Shewry, 1999; DuPont, Chan, Lopez & Vensel, 2005).
304 However, in our study, Osborne fractionation proved to be a useful tool for
305 identifying key differences between the protein contents of the samples, and
306 demonstrated the influence of certain process steps on specific protein groups, such as
307 albumins.

308 In order to further characterise the protein content of DDGS and in-process samples,
309 prolamins were sequentially extracted according to the protocol of Singh, Shepherd
310 and Cornish (1991). SDS PAGE analysis (Fig 2a) of the gliadins and glutenins present
311 in spent solids, wet solids and DDGS was conducted. Based on the molecular weight
312 ladder (lane 1), distinctive bands with molecular weight of around 50 kDa were
313 present in spent and wet solids, corresponding to ω-gliadins, as well as bands with
314 molecular weights of 40 and 30 kDa, corresponding α- and γ-gliadins, respectively. In

DDGS, only bands corresponding to ω-gliadins were distinctively present. It has been reported that high temperatures (above 100°C) can result in re-arrangements leading to the formation of new disulphide bonds among the sulphur-rich α, β- and γ-gliadins, whereas ω-gliadins do not contain cysteine residues and thus cannot form disulphide bonds (Schofield et al. 1983). This could explain the presence of only ω-gliadins in the DDGS sample, as the latter is subjected to an intensive thermal treatment at the last stage of its production (drum drying). All samples demonstrated intense bands at the top of the gel, suggesting the presence of high molecular weight aggregates of glutenin or gliadin subunits or even polymerised gliadins that were not able to enter the gel. Distinctive bands corresponding to low molecular weight glutenin subunits were present in spent and wet solids (30-60 kDa), whereas these bands were only present in traces in DDGS. The intensities of the glutenin bands on the SDS-PAGE gels confirmed the results obtained from the Osborne fractionation which indicated that glutenins were the most abundant proteins in the samples. In most of the gliadin and glutenin protein fractions, a clear band was obtained around 20 kDa. This could correspond to albumins, resulting from partial cross-contamination during Osborne fractionation, or partially hydrolysed proteins.

3.3 Extraction of proteins from DDGS and in-process samples

One of the major goals of this study was to investigate the methodology for the extraction of proteins from DDGS and in-process samples. Water-insoluble proteins (i.e. gliadins and glutenins) were mainly targeted, as these could serve as suitable starting materials for the development of biodegradable polymers for food and non-food applications, as previously shown for gliadins and glutenins derived from wheat grains (Kuktaite et al. 2011; Lagrain et al. 2010). Gliadins and reduced glutenin

subunits are both soluble in aqueous (60-70% v/v) ethanol (Shewry, 1999). Reducing agents are typically used to improve protein extraction, as they reduce the disulphide bonds present both within (intra-chain) and between (inter-chain) gluten protein subunits (Shewry & Tatham, 1997). Dithiothreitol (DDT) and β-mercaptoethanol (β-ME) are most widely utilised for this purpose. However, these chemicals are not suitable for commercial production because of their toxicity. Alternatively, sodium metabisulfite is a preferable reducing agent, as it is food grade and has lower toxicity and odour compared to other reducing agents (Park, Bean, Wilson & Schober, 2006).

The first set of extraction experiments was carried out using 70% (v/v) aqueous ethanol, in order to determine the effects of temperature and reducing agent concentration on protein extractability. The protein content of the dried extracts as determined by Kjeldalh analysis is presented in Table 2. Extraction at 50 °C resulted in low protein content, ranging between 14-32 %, in all extracts depending on the reducing agent concentration. The greatest amount of protein was present in the extracts from the wet solids (~32%) followed by spent solids (~23%) and then DDGS (~14%). Extraction at 70 °C improved significantly ($P<0.05$) the protein content of all samples compared to 50 ^{o}C, with the highest being ~45% for DDGS, ~58% for wet solids and ~62% for spent solids; the optimum reducing agent concentration was in most cases 1%. At 90 ^{o}C the protein content of the extracts decreased significantly ($P<0.05$) compared to that at 70 ^{o}C for all samples. In terms of the protein extraction yield (% of protein per total protein of original sample), the best extraction conditions were identified as 70 °C and 1% reducing agent, resulting in protein extraction yields of 30.1% (w/w) for DDGS, 55.3% (w/w) for wet solids and 52.1% (w/w) for spent solids.

In the presence of ethanol, only the hydrophobic fraction of wheat protein is solubilised, as a result of the disruption of low-energy hydrogen bonds in the decreased dielectric constant of the medium. Reduction of the disulphide bonds is responsible for the solubilisation of small amounts of ω-gliadins that are present in glutenin (the D type low molecular weight subunits) and some low molecular weight glutenin subunits, which in turn renders the remaining gluten proteins (comprising high molecular weight glutenins as well as α-, β- and γ-gliadins) soluble in hot ethanol solution (Mimouni, Robin, Azanza & Raymond, 1998). In this study, the use of reducing agent and 70 °C led to the extraction of water-insoluble prolamins, with the extraction efficiency being dependent on the starting material. However, at elevated temperatures (around 100 °C), the rich-sulphur α-, β- and γ-gliadins undergo disulphide bond rearrangements which reduces their solubility; this could be the reason for the lower protein extraction seen in the case of DDGS compared to the other samples in all extraction temperatures, and particularly at 90 ^{o}C (Table 2). Moreover, under such conditions, glutenin polymerisation can occur via sulphydryl-disulphide inter-chain exchange reactions between polymers (Lagrain et al., 2008). The latter may be further facilitated by a temperature-dependent unfolding of the tertiary structure of the proteins. Recently, Hong and co-workers (2012) stated that the protein extraction efficiency of samples post-distillation (i.e. spent solids) is higher compared to DDGS, as a result of heat-induced protein denaturation and increased disulphide bonding within and among proteins, which occurs during the final drum drying step of the process.

The second set of extraction experiments was carried out using an alkaline-aqueous ethanol solution at 70 ^{o}C and a reducing agent concentration of 1.0%, as these were

388 shown from the previous experiments to be the optimal conditions for extraction.
389 Alkalis and acids can partially hydrolyse protein molecules into smaller peptide
390 fragments, which typically increases their solubility and extractability. In these
391 experiments, the proteins were extracted with 45% or 70% (v/v) aqueous ethanol in
392 the presence of 0.05M or 0.1M of NaOH. As shown in Table 2, 45% ethanol
393 combined with 0.05M NaOH resulted in low protein contents in the extracts derived
394 from wet and spent solids (~ 13% and 20%, respectively) and DDGS (~21%). The
395 extractability of proteins was significantly ($P<0.05$) increased with 70% (v/v) ethanol,
396 in particular in the presence of relatively high concentrations of alkali (0.1M); the
397 protein content of the dried extracts was ~39% for DDGS, ~49% for wet solids and
398 ~52% for spent solids, whereas the extraction yields were 27.1% for DDGS, 33.4%
399 for wet solids and 31.2% for spent solids, respectively.

400 SDS-PAGE analyses of the proteins in the aqueous ethanol and alkaline-aqueous
401 ethanol extracts are shown in Fig 2b. For both extraction methods, distinctive bands
402 were obtained for all samples in the range of 35-50 kDa, most likely corresponding to
403 a mixture of α-, γ- and ω-gliadins and low molecular weight glutenin subunits.
404 Visualisation of the gels suggests that the profile of the extracted proteins was not
405 considerably affected by the extraction conditions, and that the main differences in the
406 protein content of the extracts obtained under the different conditions were primarily
407 quantitative rather than qualitative.

408 Taking the above results into account, it can be deduced that aqueous ethanol
409 extraction (pH~10) was a more efficient method for the extraction of proteins from
410 the wheat DDGS and in-process samples compared to alkaline-aqueous ethanol
411 extraction (pH~12). Utilising aqueous ethanol for extraction of proteins from DDGS

412 or in-process samples would be particularly attractive for distilleries and bioethanol
413 plants. Moreover, the presence of alkali in the extraction process could result in
414 corrosion of equipment in the long-term.

415 Although the literature on the extraction of proteins from wheat DDGS is limited, a
416 few studies have studied the extraction of proteins from DDGS from other cereals. Xu
417 et al. (2007) reported an extraction yield of 44% with 90% protein content for corn
418 DDGS using 70% ethanol and 0.25% sodium sulfite at acidic pH. More recently, in a
419 two fraction extraction process with 70% (v/v) aqueous 2-propanol and 70% (v/v)
420 aqueous ethanol, Anderson, Ilankovan & Lamsai (2012) achieved an extraction yield
421 of 70% of α-zein from maize DDGS. In another study, Wang, Tilley, Bean, Sun &
422 Wang (2009) investigated the extraction efficiency of kafirin proteins (prolamins)
423 from sorghum DDGS and reported an extraction yield of 44% with a kafirin content
424 of 98.8% using acetic acid under reducing conditions. In the same context, Bandara et
425 al. (2011) studied the efficiency of protein extraction from triticale DDGS and
426 demonstrated that alkaline-ethanol conditions gave extraction yields between 21-30%
427 (w/w) and a maximum protein content of ~66% (w/w). The present study is the first to
428 investigate the extraction of proteins from in-process samples produced during the
429 wheat DDGS production. Comparison of the extractability of proteins within samples
430 shows that wet solids are the most appropriate starting material for protein extraction.
431 Under optimal extraction conditions, 55.3% of the total protein was recovered from
432 wet solids, with a protein content of 58% (w/w). From an industrial perspective,
433 protein recovery and purity are very important for the translation of the process to
434 large scale extraction. Commercial gluten products extracted from wheat contain
435 around 75% protein. Therefore, efficient extraction using DDGS or in-process

samples as starting material should ideally result in a protein-rich extract with a similar purity. To this end, the addition of an ultrafiltration step post-reduction would reduce the amounts of carbohydrates and other non-protein components in the protein extracts and increase their purity.

3.4 Composition of protein extracts and solid residues

Table 3 shows the compositions of the protein extracts and their respective solid residues after ethanol extraction of DDGS, spent and wet solids samples. Very small amounts of water-soluble carbohydrates were detected in all aqueous ethanol extracts (2.4-5.1%, w/w), with the spent solids containing the smallest amount. Glucose was the major monosaccharide determined after hydrolysis, indicating the presence of starch followed by xylose and arabinose, the latter indicating the presence of soluble arabinoxylans, which are the major non-starch polysaccharides in wheat grain (Saulnier, Peneau & Thibault, 1995). On the other hand, the solid residue after extraction had a high content of water unextractable polysaccharides which was around 49% (w/w) for all samples. The monosaccharides composition (Table 3) indicated the presence of insoluble β-glucan, cellulose and water unextractable arabinoxylan. The protein content of the solid residues was ~4.7% for spent solids, ~7.4% for wet solids and ~11.6% for DDGS, i.e. the reverse ranking of that obtained for protein extractability.

Moreover, the mass balances for the principal components (i.e. protein and carbohydrates) were calculated. It should be noted that because the current study focused on the extraction of water-insoluble proteins, the contents of gliadins and glutenins determined by Osborne analysis (Fig 1) were taken into account for calculating the protein mass balance. Based on the data in Table 1, only ~69% of the

initial protein (gliadins and glutenins) content was recovered from DDGS. This could be attributed to only partial precipitation of the low molecular weight proteins in the extraction liquid, as well as to the thermal denaturation of DDGS proteins during the drying stage. At the drying stage DDGS is subjected to intense and prolonged thermal treatment (higher than 100°C). Under these conditions, the disulphide bonds present in all wheat gluten proteins (except ω-gliadins) may undergo rearrangements to form cross-links in highly insoluble denatured aggregates (Wang, Wei, Li, Bian & Zhao, 2009; Hong et al. 2012). On the other hand, protein recoveries from wet and spent solids were noticeably higher (89-93%), reflecting the higher protein extractability of these samples. In terms of the carbohydrate mass balances, the recoveries ranged from 86 to 94% for all samples. These values are very good considering that approximately 5% of the material could be lost during the intermediate washing steps. The high carbohydrate contents of the solid residues after protein extraction indicate that these materials could be a potential source of non-starch polysaccharides, and if processed to oligosaccharides could provide functional ingredients (prebiotics, stabilisers, emulsifiers) for food and non-food applications.

Further information on the proteins present in the extracts of DDGS and wet solids was provided by thermogravimetric analysis (TGA). Degradation of the samples was carried out under nitrogen and the observed peaks are presented as derivatives of the weight loss as a function of temperature. As depicted in Fig 4, a peak was identified for both samples at around 53-60°C. This was more intense in the case of wet solids and corresponded to the loss of free and bound water. Extracts of both DDGS and wet solids exhibited a prominent broad peak in the range of 230-370°C, which was attributed to the breakage of the covalent peptide bonds of amino acids, as well as to

484 the cleavage of disulphide, O-N and O-O bonds in protein molecules (Sun, Song &
485 Zheng, 2007). Moreover, the analysis showed an additional peak for the DDGS
486 extract at about 730°C, which is probably associated with the degradation of lignin
487 components (Sahoo, Seydibeyoğlu, Mohanty & Misra, 2011).
488
489 **3.5 Amino acid compositions of solid samples and protein extracts**
490 Fig 5a shows the relative concentrations of amino acids in hydrolysates of the DDGS,
491 wet and spent solids samples. Glutamic acid (which is mostly derived from the
492 deamidation of glutamate), proline, leucine and phenylalanine were the major amino
493 acids in the samples and are representative of wheat gluten proteins (Wieser, 2007). It
494 is worth noting the reduced concentration of lysine in the DDGS sample, as lysine is
495 the limiting essential amino acid in wheat grain proteins for the nutrition of humans
496 and monogastric livestock (Shewry, 2007) but is labile to heating (Almeida, Htoo,
497 Thomson & Stein, 2013). Fig 5b shows the relative concentration of individual amino
498 acids in the wet solid and DDGS protein samples, obtained after aqueous ethanol and
499 alkaline-aqueous ethanol extraction. The increased amino acid content of the wet solid
500 extracts reflects the increased protein extractability of wet solids compared to the
501 intensively thermally treated DDGS sample. Comparing the amino acid profile
502 obtained between the two extraction methods, aqueous ethanol conditions showed
503 increased specificity towards glutamic acid, phenylalanine and proline. These amino
504 acids are present in α-, γ-, ω-gliadins and low molecular subunits of glutenin (Shewry,
505 Tatham, Forde, Kreis & Miflin, 1986) and as shown by SDS-PAGE, these were the
506 major protein groups in the extracts. Apart from the potential utilisation of protein
507 extracts as starting material for biodegradable plastics, the high content of glutamic
508 acid could justify its extraction and utilisation as building block for chemical

compounds such as succinonitrile or acrylonitrile (Lammens, Franseen, Scott & Sanders, 2012). To this end, glutamic acid is a non-essential amino acid and its extraction would not compromise the nutritional value of DDGS used as livestock feed.

4. Conclusions

Aqueous ethanol extraction was more effective than alkaline-aqueous ethanol for extracting water-insoluble proteins from DDGS and in-process samples. The extractability of the proteins and their compositional characteristics were highly influenced by the starting raw material, i.e. wet solids, spent solids or DDGS. Protein was less efficiently extracted from DDGS, probably due to the decreased solubility of protein aggregates formed during the intensive thermal treatment during the drum drying stage. This is also indicated by the low recovery of α- and γ-gliadins. The wet solids exhibited the highest protein extractability (gliadins and glutenins), with a maximum recovery yield of 55% (w/w) (on the basis of total protein) and a protein content of 58% (w/w), and hydrolysates were particularly rich in glutamic acid and proline. The solid residues after extraction had a high carbohydrate content, which renders them amenable to enzymatic processing for the production of bioactive carbohydrates, such as prebiotic oligosaccharides, or for use as fibre-rich livestock feed. Overall, the research demonstrated the feasibility of utilising in-process samples from the DDGS production process for the extraction of proteins with good commercial potential.

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661 Xu, W., Reddy, N., Yang, Y. (2007). An acidic method of zein extraction from
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663

664 Table 1. Chemical composition of DDGS and in-process samples

(in %, db)	DDGS	Wet solids	Spent solids
Dry matter	96.6±0.7	33.2±1.1	22.1±1.2
Crude protein	29.1±1.7	19.8±1.2	25.6±1.1
Crude fat	3.4±0.1	2.9±0.9	5.4±0.4
Cellulose and *β*-glucan	14.9±0.4	15.1±0.6	16.3±1.1
Starch	2.6±0.10	2.0±0.3	1.4±0.19
Hemicellulose	25.1±1.6	28.0±1.3	25.5±0.9
Xylose	(16.7±0.9)	(18.4±0.7)	(16.7±0.6)
Arabinose	(8.3±0.8)	(9.6±0.6)	(8.9±0.3)
Lignin	5.3±0.7	4.1±0.5	3.8±0.3
Ash	3.9±0.5	2.1±0.3	4.4±0.6

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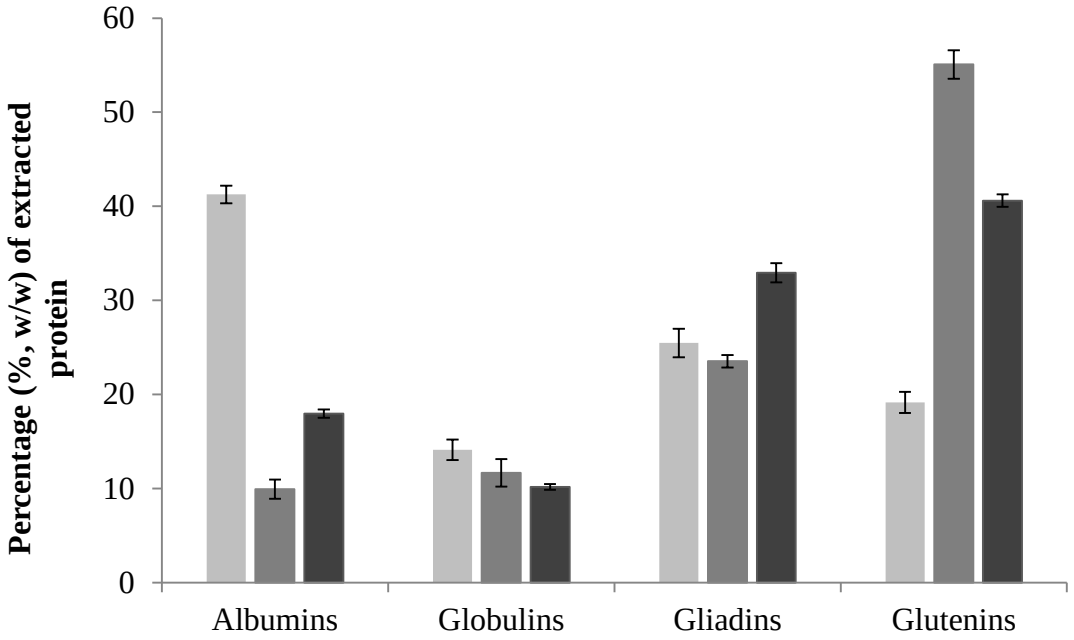
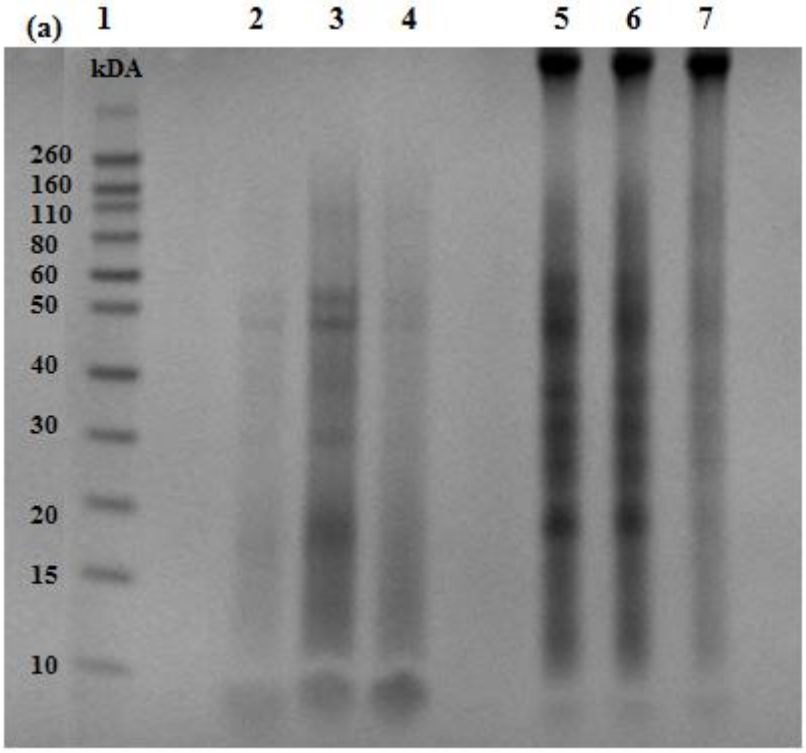


Fig. 1 Protein composition of fractionated spent solids (grey), wet solids (dark grey) and DDGS (black), based on Osborne protocol

Table 2. Protein content of isolates (%, w/w) derived from DDGS and in-process samples during different extraction conditions, as determined by Kjeldahl analysis

				Protein content of isolates (%, w/w)		
Extraction Temperature (°C)	**SMB (%, w/w)**	**EtOH (%, v/v)**	**NaOH (M)**	**DDGS**	**Wet solids**	**Spent solids**
50	0.5	70	-	14.0 (0.8)	27.0 (1.3)	23.5 (1.2)
	1.0			14.5 (0.7)	31.7 (1.7)	22.9 (0.8)
	1.5			14.9 (0.5)	29.9 (1.7)	23.5 (0.9)
70	0.5	70	-	34.1 (3.2)	47.8 (2.9)	53.6 (2.8)
	1.0			42.7 (2.1)	55.6 (2.9)	62.4 (0.5)
	1.5			44.9 (1.5)	58.2 (0.5)	54.7 (0.3)
90	0.5	70	-	38.1 (1.3)	43.7 (0.3)	42.4 (0.3)
	1.0			29.6 (0.1)	42.6 (0.1)	45.8 (0.3)
	1.5			27.2 (0.2)	39.1 (0.2)	40.2 (0.3)
70	1.0	45	0.10	22.1 (0.6)	19.7 (1.7)	13.5 (1.4)
		70	0.05	27.5 (1.4)	25.9 (0.8)	36.6 (1.8)
		70	0.10	39.5 (2.1)	49.1 (0.7)	51.8 (2.2)

Data in parenthesis represent standard deviation values



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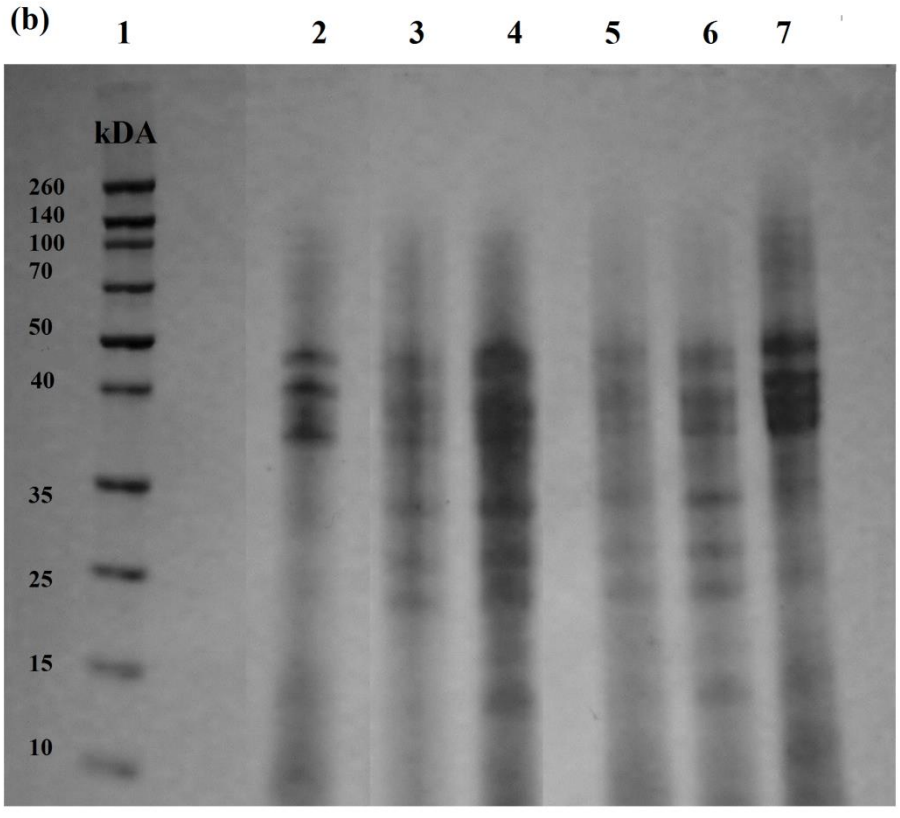

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684 Fig 2. (a) SDS-PAGE according to the protocol by Singh et al. (1991) of original
685 samples: Lane 1, Molecular weight marker; lanes 2-4, Gliadin proteins of: spent solids
686 (lane 2), wet solids (lane 3) and DDGS (lane 4), respectively; Lanes 4-6, Glutenin

687 proteins of: spent solids (lane 4), wet solids (lane 5) and DDGS (lane 6). (b) SDS-
688 PAGE of proteins extracted in aqueous-ethanol or alkaline-ethanol solutions: Lane 1,
689 Molecular weight marker; lanes 2-4, Ethanol extracted proteins of wet solids (lane 2),
690 spent solids (lane 3) and DDGS (lane 4); lanes 5-7, Alkaline (0.1M)-ethanol (70%,
691 v/v) extraction of wet solids (lane 5), spent solids (lane 6) and DDGS (lane 7).

692

693 Table 3 Protein and carbohydrate content of ethanol extracted proteins and their solid
694 residues and mass balance calculations compared to the starting raw materials
695 (DDGS, wet solids, spent solids)

	Protein extracts (%, db)			Solid residues (%, db)			Mass balance[b] (%, per 100 g)		
	DDGS	**Wet solids**	**Spent solids**	**DDGS**	**Wet solids**	**Spent solids**	**DDGS**	**Wet solids**	**Spent solids**
Protein[a]	44.7	55.6	62.4	11.6	7.4	4.7	68.7	89.2	92.8
Carbohydrates	4.2	2.4	5.1	49.7	49.0	49.1	94.2	85.8	92.8
Glucose	2.1	1.2	2.8	20.1	18.6	19.8	87.3	83.1	89.2
Xylose	1.5	0.9	1.1	17.3	19.1	18.4	90.7	78.8	86.8
Arabinose	0.6	0.3	1.1	12.3	11.3	10.9	101.8	92.6	108.9

696 Data presented as mean values

697 [a]: Protein content measured by Kjeldahl

698 [b]: Mass balance for protein calculated by taking into account Osborne analysis results for gliadin and
699 glutenin content (45% in spent solids, 78% in wet solids and 73% in DDGS).

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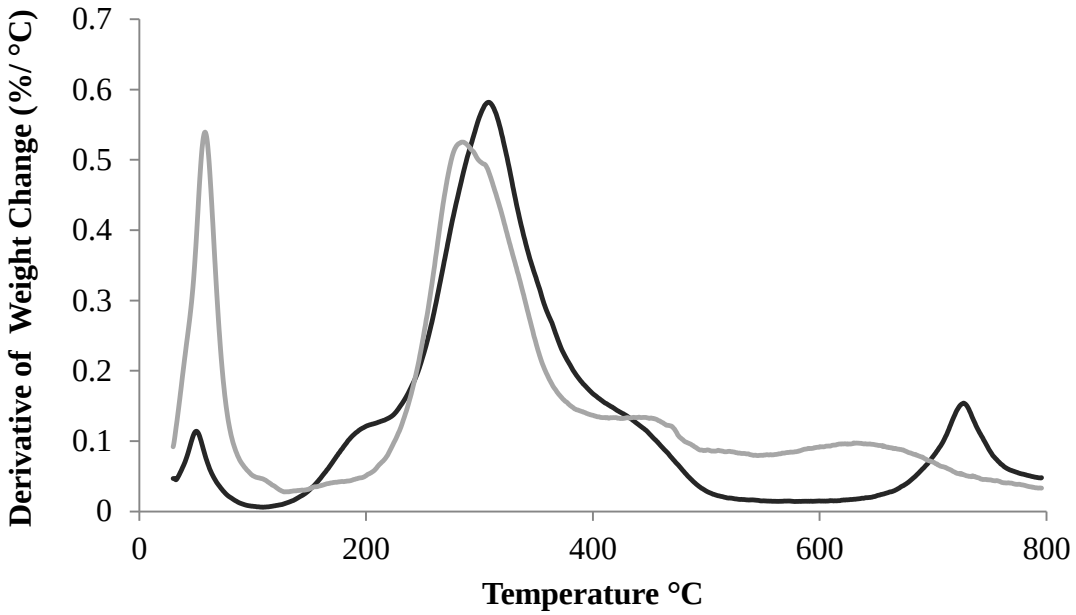
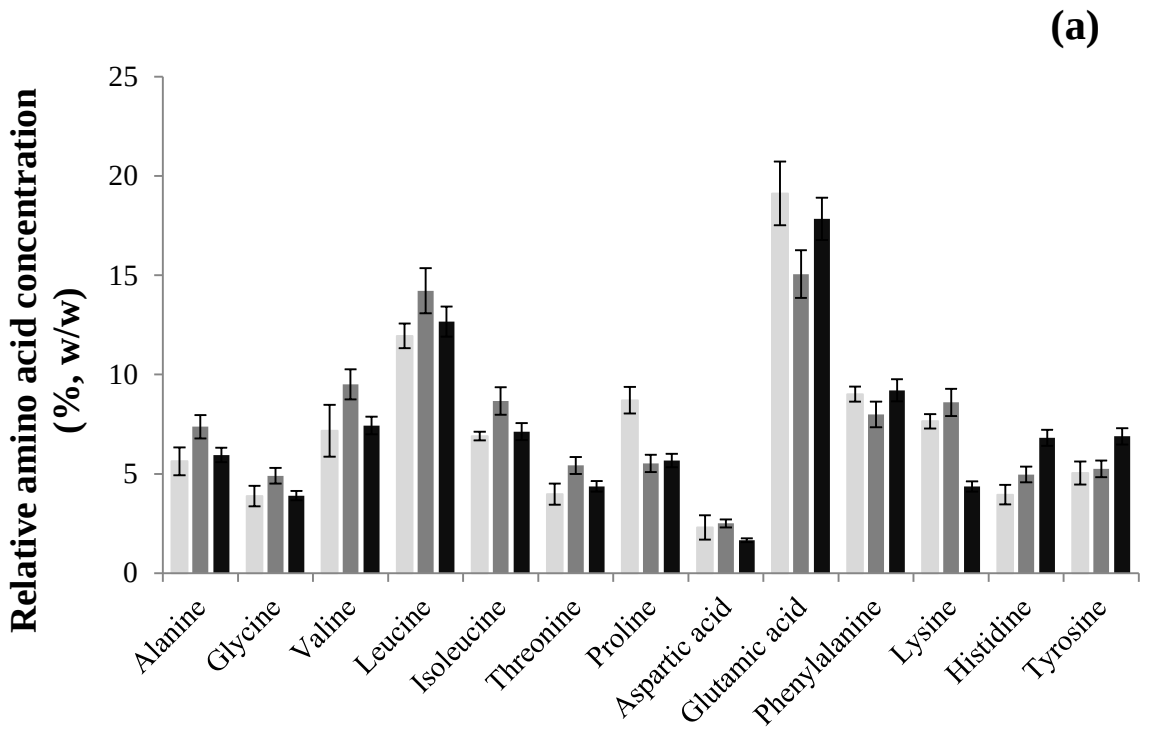


Fig 4. TGA analysis of ethanol extracted proteins from DDGS (black line) and wet solids (grey line)



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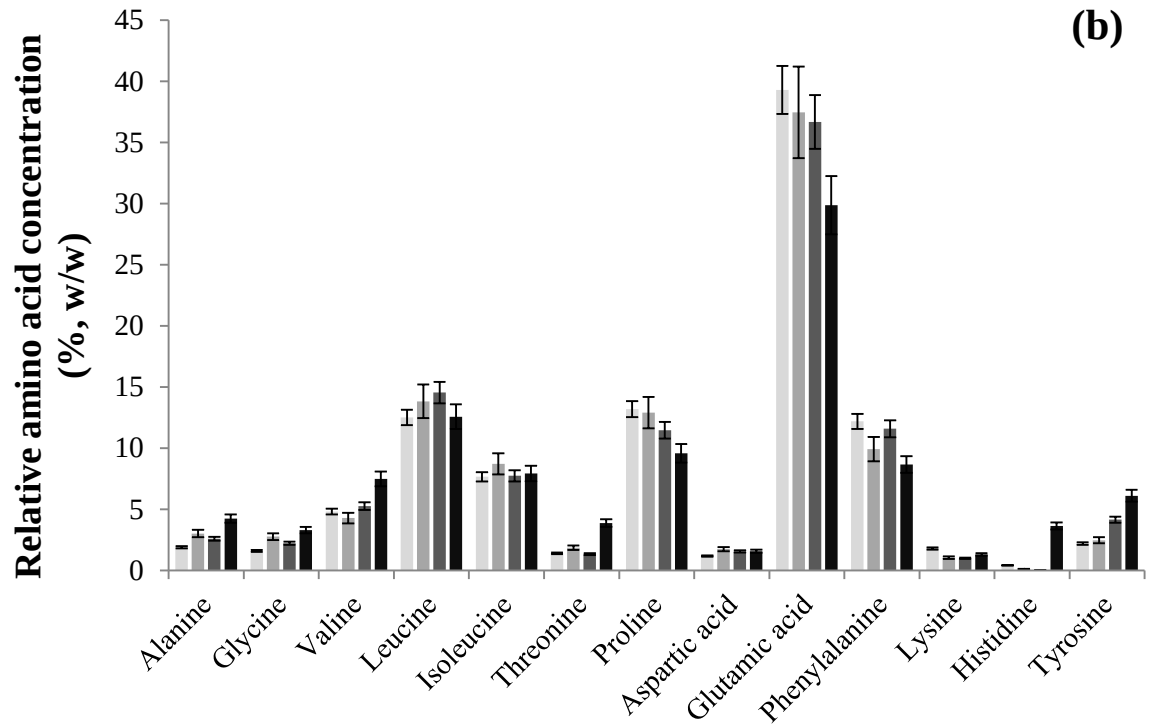

709

710 Fig 5. Amino acid analysis of samples: (a) Relative amino acid concentration of spent
711 solids (grey), wet solids (dark grey) and DDGS (black) after acid hydrolysis; (b)
712 Relative amino acid concentration in wet solid protein extracted with ethanol, (light
713 grey), wet solid protein extracted with alkaline-ethanol (grey), DDGS protein

714 extracted with ethanol (dark grey) and DDGS protein extracted with alkaline-ethanol
715 (black).