

# *Extractability and characteristics of proteins deriving from wheat DDGS*

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1 **Extractability and characteristics of proteins deriving from wheat**

2 **DDGS**

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11

12 **Abstract**

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

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

28

29 **1. Introduction**

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

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

53 DDGS (Kingsly et al. 2010). Overall, for 100 kg of grain approximately 40 litres of  
54 ethanol, 32 kg of DDGS and 32 kg of CO<sub>2</sub> are generated (Schingoethe, 2006).

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

69 In contrast to the literature on maize DDGS, a limited number of studies have  
70 investigated the extraction of protein from wheat-based DDGS (Bandara, Che & Wu,  
71 2011; Hong, Avramenko, Stone, Abbott, Classen & Nickerson, 2012; Xu, Reddy  
72 & Yang, 2007). Wheat grains contain gluten proteins that account for 80% of the total  
73 wheat protein, with the remaining 20% corresponding to a heterogeneous group of  
74 structural and metabolic proteins, including a major group of water soluble  
75 components with molecular weight (MW) lower than 25 kDa (Veraverbeke &  
76 Delcour, 2002). By contrast, gluten proteins are largely insoluble in water due to their

77 high non-polar amino acids content (in particular proline and glutamine) and serve as  
78 storage reserves in the wheat grain (prolamins) (Shewry, 1999). Prolamins comprise  
79 both alcohol-soluble monomers (gliadins) and alcohol-insoluble polymers (glutenins)  
80 with the individual glutenin subunits being alcohol-soluble in their reduced state.  
81 Prolamin monomers and subunits show considerable diversity in MW, ranging from  
82 10 to 100 kDa (Shewry & Halford, 2002). The extraction of proteins from DDGS at  
83 high yield and purity remains a challenge; DDGS proteins often show low  
84 extractability possibly due to the intensive heating applied at the final stage of the  
85 production process. Looking towards potential applications, DDGS proteins can be  
86 exploited for the production of biodegradable films, coatings and biodegradable  
87 plastics, which can be used for food, agricultural and industrial applications (Day,  
88 Augustin, Batey & Wrigley, 2006). Wheat protein (gluten) has therefore been  
89 extensively studied as a natural starting material for the development of biodegradable  
90 films, due to its remarkable cohesive and elastic properties, as well as its susceptibility  
91 to chemical modifications (Irissin-Mangata, Bauduin, Boutevin & Gontard, 2001;  
92 Kuktaine et al. 2011). Further applications of gluten include in aquaculture feed and in  
93 pet food, as an adhesive material in tapes and medical bandages, or as a biodegradable  
94 polymer material for the slow release of pesticides or fertilising agents (Day et al.  
95 2006; Majeed, Ramli, Mansor & Man, 2015).

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

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

## 102 **2. Materials and methods**

### 103 **2.1 Raw materials**

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

### 111 **2.2 Compositional analysis of samples**

112 All samples were milled using a conventional coffee grinder in order to reduce their  
113 particle size to less than 0.5 mm. The moisture content was determined by drying at  
114 105 °C until a constant weight was reached (at least 18 h of drying needed). Ash was  
115 determined after drying the samples in a muffle furnace at 550±10 °C for at least 6 h  
116 until a constant weight was reached. Kjeldahl analysis was used to determine total  
117 protein using N×5.7 as the conversion factor. Starch content was measured using the  
118 Megazyme total starch assay kit (Megazyme International, Ireland). The lipid content  
119 was measured gravimetrically after extraction with a Soxhlet apparatus using  
120 petroleum ether (Merck, Germany) as solvent.

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



123 protocol (NREL/TP-510-42618). The material (300 mg) was first hydrolysed with  
124 72% v/v of sulphuric acid at 30 °C for 1 h and then in diluted acid (4%, v/v) at 121 °C  
125 for 30 min. During hydrolysis the polysaccharides are hydrolysed into  
126 monosaccharides (glucose derived from cellulose and  $\beta$ -glucan, and xylose and  
127 arabinose derived from hemicellulose) which were quantified by HPLC (Agilent,  
128 1100 series) with an Aminex HPX-87H column (300 mm  $\times$  7.8 mm, Bio-Rad,  
129 California, USA) and a refractive index detector. The operating conditions were:  
130 sample volume 20  $\mu$ L; mobile phase 0.005 M H<sub>2</sub>SO<sub>4</sub>; flow rate 0.6 mL/min; column  
131 temperature 65 °C. According to the NREL protocol, during acid hydrolysis lignin is  
132 fractionated into acid soluble and acid insoluble material. Acid-soluble lignin was  
133 measured with a UV-Vis spectrometer at 320 nm and acid-insoluble lignin  
134 gravimetrically after subtracting the ash and protein contents of the samples. The  
135 lignin content of samples is presented as the sum of acid soluble lignin and acid  
136 insoluble residue.

### 137 **2.3 Osborne fractionation of DDGS and in-process samples**

138 DDGS, wet and spent solid samples were subjected to Osborne fractionation  
139 according to the method of Lookhart and Bean (1995). Briefly, 100 mg of sample  
140 were sequentially extracted with deionised water, 0.5 M NaCl (Sigma, UK), 70%  
141 (v/v) aqueous ethanol (Sigma, UK) and 50% (v/v) 1-propanol (Merck, Germany) with  
142 1% (w/v) dithiothreitol (DTT) (Sigma, UK), in order to extract the water-soluble  
143 albumins, salt-soluble globulins, ethanol-soluble prolamins and ethanol-insoluble  
144 prolamins (as reduced subunits), respectively. A 1:10 (w/v) solids-to-liquid ratio was  
145 used for the extractions, which were performed in a thermomixer (Eppendorf, UK)  
146 with constant mixing (1400 rpm), at 60 °C for 30 min. Extractions for each sample

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

## 152 **2.4 Protein extraction**

### 153 **2.4.1 Aqueous-ethanol extraction of proteins**

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

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

#### 173 **2.4.2 Alkaline-ethanol extraction of proteins**

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

$$186 \text{ Protein content of extract (\%)} = \frac{\text{Total Kjeldahl Nitrogen} \times 5.7}{\text{Dry weight of extract}} \times 100$$

$$187 \text{ Protein yield (\%)} = \frac{\text{Protein content of extract}}{\text{Protein concentration in original sample}} \times 100$$

#### 188 **2.5 SDS-PAGE of samples and protein isolates**

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

194 and pooled together. The solid residues, free of gliadins, were incubated with 50%  
195 (v/v) 1-propanol in 0.08M Tris-HCl (pH 8.0) with 1% (v/v) p-mercaptoethanol  
196 (Sigma, UK) as reducing agent and 1.4% (v/v) 4-vinylpyridine (Sigma, UK) as  
197 alkylating agent of sulfhydryl groups, in order to extract glutenin subunits; the  
198 supernatant containing the glutenin fraction was collected by centrifugation (3,000×g  
199 for 2 min). The supernatants containing the gliadin and glutenin fractions,  
200 respectively, were diluted in sample buffer [2% (v/v) SDS, 40% (w/v) glycerol,  
201 0.02% (w/v) bromoethyl-blue in 0.08M Tris-HCl (pH=8.0)] and loaded onto a 1.0 mm  
202 4-12% Bis-Tris pre-casted gel (NuPAGE Novex, UK). Proteins were separated in an  
203 XCell Surelock™ unit (Invitrogen, UK) at constant voltage (200 V) for 35 min. Gels  
204 were washed three times with purified water, stained with SimplyBlue SafeStain  
205 buffer (Life Technologies, UK) for 1 h at room temperature and washed with distilled  
206 water to obtain a clear background. The molecular weights of the visualised bands  
207 were estimated using Novex Sharp pre-stained protein standards (Invitrogen, UK).

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

## 214 **2.6 Amino acid analysis**

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

218 neutralised and derivatised using the EZ-Faast amino acid derivatisation kit  
219 (Phenomenex, UK). The kit is based on a solid-phase extraction that binds amino  
220 acids and enables the derivatisation in aqueous solution of both the amine and  
221 carboxylic groups of amino acids at room temperature. Amino acid profiles were  
222 determined using a Gas Chromatography-Mass Spectrometry instrument (Agilent  
223 6890/5975) as described by Elmore, Koutsidis, Dodson, Mottram & Wedzicha (2005).  
224 Norvaline was used as internal standard and detected amino acids were quantified  
225 according to standard solutions supplied by the manufacturer. Methionine, cysteine  
226 and tryptophan were not detected as they were degraded by acid hydrolysis.

## 227 **2.7 Thermogravimetric analysis (TGA)**

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

## 232 **2.8 Statistical analysis**

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

236

## 237 **3. Results and discussion**

### 238 **3.1 Composition of DDGS, wet solids and spent solids**

239 The compositions of DDGS, wet solids and spent solids are shown in Table 1 as  
240 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  $\omega$ -gliadins, as well as bands with  
314 molecular weights of 40 and 30 kDa, corresponding  $\alpha$ - and  $\gamma$ -gliadins, respectively. In



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

332

### 333 **3.3 Extraction of proteins from DDGS and in-process samples**

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

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

348 The first set of extraction experiments was carried out using 70% (v/v) aqueous  
349 ethanol, in order to determine the effects of temperature and reducing agent  
350 concentration on protein extractability. The protein content of the dried extracts as  
351 determined by Kjeldahl analysis is presented in Table 2. Extraction at 50 °C resulted  
352 in low protein content, ranging between 14-32 %, in all extracts depending on the  
353 reducing agent concentration. The greatest amount of protein was present in the  
354 extracts from the wet solids (~32%) followed by spent solids (~23%) and then DDGS  
355 (~14%). Extraction at 70 °C improved significantly ( $P<0.05$ ) the protein content of all  
356 samples compared to 50 °C, with the highest being ~45% for DDGS, ~58% for wet  
357 solids and ~62% for spent solids; the optimum reducing agent concentration was in  
358 most cases 1%. At 90 °C the protein content of the extracts decreased significantly  
359 ( $P<0.05$ ) compared to that at 70 °C for all samples. In terms of the protein extraction  
360 yield (% of protein per total protein of original sample), the best extraction conditions  
361 were identified as 70 °C and 1% reducing agent, resulting in protein extraction yields  
362 of 30.1% (w/w) for DDGS, 55.3% (w/w) for wet solids and 52.1% (w/w) for spent  
363 solids.

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

386 The second set of extraction experiments was carried out using an alkaline-aqueous  
387 ethanol solution at 70 °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  $\alpha$ -,  $\gamma$ - and  $\omega$ -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  $\alpha$ -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

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

#### 440 **3.4 Composition of protein extracts and solid residues**

441 Table 3 shows the compositions of the protein extracts and their respective solid  
442 residues after ethanol extraction of DDGS, spent and wet solids samples. Very small  
443 amounts of water-soluble carbohydrates were detected in all aqueous ethanol extracts  
444 (2.4-5.1%, w/w), with the spent solids containing the smallest amount. Glucose was  
445 the major monosaccharide determined after hydrolysis, indicating the presence of  
446 starch followed by xylose and arabinose, the latter indicating the presence of soluble  
447 arabinoxylans, which are the major non-starch polysaccharides in wheat grain  
448 (Saulnier, Peneau & Thibault, 1995). On the other hand, the solid residue after  
449 extraction had a high content of water unextractable polysaccharides which was  
450 around 49% (w/w) for all samples. The monosaccharides composition (Table 3)  
451 indicated the presence of insoluble  $\beta$ -glucan, cellulose and water unextractable  
452 arabinoxylan. The protein content of the solid residues was ~4.7% for spent solids,  
453 ~7.4% for wet solids and ~11.6% for DDGS, i.e. the reverse ranking of that obtained  
454 for protein extractability.

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

460 initial protein (gliadins and glutenins) content was recovered from DDGS. This could  
461 be attributed to only partial precipitation of the low molecular weight proteins in the  
462 extraction liquid, as well as to the thermal denaturation of DDGS proteins during the  
463 drying stage. At the drying stage DDGS is subjected to intense and prolonged thermal  
464 treatment (higher than 100°C). Under these conditions, the disulphide bonds present  
465 in all wheat gluten proteins (except  $\omega$ -gliadins) may undergo rearrangements to form  
466 cross-links in highly insoluble denatured aggregates (Wang, Wei, Li, Bian & Zhao,  
467 2009; Hong et al. 2012). On the other hand, protein recoveries from wet and spent  
468 solids were noticeably higher (89-93%), reflecting the higher protein extractability of  
469 these samples. In terms of the carbohydrate mass balances, the recoveries ranged from  
470 86 to 94% for all samples. These values are very good considering that approximately  
471 5% of the material could be lost during the intermediate washing steps. The high  
472 carbohydrate contents of the solid residues after protein extraction indicate that these  
473 materials could be a potential source of non-starch polysaccharides, and if processed  
474 to oligosaccharides could provide functional ingredients (prebiotics, stabilisers,  
475 emulsifiers) for food and non-food applications.

476 Further information on the proteins present in the extracts of DDGS and wet solids  
477 was provided by thermogravimetric analysis (TGA). Degradation of the samples was  
478 carried out under nitrogen and the observed peaks are presented as derivatives of the  
479 weight loss as a function of temperature. As depicted in Fig 4, a peak was identified  
480 for both samples at around 53-60°C. This was more intense in the case of wet solids  
481 and corresponded to the loss of free and bound water. Extracts of both DDGS and wet  
482 solids exhibited a prominent broad peak in the range of 230-370°C, which was  
483 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  $\alpha$ -,  $\gamma$ -,  $\omega$ -gliadins and low molecular subunits of glutenin (Shewry,  
505 Tatham, Forde, Kreis & Mifflin, 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



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

#### 513 **4. Conclusions**

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

530

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536 functional products from DDGS” (BB/J019429/1–University of Reading;  
537 BB/J019380/1 – Rothamsted Research).

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- 663

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

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

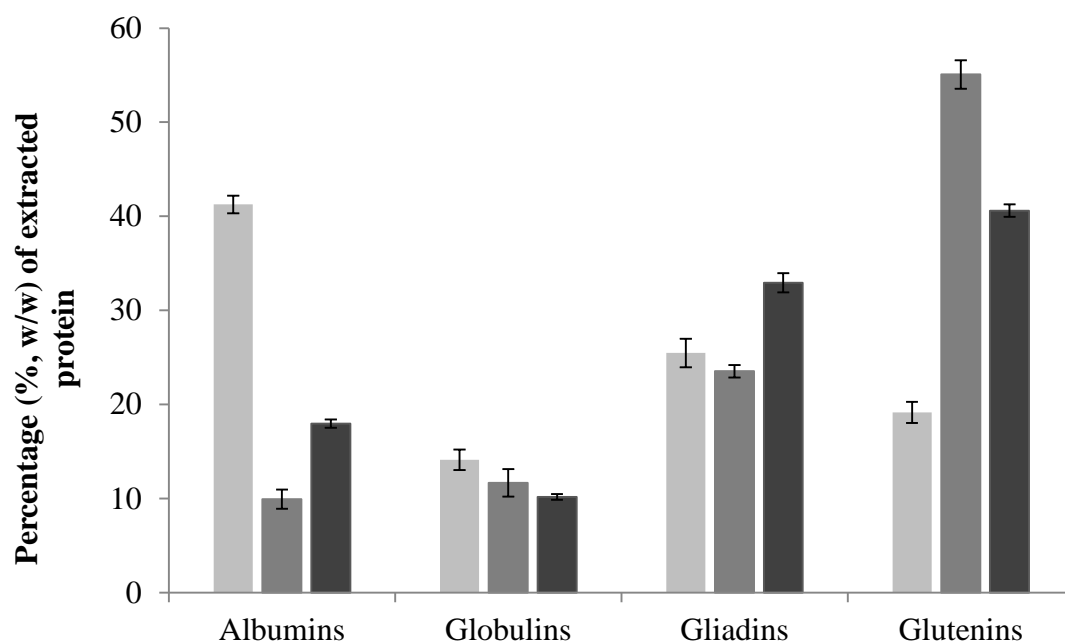
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670 Fig. 1 Protein composition of fractionated spent solids (grey), wet solids (dark grey)  
671 and DDGS (black), based on Osborne protocol

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

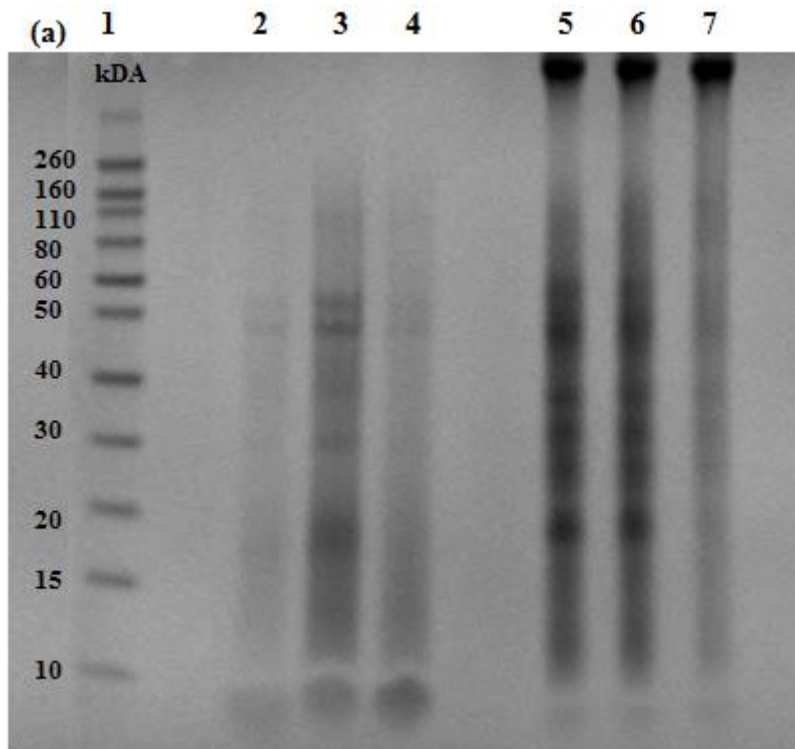
Extraction Temperature (°C)	Protein content of isolates (% w/w)					
	SMB (%, w/w)	EtOH (%, v/v)	NaOH (M)	DDGS	Wet solids	Spent solids
50	0.5			14.0 (0.8)	27.0 (1.3)	23.5 (1.2)
	1.0	70	-	14.5 (0.7)	31.7 (1.7)	22.9 (0.8)
	1.5			14.9 (0.5)	29.9 (1.7)	23.5 (0.9)
70	0.5			34.1 (3.2)	47.8 (2.9)	53.6 (2.8)
	1.0	70	-	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			38.1 (1.3)	43.7 (0.3)	42.4 (0.3)
	1.0	70	-	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		45	0.10	22.1 (0.6)	19.7 (1.7)	13.5 (1.4)
	1.0	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)

678 Data in parenthesis represent standard deviation values

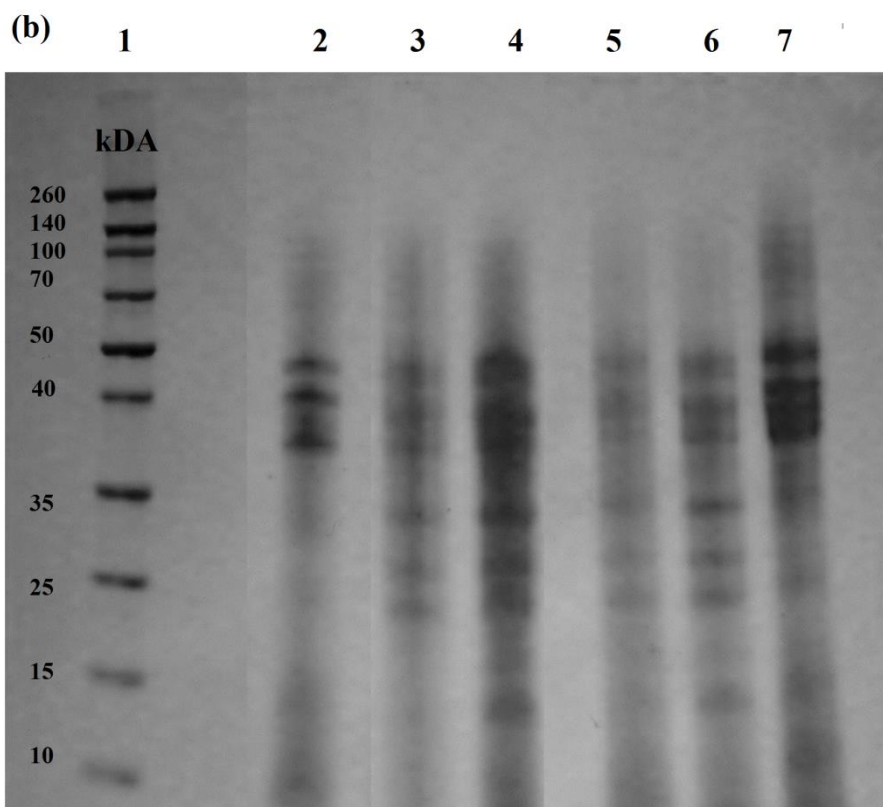
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684 Fig 2. (a) SDS-PAGE according to the protocol by Singh et al. (1991) of original  
 685 samples: Lane 1, Molecular weight marker; lanes 2-4, Gliadin proteins of: spent solids  
 686 (lane 2), wet solids (lane 3) and DDGS (lane 4), respectively; Lanes 5-7, 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 <sup>b</sup> (%, per 100 g)		
	DDGS	Wet solids	Spent solids	DDGS	Wet solids	Spent solids	DDGS	Wet solids	Spent solids
<b>Protein<sup>a</sup></b>	44.7	55.6	62.4	11.6	7.4	4.7	68.7	89.2	92.8
<b>Carbohydrates</b>	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

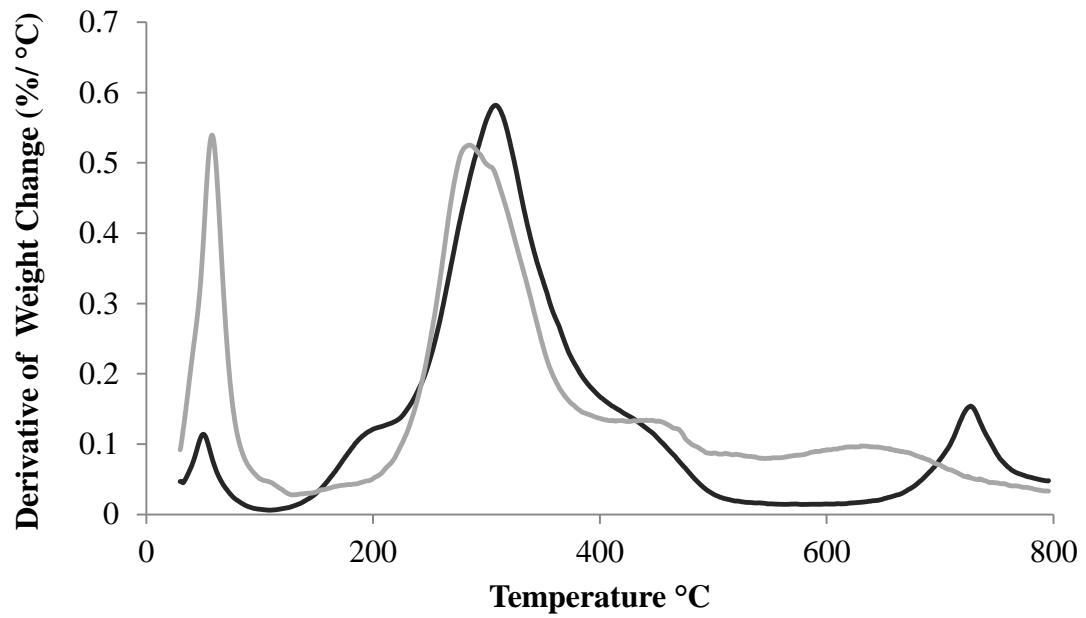
697 <sup>a</sup>: Protein content measured by Kjeldahl

698 <sup>b</sup>: 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).

700

701

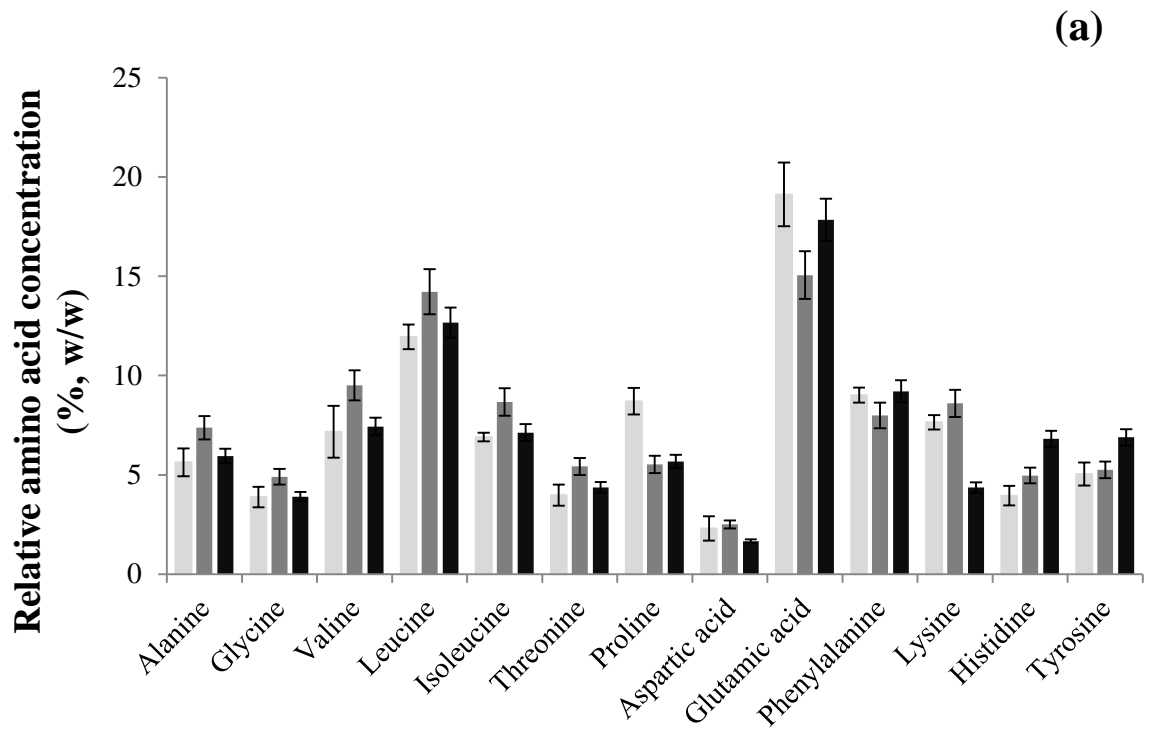
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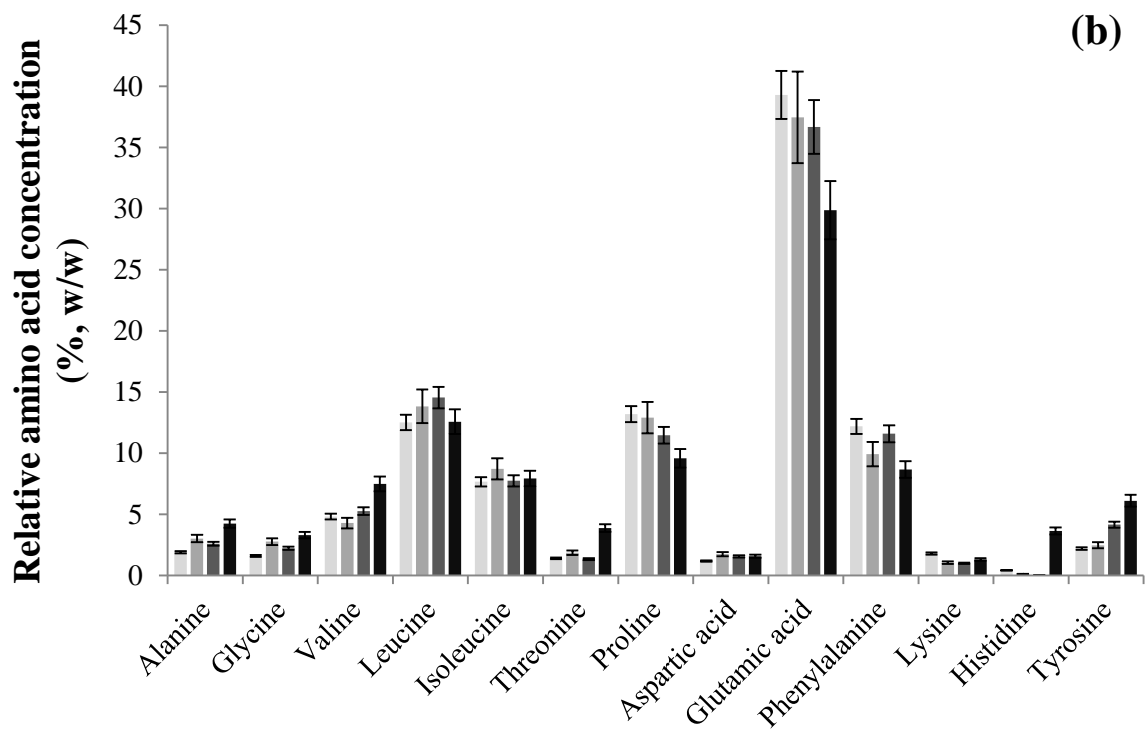
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704 Fig 4. TGA analysis of ethanol extracted proteins from DDGS (black line) and wet  
705 solids (grey line)

706



707  
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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).