



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 α - and γ -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 amylolytic 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 CO₂ 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 β -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 μ L; mobile phase 0.005 M H₂SO₄; 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 ω -gliadins, as well as bands with
314 molecular weights of 40 and 30 kDa, corresponding α - and γ -gliadins, respectively. In

315 DDGS, only bands corresponding to ω -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 α , β - and γ -gliadins,
318 whereas ω -gliadins do not contain cysteine residues and thus cannot form disulphide
319 bonds (Schofield et al. 1983). This could explain the presence of only ω -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 β -mercaptoethanol (β -
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 ω -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 α -, β - and γ -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 α -, β - and γ -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 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

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 β -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 ω -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 α -, γ -, ω -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 α - and γ -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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Table 1. Chemical composition of DDGS and in-process samples

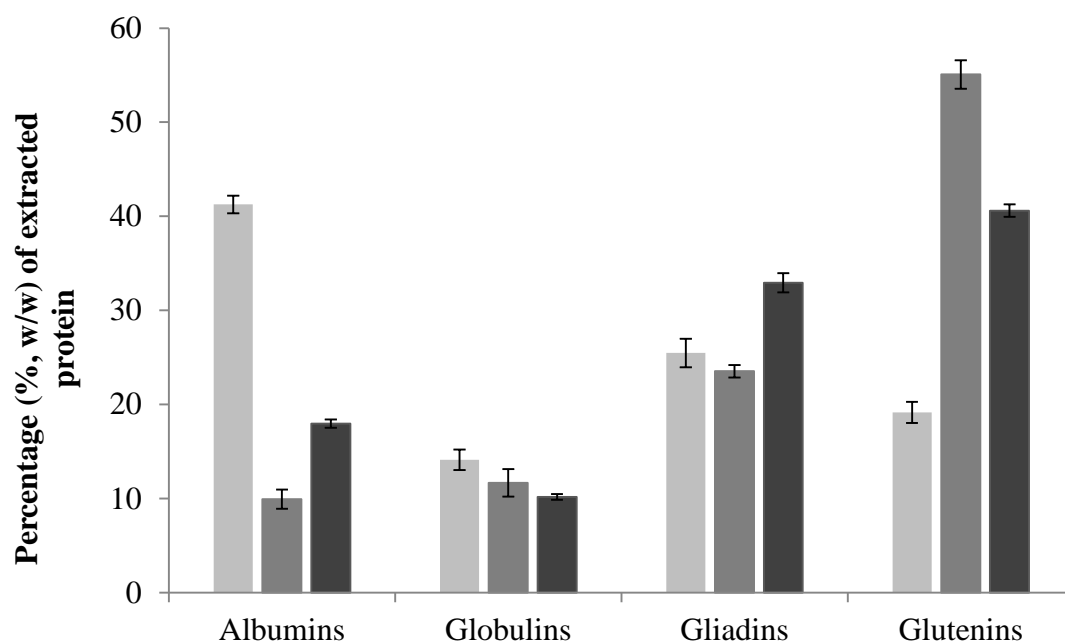
(in %, db)	DDGS	Wet solids	Spent solids
Dry matter	96.6±0.7	33.2±1.1	22.1±1.2
Crude protein	29.1±1.7	19.8±1.2	25.6±1.1
Crude fat	3.4±0.1	2.9±0.9	5.4±0.4
Cellulose and β-glucan	14.9±0.4	15.1±0.6	16.3±1.1
Starch	2.6±0.10	2.0±0.3	1.4±0.19
Hemicellulose	25.1±1.6	28.0±1.3	25.5±0.9
Xylose	(16.7±0.9)	(18.4±0.7)	(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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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

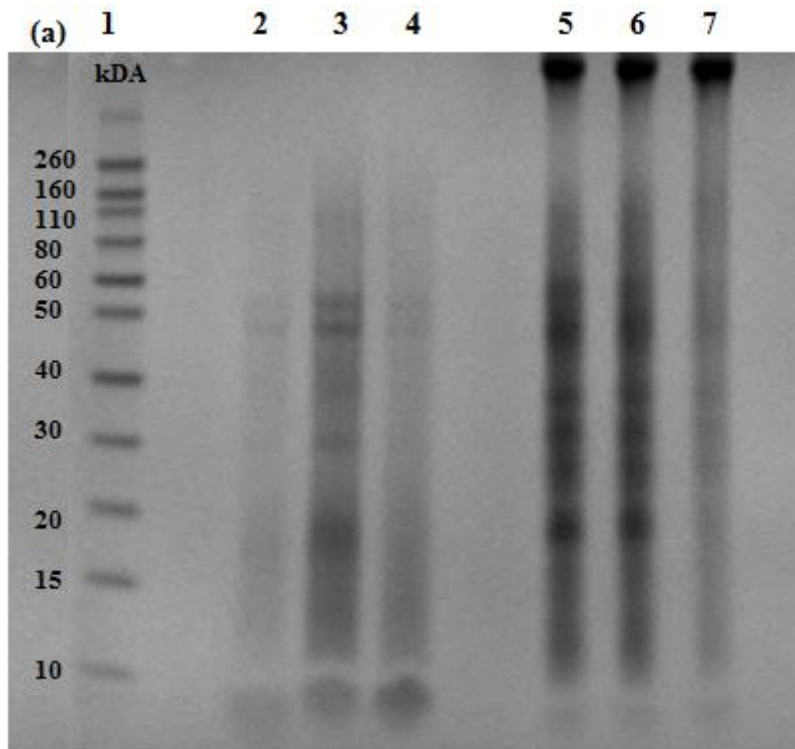
Extraction Temperature (°C)	Protein content of isolates (% w/w)					
	SMB (%, w/w)	EtOH (%, v/v)	NaOH (M)	DDGS	Wet solids	Spent solids
50	0.5			14.0 (0.8)	27.0 (1.3)	23.5 (1.2)
	1.0	70	-	14.5 (0.7)	31.7 (1.7)	22.9 (0.8)
	1.5			14.9 (0.5)	29.9 (1.7)	23.5 (0.9)
70	0.5			34.1 (3.2)	47.8 (2.9)	53.6 (2.8)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

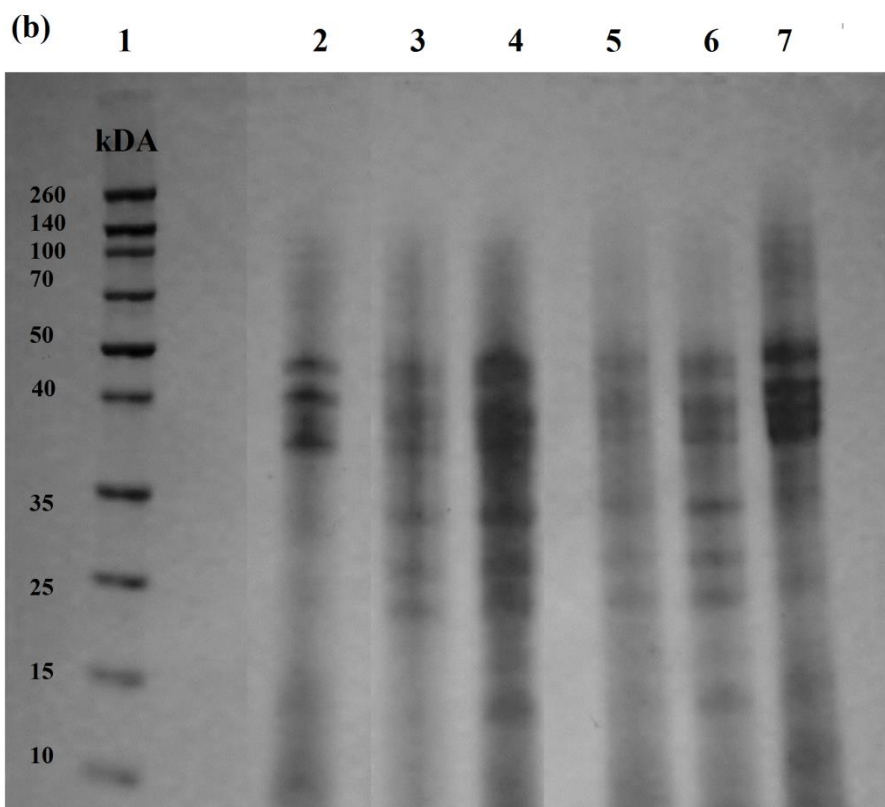
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684 Fig 2. (a) SDS-PAGE according to the protocol by Singh et al. (1991) of original
 685 samples: Lane 1, Molecular weight marker; lanes 2-4, Gliadin proteins of: spent solids
 686 (lane 2), wet solids (lane 3) and DDGS (lane 4), respectively; Lanes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

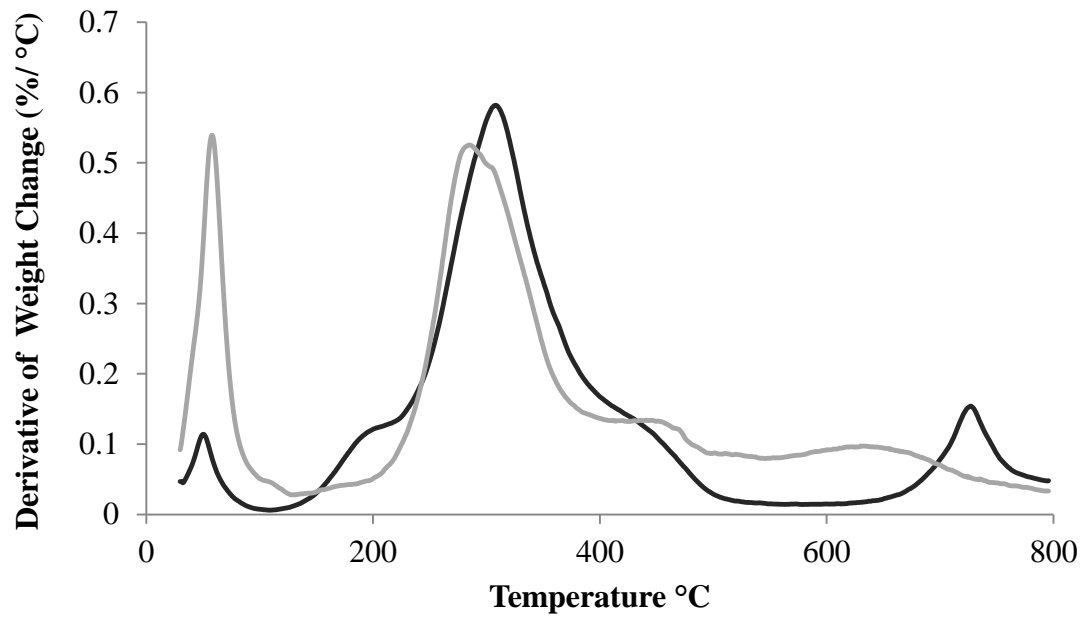
697 ^a: Protein content measured by Kjeldahl

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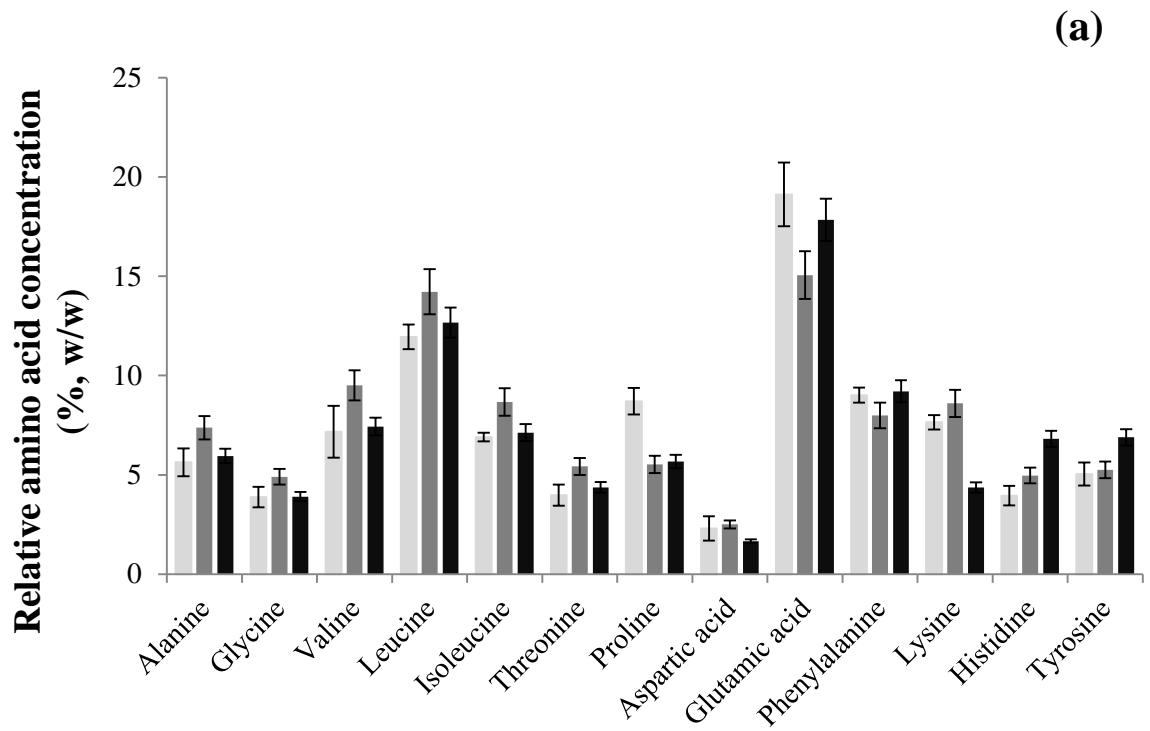
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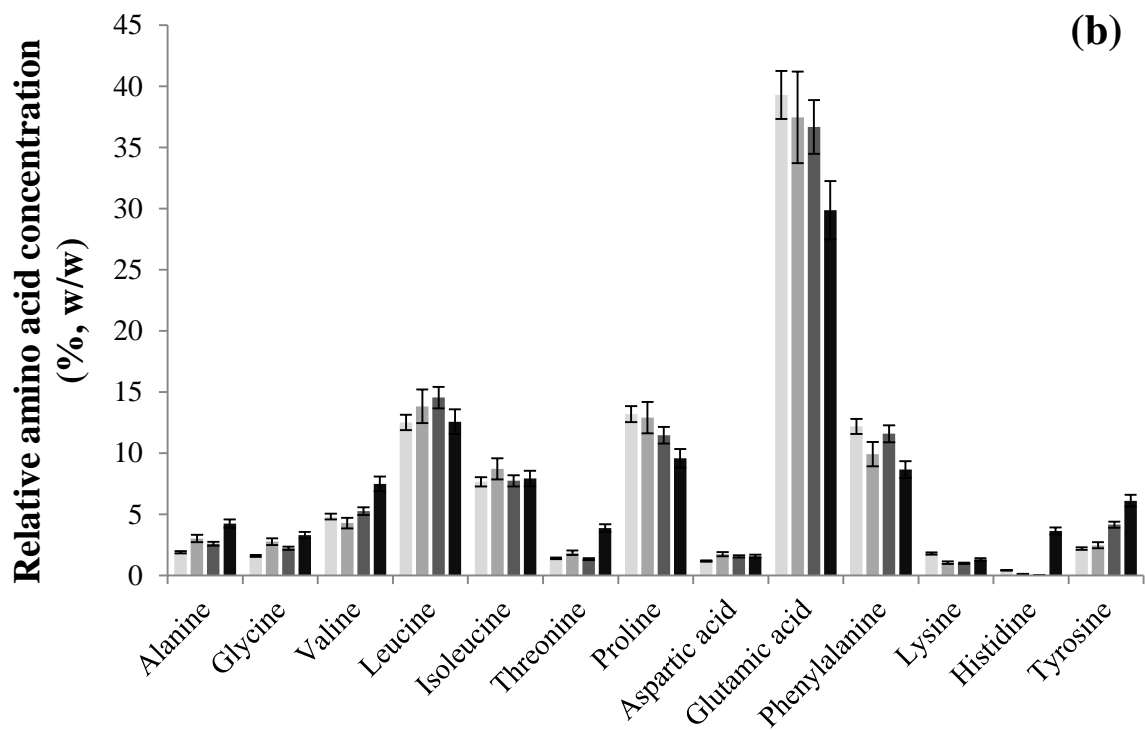
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704 Fig 4. TGA analysis of ethanol extracted proteins from DDGS (black line) and wet
705 solids (grey line)

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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).