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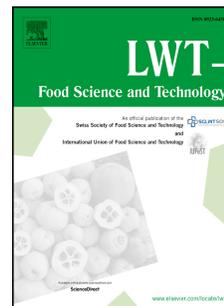
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# Changes in protein size distribution during wheat flour cake processing

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**Abbreviations:** BCS: sodium bicarbonate, SAPP: sodium acid pyrophosphate, SDS: Sodium Dodecyl Sulfate, IAM: iodoacetamide, DTE: dithioerythritol

25 **ABSTRACT:**

26 Aggregation of egg and wheat proteins during cake mixing and baking was monitored by SE-  
27 HPLC after sequential extraction of dough and baked cakes in SDS-buffer first and then in  
28 SDS/DTE buffer. Three cake recipes were compared, including either only egg, only flour, or  
29 both flour and egg proteins. Dough mixing did not result in any changes in protein solubility or size  
30 distribution. Baking promoted protein aggregation and quickly resulted in the solubility loss of all  
31 proteins within the first extracting solvent with the exception of wheat omega gliadins. Upon baking  
32 similar kinetics of proteins solubility loss in SDS-buffer were observed regardless of cake recipes.  
33 Extraction of the remaining SDS-insoluble proteins with SDS/DTE buffer allowed total protein recovery  
34 but only in the case of cakes made on the basis of only flour. For cakes including eggs and despite the  
35 presence of DTE a disulfide reducing agent, very large polymers were release into solution, contrarily to  
36 the only flour cakes where only small polypeptides (>70 000 g/mol) were mostly recovered. Protein  
37 sequential extraction combined with SE-HPLC analysis highlighted the critical role of egg proteins in  
38 triggering wheat and egg proteins complexation into SDS-insoluble aggregates stabilized through  
39 disulfide but also non-reducible covalent bonds.

40

41

42 **Keywords:** soft cake, baking, protein thermal aggregation, protein SE-HPLC, iso-peptide bonds.

43

44

## 45 1. INTRODUCTION

46 Like bread, cakes are among the cereal products mainly composed of wheat flour (30-40% dry weight),  
47 and characterized by a soft and elastic alveolar crumb. The setting of the alveolar structure takes place  
48 during baking, as the viscous and foamy cake batter transforms into an expanded and porous soft gel.

49 Although it has long since been established that both proteins and starch play a major role in cereal  
50 product texture (Donelson and Wilson, 1960), their respective roles still require investigation to fully  
51 understand the mechanisms involved.

52 In bread-making, proteins only come from wheat flour, and interact during mixing, resting and baking to  
53 form a gluten network. Mixing flour/water blends allows for protein hydration while mechanical shear  
54 enables gluten network development by stretching and distributing the gluten evenly within the dough.  
55 Gluten network is mainly responsible for dough viscoelasticity, gas retention capacity and, after cooling,  
56 crumb elasticity. Dough mixing energy input, as well as the quality of the flour proteins, impacts the  
57 structure of the gluten network and the subsequent physical and chemical properties of the dough.  
58 Several authors have shown that gluten proteins, *i.e.* gliadins and glutenins, are responsible for the  
59 bread-making quality of flour. Since gluten proteins represent 80—85% of the total flour proteins, the  
60 flour protein content is most often considered as the first wheat flour quality criterion in bread-making.  
61 However, the glutenin macropolymer content would be a better parameter to predict the bread-making  
62 quality of flour (Weegels et al., 1996).

63 In cake batter, gluten proteins are more diluted into the matrix, and the mixing step is far shorter. Thus,  
64 it is still unclear if a gluten network is formed or even initiated in cake batter during mixing. In particular  
65 the impact of cake batter mixing on flour protein size distribution remains undocumented, while for  
66 bread-making a strong depolymerization effect of mixing is well established (Weegels et al., 1997, 1996).  
67 Moreover, in cake-making, wheat flour is not the only ingredient providing proteins: eggs also matter  
68 and represent from 30 to 50% of the total protein content of pound cakes. Egg proteins are known to be  
69 thermosensitive, and thus are supposed to play a major role in cake texture setting. In 1976, Shepherd &  
70 Yoell proposed a model cake structure in which starch granules play the role of bricks, and egg proteins  
71 the role of mortar. This mortar would be a composite, involving ovalbumin as a flexible cementing agent,  
72 and ovomucin as a non-denaturable fibrous protein, but its structure remains unclear (Donovan, 1977).  
73 More recently, it has been shown that cake cell walls are made of a combination of a protein network  
74 involving both egg and gluten proteins, built upon baking, and a starch gel, set during cooling (Deleu et  
75 al., 2015; Wilderjans et al., 2010). However, the specific roles of egg proteins and gluten in cake batter  
76 and then crumb structure are still currently unclear.

77  
78 The SE-HPLC technique, combined to protein SDS extraction, was successfully developed by Singh,  
79 Donovan, Batey, & MacRitchie (1990) for wheat flour protein characterization. Morel et al. (2000),  
80 improved the technique's reproducibility and proposed to divide the SE-HPLC profile of flour SDS soluble  
81 proteins into 5 fractions, which are respectively large-size glutenin macropolymers (F1 : 800,000-  
82 1,700,000 g/mol), small glutenin macropolymers (F2 : 800,000-120,00 g/mol),  $\omega$  gliadin (F3), and then  
83  $\gamma$  and  $\alpha/\beta$  gliadins (F4), and latterly salt-water-soluble wheat proteins (Morel et al., 2000). In 1999,  
84 Huebner, Bietz, Nelsen, Bains, & Finney used SE-HPLC to evaluate the cookie-making quality of several  
85 soft wheat flours. They assumed that, despite finding no strong correlation between flour protein  
86 composition and cookie quality, soft flours with higher contents of glutenin macropolymers give cookies  
87 an overall better quality. As observed for bread, glutenin macropolymers may play a role in biscuit  
88 quality. In 2008, Wilderjans, Pareyt, Goesaert, Brijs, & Delcour used SE-HPLC to compare the protein  
89 extractabilities of batter and cake according to their gluten contents. They found that when more gluten  
90 is added to cake batter, protein SDS extractability decreases further after baking while cake quality  
91 increases. Gluten may therefore be presumed to play a key role in cake texture.

92  
93 In light of these investigations, SE-HPLC appears to be a promising tool for monitoring protein changes  
94 (polymerization and aggregation) during soft cake processing steps (mixing and baking) and therefore, a  
95 better understanding of the specific roles of flour and egg proteins in cake structure building. The aim of  
96 this study was to validate SE-HPLC analysis of cake proteins as an effective tool to study the interactions  
97 between egg and flour proteins during cake batter mixing and baking.

98

## 99 **2. MATERIALS AND METHOD**

100

### 101 **2.1. Materials**

102 Apache bread wheat flour (13.5% moisture content, 8.6% protein content d.b., 0.42% ash content),  
103 pasteurized eggs (10.6% protein w.b., 76% water content), emulsifiers (containing 9% of milk proteins,  
104 d.b.), sucrose, glucose syrup (19.7% moisture content), rapeseed oil, glycerol (0.5% moisture content),  
105 salt and raising agents were all provided by Mondelēz International. Native wheat starch (12.2%  
106 moisture content, 0.4% gluten) was supplied by Cargill, USA. Moisture contents were determined  
107 according to AACC Approved Method 44-19 (AACC, 1983). The protein contents of flour, eggs and  
108 emulsifiers were determined from triplicate measurements using Kjeldhal procedure. Nitrogen-to-

109 protein conversion factors of 5.7 for wheat flour samples and 6.25 for egg and emulsifier samples were  
110 applied.

111

## 112 **2.2. Cake batter preparation**

113 The standard recipe was prepared as follows: 35.0% of wheat flour, 13.5% of eggs, 8.0% of rapeseed oil,  
114 10.5% of additional water, 12% of sucrose, 12% of glucose syrup, 6% of glycerin, 1.6% of emulsifiers,  
115 0.2% of salt and 1.2% of raising agents. The egg-free cake batter was prepared with the same ingredients  
116 except that eggs were replaced by their water equivalents in order to keep constant the batter moisture  
117 content. The gluten-free recipe was prepared by replacing the 350g of flour by 11g of water and 312g of  
118 native wheat starch, in order to keep constant both starch and water contents in the gluten-free cake  
119 batter. All the cake batters were prepared at 20°C using a planetary Hobart N50 5-Quart mixer with a flat  
120 beater paddle (Hobart Corporation, Troy, OH, USA). Powders were homogenized before adding all  
121 soluble ingredients except rapeseed oil. Mixing was started for 2.5min at medium speed, before adding  
122 the oil and mixing one minute more.

123

## 124 **2.3. Cake batter baking kinetics**

125 After a resting time of 30min, the cake batter was poured into 15 metallic pans of 80x45 mm (40g of  
126 batter each) and baked at 180°C for 25min. The batters' core temperatures all increased from 20°C to  
127 90°C in approximately 10min, then remained at 90°C due to their high internal moisture contents. The  
128 crumb temperature increased a little faster in the case of the gluten-free batter (90°C after 7.5min of  
129 baking), probably due to the lower batter viscosity and the lower air bubble content. Every 5min, 3  
130 cakes were removed from the oven, one for temperature measurements and two for HPLC analysis  
131 sampling. In order to limit thermal fluctuation during baking, the number of sampling (and so the oven  
132 door opening) was limited to five. Two different batter batches were prepared in order to obtain 3X10  
133 samples, covering a 25 min baking period

134

## 135 **2.4. SE-HPLC characterization**

136 The batter protein extraction procedure was adapted from Morel et al. (2000). Eighty milligrams of  
137 freeze-dried and grinded cake batter were dispersed for 80 min at 20°C, in 10ml of 1% SDS-phosphate  
138 buffer (pH 6.8) containing 5mM iodo-acetic amide (IAM). After centrifugation (30 min, 18 000rpm) the  
139 supernatant (SDS-soluble protein extracts) was collected and stored at -20°C before SE-HPLC analysis.  
140 The pellet was re-suspended in 5ml of 1% SDS-phosphate buffer (pH 6.8) including 40mM DTE for 1h at

141 60°C and sonicated for 5 minutes (30% power). The supernatant (DTE-soluble protein extracts) were  
142 collected after centrifugation and stored as above. SE-HPLC analysis of the samples (20µL) was  
143 performed on an Alliance system (Waters) using a tandem of TSK G4000-SWXL (7.8 × 300mm) and TSK  
144 SWXL (6 × 40mm) columns, both from Tosoh Biosep. Elution was performed at 0.7 mL/min with 0.1%  
145 SDS, 0.1M sodium phosphate buffer (pH 6.8) and 214 nm was used for protein detection. The total  
146 protein extraction yield was estimated from the sum of the area of the SDS-soluble and the DTE-soluble  
147 chromatography profiles after correction for the difference in solid to solvent ratios. The un-extractable  
148 protein fraction remaining in the last pellet was estimated in reference to the results obtained for the  
149 mixed batter for which the extraction yield was set to 100%. Cake ingredients (flour, egg and emulsifier)  
150 were similarly analyzed, except that solid to solvent ratios corresponding to their specific contribution to  
151 the total dry mass of the standard batter recipe were used for extraction. The apparent molecular  
152 weights of the major fractions were estimated by calibrating the column with various protein standards  
153 according to Morel et al. (2000).

### 156 3. RESULTS AND DISCUSSION

#### 158 3.1. SE-HPLC protein profiles of cake ingredients

159 Figure 1a shows the SE-HPLC profiles of the SDS-soluble proteins from flour. The chromatogram is  
160 typically divided into 5 fractions, namely F1 and F2 that include glutenin macropolymers, F3 and F4 that  
161 include mostly gliadins and fraction F5 that gathers small water-soluble proteins such as albumin and  
162 globulin (Morel et al. , 2000). The remaining proteins were then solubilized with a buffer including SDS  
163 and DTE in order to reduce the intermolecular disulfide bonds. SE-HPLC profiles of this second extract  
164 shows 3 mains peaks (Figure 1.b). The first one (F'3) can be assimilated to high-molecular-weight  
165 glutenin subunits (HMW-GS) and the second one (F'4) to the low-molecular-weight glutenin subunits  
166 (LMW-GS). Fraction F'5 would include water-soluble proteins, either chemically or physically linked to  
167 the SDS-insoluble glutenin polymers network before its reduction by DTE.

168 The SDS-soluble whole egg protein chromatogram (Figure 1.c) presents 7 peaks but was arbitrarily  
169 divided into the same 5 fractions used above, allowing for an estimate of the relative quantitative  
170 contribution of egg and wheat proteins in the standard cake recipe, within the same molecular weight  
171 ranges. The nature of the egg proteins as deduced from their molecular weight is presented in Table1.

172 As shown in figure 1.e, emulsifier mostly includes water-soluble whey proteins and contributes very  
173 faintly to the total protein profile of the recipe.

174

### 175 **3.2. SE-HPLC protein profiles of cake batter**

176 Experimental profiles of the first and second sequential protein extracts of standard batter and each  
177 ingredient are represented on figure 2a,b. Theoretical first and second SE-HPLC profiles were obtained  
178 by summing the profiles of the ingredients containing proteins (Figure 1).

179 For both SDS-soluble and SDS-insoluble extracts, the real chromatogram superimposed with the  
180 theoretical one, except for the exclusion peaks (F1 and F'1, eluting at 8.5 min). Formation of some  
181 protein aggregates may have occurred during the freeze-drying step that was used to prepare dry  
182 extracts of batter and egg but omitted for flour and emulsifier.

183 Since the cake batter SE-HPLC profile coincided with the sum of the SE-HPLC profiles of its protein-  
184 contributing ingredients, it can be deduced that neither wheat nor egg proteins underwent  
185 polymerization or depolymerization during the mixing step. No covalent bond formation or rupturing  
186 occurred during mixing. In addition, no SE-HPLC profile change occurred during batter resting prior to  
187 baking (results not shown). During batter mixing and resting, glutenin macropolymers initially contained  
188 in flour remained unchanged in terms of molecular size distribution in contrast to bread dough  
189 (Mecham et al., 1962; Weegels et al., 1997). The lower mixing energy input (lower mixing speed, time,  
190 and dough viscosity compared to bread dough) and the fact that gluten proteins are far more diluted in  
191 cake batter may account for this lack of significant effect.

192

### 193 **3.3. Evolution of cake batter protein profile during the baking step**

194

#### 195 **3.3.1. Evolution of cake batter protein SDS extractability during baking.**

196 Proteins from egg-free, flour-free and standard batters samples collected during baking were  
197 sequentially extracted and analyzed by SE-HPLC. Sequential extraction enables protein to be classified  
198 into SDS-soluble and DTE-soluble proteins while the last pellet gathers the SDS-unextractable proteins  
199 (Fi). Figure 3 presents the evolutions of these three protein fractions during baking for the three cake  
200 recipes.

201 SDS-extractable proteins follow a three-phase evolution during baking. First, from room temperature up  
202 to 60–70°C, whatever the batter formula, the total amounts of SDS-soluble and SDS-insoluble proteins

203 remain stable. Then, between 60–70°C and 90°C, SDS-soluble protein content drops, while both SDS-  
204 insoluble and unextractable protein contents increase. Finally, once the baking temperature exceeds  
205 90°C, the SDS-soluble protein content remains stable.

206 Wilderjans, Pareyt, Goesaert, Brijs, & Delcour (2008) also found that cake batter baking resulted in loss  
207 of protein extractability in SDS buffer. They assumed that in cake batter, thermal aggregation starts  
208 between 60 and 70°C and achieved as temperature exceeds 90°C.

209 Many authors reported the decrease in protein SDS extractability of wheat and egg proteins during  
210 thermal treatments. The decrease is attributed to protein unfolding, exposure of hydrophobic zones and  
211 formation of covalent bonds, mostly disulfide bonds (Ma and Holme, 1982; Singh and MacRitchie, 2004;  
212 Singh, 2005; Van der Plancken et al., 2006). Adding DTE to the extracting solvent allowed almost total  
213 protein extraction, which is consistent with the prominent role play by disulfide bonds during baking.  
214 Hence in the case of standard batter (Figure 3.a and 3.c), whatever the baking time, the un-extractable  
215 protein fraction remains small, and almost all the aggregated protein can be recovered after sonication,  
216 disulfide bond reduction and protein denaturation with SDS.

217 In the case of the gluten-free cake recipe (Figure 3.b) even after both sonication and DTE reduction,  
218 proteins become less and less extractable as baking time increases while the unextractable protein  
219 fraction increases. It may indicate that, when cake batter contains only egg proteins, disulfide bonds are  
220 not the only covalent bonds at play during baking, and/or that the interactions are so tight that the  
221 reducing conditions do not allow the formation of small SDS-soluble aggregates. Baking of the standard  
222 and gluten-free batters did not lead to similar accumulation of unextractable proteins suggesting the  
223 formation of flour and egg proteins mixed aggregates.

224

### 225 3.3.2. Proteins involved in cake protein aggregates.

226 Figure 4.a shows the SE-HPLC profiles of the SDS-soluble proteins from egg-free cakes sampled at  
227 different baking times. After 12.5min of baking, no more change in SDS-soluble profiles occurs and only  
228 traces of protein from F3, F4 and F5 fractions remain. All fractions drop during baking, but at different  
229 extents and rates (Fig. 4b). Above 60–70°C, fraction F2 rapidly decrease and disappear above 90°C.  
230 Fraction F1 starts to decrease above 80°C, and totally disappears above 92°C. Fractions F3 and F5  
231 decrease between 66°C and 92°C but do not completely disappear. Fraction F4 decrease is slower,  
232 incomplete as well, and ends later than the other fractions do. These results are consistent with those  
233 reported in the literature for heated hydrated gluten (Schofield et al., 1983; Singh and MacRitchie, 2004).

234 Glutenin polymers (F1+F2) are found to be the first to aggregate while gliadin (F4), except omega-gliadin  
235 (F3), reacts at higher temperature (>90°C). Omega-gliadins which are devoid of cysteine residues remain  
236 SDS-soluble even after prolonged heating.

237 Slightly higher aggregation temperatures was observed for egg-free batter compared to heat treated wet  
238 gluten. It is likely that flour dilution with large amounts of sugar and water lower protein reactivity in  
239 cake batter (Donovan, 1977). Although Schofield, Bottomley, Timms, & Booth (1983) did not observe  
240 globulin/albumin aggregation during heating of wet gluten, Rosell et al. (2013) found that albumin and  
241 globulin partially co-aggregate with gluten proteins in flour dough during baking, which is consistent with  
242 the current results.

243 Results obtained for the gluten-free cake are showed in figures 4.c and 4.d. The protein signal comes  
244 from flour and egg proteins. The low density lipoprotein (LDL) amounts for more than 50% of the total  
245 egg proteins followed by ovalbumin (15%), high density lipoprotein (HDL, 12.5%) and livetin (7.5%).  
246 Phosvitin, ovotransferin and ovomucoid amount roughly for 3% while lysozyme and ovomucin contribute  
247 for less than 1% of total egg proteins.

248 All protein fractions decrease during baking, except fraction F1 which shows a transient increase around  
249 65°C. Fraction F2, which may correspond to both yolk high density lipoprotein (HDL) and low density  
250 lipoprotein (LDL), disappears in two steps; the LDL peak starts to decrease between 65°C and 83°C while  
251 the HDL peak starts to decrease above 83°C. Both totally disappear above 90°C. Ovotransferrin (F3),  
252 starts to decrease between 65°C and 83°C and totally disappears above 90°C. Fraction F4, which  
253 probably corresponds to ovalbumin, ovoglobulin, livetin and ovomucoid, disappears in two steps; first  
254 the ovalbumin-ovoglobulin-livetin peak rapidly decreases when temperature reaches 65°C, as observed  
255 for F3, while the ovomucoid peak remains stable up to 83°C before totally disappearing once  
256 temperature exceeds 90°C. F1, which corresponds to the larger soluble egg proteins such as ovomucin,  
257 acts the same way, being stable up to 83°C before totally disappearing beyond 90°C. F5, which  
258 corresponds to the smaller egg proteins such as lysozyme, only changes slightly. For all fractions, after  
259 15min of baking (*i.e.* 8 min spent at 90°C), no more change in SDS-soluble profiles was observed.

260 Although, to our knowledge, no aggregation kinetics study was made on whole eggs, these results are  
261 consistent with those found in the literature on, respectively, egg yolk and albumen: Donovan (1977),  
262 found that egg albumen aggregation is mainly driven by ovotransferrin insolubilization first and, later,  
263 ovalbumin. More recently, Kiosseoglou & Paraskevopoulou (2005) have reported that, in yolk, LDL and  
264 livetin are more thermosensitive than HDL.

265 Figures 4e and 4f show the results obtained for the standard cake batter. As before all protein fractions  
266 tend to disappear during baking, but not at the same rate. Fraction F2 rapidly decreases above 60°C to  
267 totally disappear beyond 90°C. Fractions F3, F4 and F5 severely drop between 60 and 90°C as well but do  
268 not disappear at the end of baking. Fraction F1 decreases later, once temperature rises above 81°C, and  
269 a few SDS-soluble proteins remain present after baking. Above 90°C, all remaining fractions do not  
270 evolve or drop slightly. After baking, the SDS-soluble protein extract mostly includes fractions F3, F4 and  
271 F5, as observed for the egg-free recipe.

272 Whatever the recipe, most of the changes in protein SDS extractability occur between 65 and 90°C. In  
273 the case of the standard cake, protein SDS solubility might start slightly earlier, as soon as the  
274 temperature reaches 60°C. The early stages of cake protein aggregation mostly involved glutenin,  
275 ovalbumin, ovotransferrin, LDL, livetin and lysozyme. Gliadin, HDL and ovomucin then aggregated as  
276 well. Once the temperature reaches 90°C, a comparison of the baking profiles of standard cake batter  
277 formula and egg-free batter formula (Figures 4.a and 4.e) shows that the SDS-soluble protein profiles are  
278 quite similar. Regardless of the presence or absence of egg proteins, the same flour proteins, in the same  
279 temperature/time ranges will aggregate.

280

### 281 3.3.3. Protein aggregate size evolutions during baking.

282 Figure 5 shows the SE-HPLC SDS-insoluble protein profiles (second extract in SDS/DTE) after different  
283 baking times for the egg-free (a), gluten-free (c) and standard (e) cake recipes. Evolutions of the SE-HPLC  
284 fractions are represented as well (figures 5b, 5d, and 5f). Areas numbered from F'1 to F'5 consist in the  
285 DTE-reduced protein fractions as defined in section 3.1, and Fi represents unextractable proteins. For  
286 sake of clarity and since both F'1 and F'2 fractions include covalently linked protein species in contrast  
287 with F'3, F'4 and F'5 which include the constitutive egg or gluten protein monomers, their evolutions are  
288 grouped in a F'1+F'2 curve.

289 Figure 5 reveals strong differences between the three different recipes in contrast with what was  
290 observed for the SDS-soluble proteins (Figure 4). After extraction with DTE and sonication, the SDS-  
291 insoluble proteins from egg-free samples are at first brought into solution in the form of monomeric  
292 polypeptides: HMW-glutenin subunits (F'3), LMW-glutenin subunits and gliadin (F'4) and smaller  
293 polypeptides (see section 3.1) (Figure 5.a). Nevertheless, between 12 to 17 min baking and while the  
294 majority of the SDS-soluble proteins have been insolubilized (Figure 4b), the SE-HPLC profile continues to  
295 evolve before stabilizing. Accumulation of large protein species (F'1+F'2) occur at the expense of  
296 monomeric species from fractions F'4 and F'5 (Figure 5b). This is consistent with the multi-steps

297 aggregation mechanism of gluten protein postulated by several authors (Weegels et al., 1997; Singh &  
298 MacRitchie, 2000). First, heating would induce the formation of disulfide bonds by oxidation of thiol  
299 groups, mostly between glutenin polymers, resulting in the formation of SDS-insoluble aggregates that  
300 could entrap some gliadin. Then gliadin, except  $\omega$ -gliadin which have no cysteine residue, will unfold and  
301 form disulfide bonds through sulfhydryl-disulphide exchanges with the firstly formed SDS-insoluble  
302 glutenin aggregates (Domenek et al., 2002). Aggregates grow in size since the number of disulfide bonds  
303 stabilizing their structure increases. These highly crosslinked aggregates would resist more to the  
304 disrupting effect of DTE and sonication leading to an increase of the (F'1+ F'2) fraction.

305 Egg proteins behave differently (Figures 5.c, 5d). In the very early stage of baking, *i.e.* from 65 to 80°C,  
306 monomeric polypeptides (F'3, F'4, F'5) are recovered, indicating that disulfide bond formation is at first  
307 involved in egg protein thermal aggregation (Figures 4c, 4d). This is consistent with the literature on both  
308 egg yolk and albumen (Kiosseoglou and Paraskevopoulou, 2005; Van der Plancken et al., 2006). Since  
309 ovalbumin is the only egg protein to present free sulfhydryl groups, but not the only one to be  
310 insolubilized in this temperature range (see section 3.2), it can be assumed that sulfhydryl/disulfide bond  
311 exchanges occur and mixed egg proteins aggregates are formed. Then, above 80°C, large DTE-resistant  
312 aggregates accumulates (F'1 + F'2), at the expense of the monomeric species (F'3, F'4, F'5). Further  
313 baking leads to the drop of these large aggregates while the amount of unextractable protein (Fi) sharply  
314 increases. This proves the formation of DTE and sonication resistant aggregates connected through  
315 covalent bonds possibly of iso-peptide type (Mohammadi Nafchi et al., 2013). Indeed, since the recipe  
316 contains glucose syrup and egg-lysine-rich proteins, Maillard reaction can occur to form cross-links which  
317 will not be broken in presence of either denaturant or reducing agents. It is noteworthy to underline that  
318 the longer the baking the more densely cross-linked the protein aggregates will be; contrarily to what  
319 was observed for egg-free recipe where an equilibrium situation was observed after 17 min baking.

320 As for the egg-free formula, DTE-soluble protein profiles of the uncooked standard cake samples (Figures  
321 5.e and 5f) contain three main peaks (F'3, F'4 and F'5), which likely derive from flour proteins, and a  
322 small exclusion peak which can be attributed to egg protein aggregates (F'1). During the early stages of  
323 baking and as observed for the previous recipes, F'3, F'4 and F'5 increase indicating that the first formed  
324 SDS-insoluble protein aggregates can be readily disrupted by DTE and sonication into the constitutive egg  
325 and flour protein monomers. Then above 90°C, F'4 (mostly ovalbumin, gliadin and LMW-glutenin) and to  
326 a smaller extent F'3 (mostly HMW-glutenin) and F'5 decrease, at the benefit of F'1 + F'2 (Figure 5f). The  
327 SDS-insoluble protein aggregates become more and more resistant to the disrupting action of DTE and  
328 sonication. However, contrarily to the gluten-free recipe, the amount of unextractable protein (Fi)

329 remained low. This would indicate that less iso-peptide bonds takes place between the egg proteins  
330 when flour proteins are also present. Contrarily to the egg-free recipe, F'1 + F'2 keeps growing all along  
331 baking, while accumulation of unextractable proteins remains even below the range observed for the  
332 egg-free samples. All these results support a mixed reactivity between wheat and egg proteins  
333 aggregates above 90°C. During standard cake baking, protein aggregation would proceed in two steps.  
334 From 65°C to 90°C, egg and flour protein would form homo-aggregates mostly stabilized by inter-chain  
335 disulfide bonds. Egg proteins would then support crosslinking between both types of proteins aggregates  
336 through the formation of iso-peptide bonds. In the full cake recipe, egg proteins will contribute to  
337 reinforce the protein connectivity and are likely to impact the crumb mechanical strength.

338

#### 339 **4. CONCLUSION**

340 Following the changes in protein solubility and size distribution during cake mixing and baking was made  
341 possible by using SE-HPLC and sequential protein extraction. Flour and egg proteins interactions during  
342 cake processing were deduced from the study of standard, egg-free and gluten-free recipes. It was  
343 shown that mixing did not result in gluten network development contrarily to what is commonly  
344 observed during bread dough mixing. Baking induced protein aggregation but gluten and egg proteins  
345 did not react in the same way when present separately or combined. Below 90°C, egg and wheat  
346 proteins aggregated mainly through a thiol/disulfide exchange mechanism but did not seem to interact  
347 together. The formed SDS-insoluble protein aggregates were brought into solution and analyzed by SE-  
348 HPLC after their disruption by sonication in the presence of SDS and DTE. By this way it was possible to  
349 demonstrate that the SDS-insoluble aggregates structure continued to evolve all along baking. Hence,  
350 the egg SDS-insoluble aggregates from the gluten-free recipe became less and less sensitive to DTE and  
351 sonication, indicating the formation of permanent iso-peptide bonds between egg proteins. In the same  
352 time/temperature baking range, the gluten SDS-insoluble aggregates from egg-free recipe remained  
353 sensitive to DTE suggesting that covalent disulfide bonds were mainly accountable for protein  
354 aggregation all along cake baking. An intermediate situation was observed for the standard recipe  
355 indicating that above 90°C egg and wheat protein involved in separated aggregates started to interact  
356 and formed mixed and DTE-resistant aggregates. In cake recipe, egg proteins would contribute to  
357 increase the crosslinking density of the gluten protein aggregates all along the baking stage.

358

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362

363

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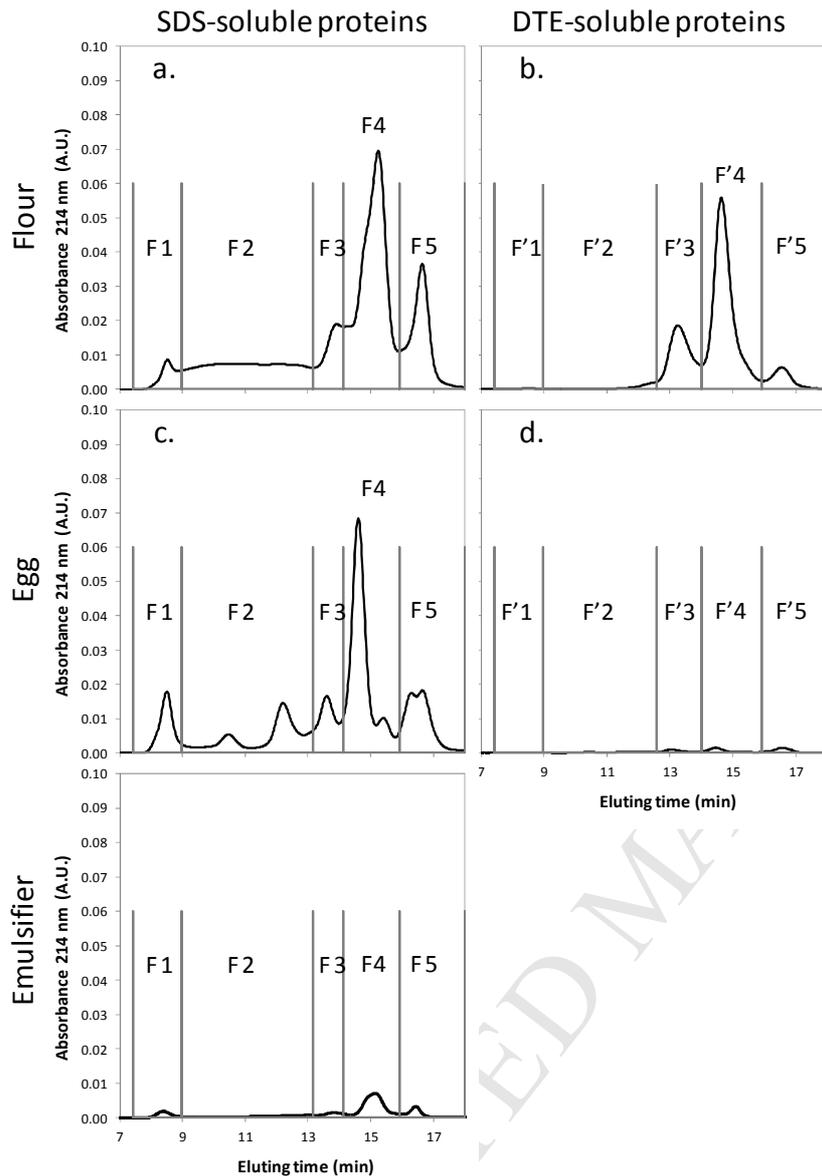


Figure 1 : SE-HPLC protein profiles of cake ingredients containing proteins. SDS-soluble (a) and DTE-soluble (b) protein extracts of Apache flour ; SDS-soluble (c) and DTE-soluble (d) extracts of pasteurized whole eggs ; SDS-soluble extract of emulsifier (e). No DTE-soluble protein extract was found for emulsifier. Sample mass was adjusted to the amount theoretically found in 80 mg of the standard cake recipe (dry basis)

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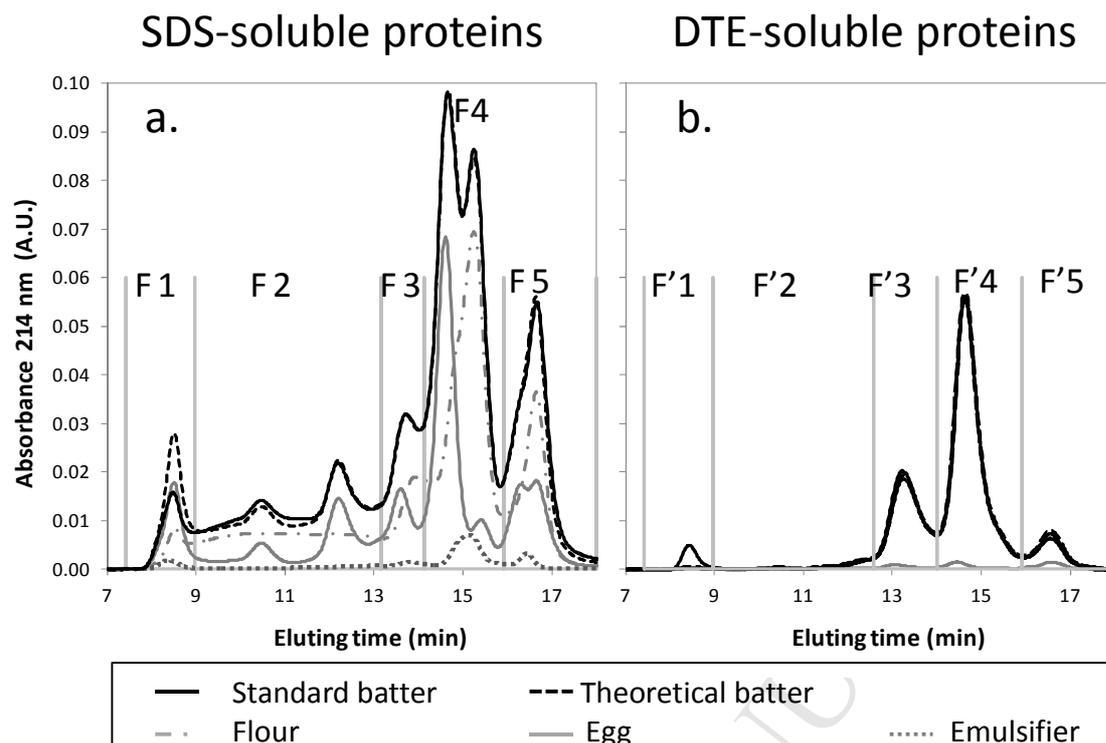


Figure 1: SE-HPLC chromatograms of SDS-soluble (a) and SDS-insoluble (b) protein extracts of standard cake batter and of the cake ingredients (flour, egg and emulsifier) that include proteins. A theoretical standard cake batter chromatogram was calculated by summing, in regards to their batter proportions, the chromatograms of egg, flour and emulsifier.

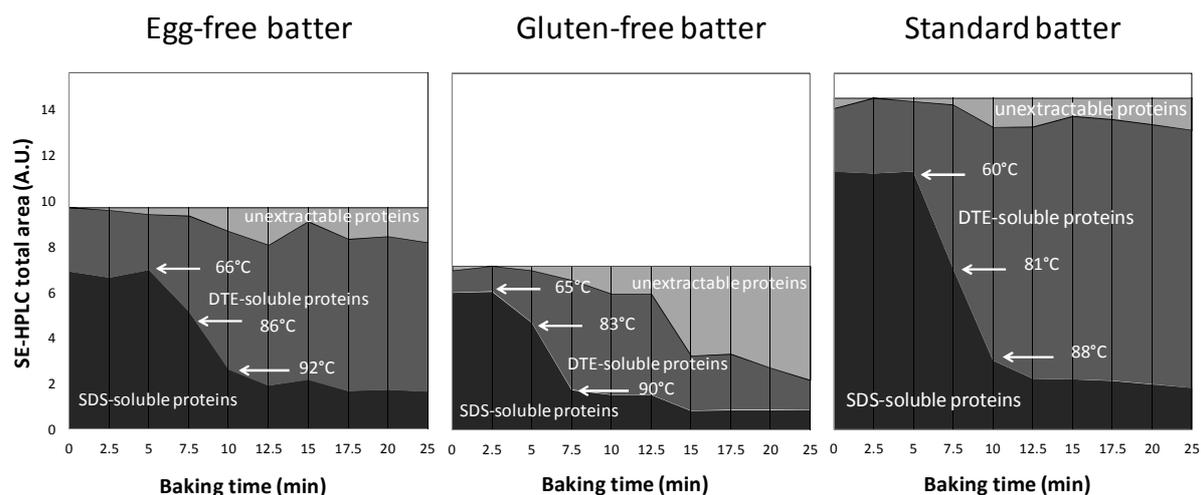


Figure 3: Cake crumb protein SDS extractability of an egg-free recipe (a), a gluten-free recipe (b) and a standard recipe (c) during baking. For each baking time, unextractable protein fraction was calculated from the difference in total SE-HPLC area between the initial un-baked batter and batter at current baking time.

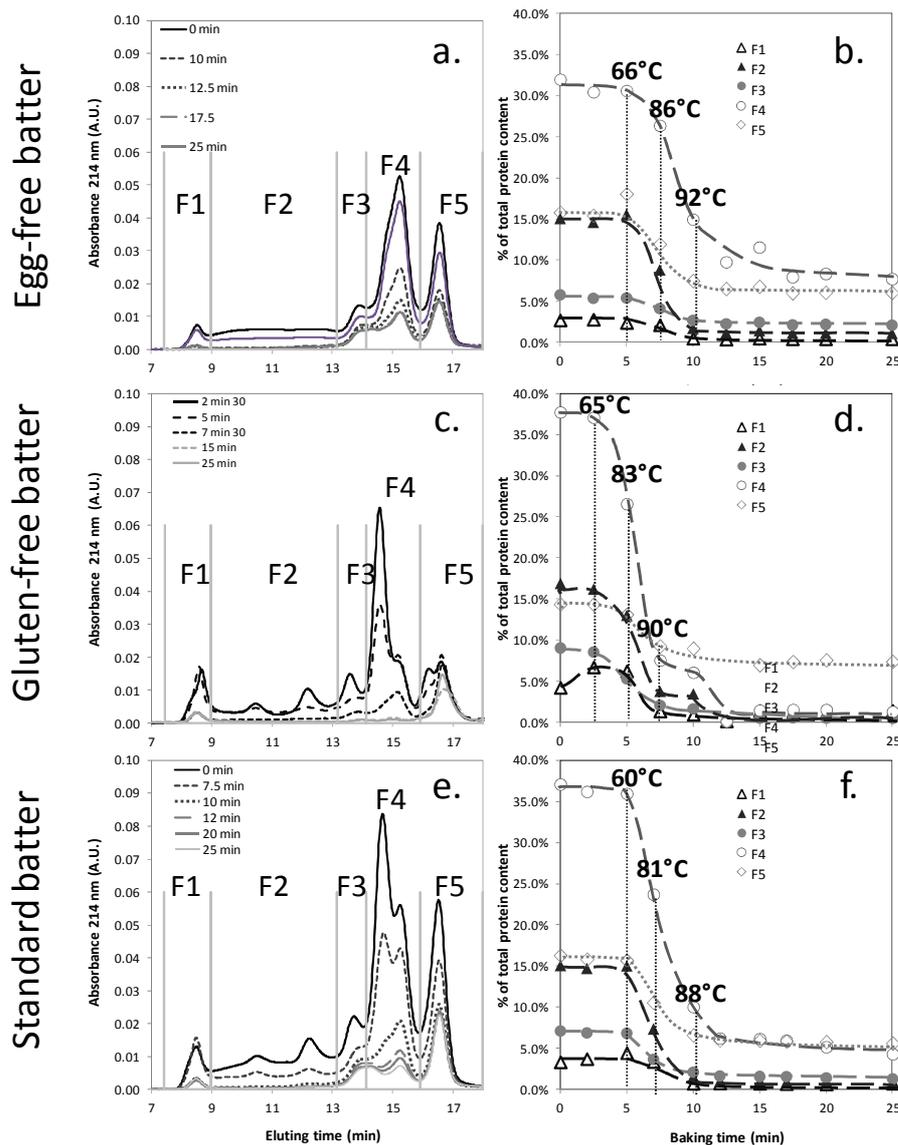


Figure 4 : SE-HPLC SDS-soluble protein profiles (a, c, e) and compositions (b, d, f) of egg-free, gluten-free and standard cake batters during baking. SE-HPLC protein fractions were obtained by calculating SE-HPLC fraction areas at different baking times.

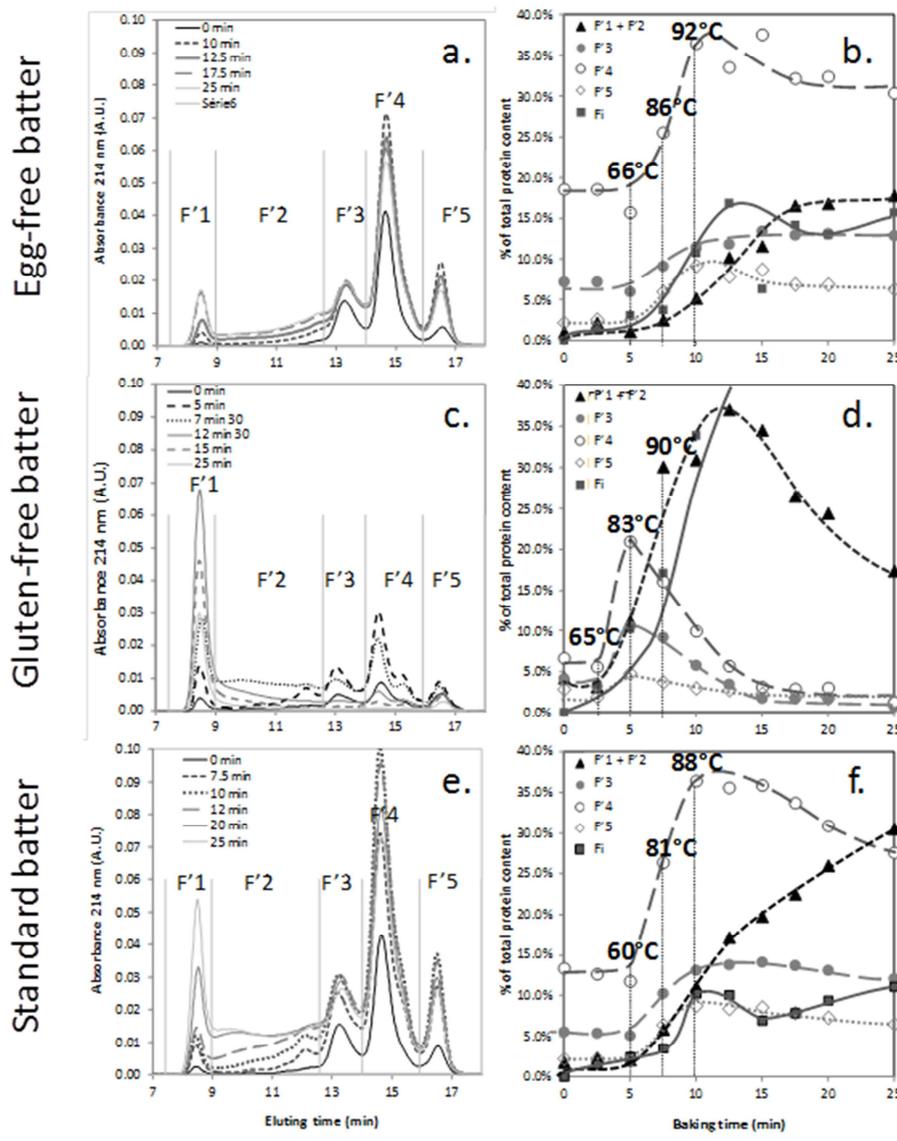


Figure 5. SE-HPLC DTE-soluble protein profiles (a, c, e) and compositions (b, d, f) of egg-free, gluten-free and standard cake batters during baking. SE-HPLC protein fractions were obtained by calculating SE-HPLC fraction areas at different baking times and were summed to evaluate DTE-soluble protein composition. The unextractable protein fraction was also estimated.

- Cake protein aggregation during processing was studied using SE-HPLC
- Dough mixing does not impact the size distribution of egg and flour proteins
- Egg and flour proteins formed mixed aggregates upon baking
- Mixed aggregates are stabilized by thiol/disulfide and iso-peptide bonds
- Eggs proteins contributes to strengthen the cake protein network

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