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# Rubisco: a promising plant protein to enrich wheat-based food without impairing dough viscoelasticity and protein polymerization

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## Highlights:

- Rubisco addition does not decrease dough stiffening during heat treatment
- Rubisco forms both weak and covalent bonds during dough hydration and mixing
- Rubisco is part of the water-insoluble protein network
- In heat-treated dough, rubisco increases the concentration of large polymers

## Abstract:

Rubisco (ribulose-1,5-bisphosphate carboxylase/oxygenase), a leaf protein, has an interesting amino acid profile and promising functional properties. Incorporated in wheat-based products, it would increase their protein content and improve their essential amino acid profile, particularly lysine. The impact of rubisco enrichment on wheat dough mechanical properties and protein-protein interactions was investigated using Dynamic Mechanical Thermal Analysis and Size-Exclusion chromatography, respectively. Experiments were also performed on gluten and pea protein enriched doughs as a comparison. Wheat doughs with increasing concentrations of rubisco, gluten or pea proteins (from 0 to 33% of total proteins) were prepared using a 2 g-mixograph at constant hydration. In contrast to pea proteins and gluten, rubisco does not reduce dough stiffening during heating, probably due to its own reactivity to temperature and to low competition with starch for water. Detailed analysis of protein interactions showed that rubisco is part of the gluten network formed during dough mixing through the establishment of weak and disulphide bonds. In addition, rubisco subunits form new covalent bonds during the heat treatment thereby increasing the concentration of SDS insoluble high molecular weight aggregates. These results suggest that rubisco actively participates in the formation of the dough protein network. The colocalization of gluten and rubisco proteins on micrographs supports the hypothesis that they form a co-protein network.

**Keywords:** Rubisco; gluten; pea proteins; protein network; protein-protein interactions; dynamic mechanical thermal analysis (DMTA)

## 30 1. Introduction

31 Foods of animal origin are a major source of proteins in today's Western diet. Nevertheless, their overconsumption  
32 raises public health (Friel et al., 2009) and environmental issues (Aiking, 2014; John Reynolds, David Buckley,  
33 Weinstein, & Boland, 2014). A panel of experts recently underlined the need to shift to a diet richer in plant  
34 proteins (Willett et al., 2019). Among other things, this food transition requires the development of alternatives  
35 to meat proteins in Western countries. Development of meat analogues and dairy substitutes is part of the solution  
36 but there are still technological challenges to address to meet consumer preferences (Wild et al., 2014). Another  
37 strategy to meet nutritional needs is to enrich traditional food with plant proteins, leading to foods with higher  
38 protein content and a balanced amino acid profile. These enriched foods are currently mainly based on a  
39 combination of proteins from legumes and wheat (ING Economics department, 2017). The association of legumes  
40 with wheat counterbalances the lack of lysine or threonine in wheat proteins (Bahnassey, Khan, & Harrold, 1986).  
41 However, adding legumes in wheat-based foods may impair the organoleptic quality of the product. As recently  
42 reviewed, fortifying durum wheat pasta with legumes increases cooking loss and reduces the cooked weight of  
43 pasta (Monnet, Laleg, Michon, & Micard, 2019). This reflects higher leaching of solids from the pasta into the  
44 cooking water due to the weakening of the gluten network (Bahnassey & Khan, 1986; Laleg et al., 2017). Weakening  
45 of the protein network is also responsible for the degradation of the textural properties of pasta enriched with  
46 legumes (Laleg et al., 2017; Petitot, Boyer, Minier, & Micard, 2010). Similarly, the addition of legume proteins in  
47 bread dough affects the volume of the loaf by reducing its gas retention capacity as recently reviewed (Boukid,  
48 Zannini, Carini, & Vittadini, 2019). The ability of wheat semolina or flour proteins to form a gluten matrix is the  
49 main factor influencing the strength and elastic properties of wheat dough that ensure the quality of wheat-based  
50 foods. However, the mechanisms through which extrinsic proteins affect the gluten network remain unclear. The  
51 addition of non-wheat components may dilute gluten proteins, thereby impairing and weakening the overall  
52 protein network (Laleg et al., 2017). It is therefore a challenge to develop protein-enriched wheat-based foods  
53 with minimized or even no gluten dilution effects.

54 Considerable attention has been paid to legume proteins for their nutritional quality and their availability. In the  
55 same way, leaf proteins have worthwhile nutritional qualities for human food (Gerloff, Lima, & Stahmann, 1965;  
56 Lexander, Carlsson, Schalén, Simonsson, & Lundborg, 1970). Leaf protein can be extracted from numerous crops,  
57 green by-products and aquatic plants (Ellis, 1979). The fractionation of wet green crops aims at recovering a juice  
58 with both high dry matter and protein content (Fiorentini & Galoppini, 1983). The soluble part is mainly composed  
59 of ribulose-1,5-bisphosphate carboxylase/oxygenase (rubisco), an enzyme involved in carbon fixation, which is  
60 richer in sulphur-containing amino acids than wheat proteins, and also rich in lysine, threonine and tryptophan  
61 (Gerloff et al., 1965). Although several extraction processes have been described (R. H. Edwards et al., 1975;  
62 Knuckles, Bickoff, & Kohler, 1972; Knuckles, De Fremery, Bickoff, & Kohler, 1975), rubisco is still not used as an  
63 ingredient in human food because of the difficulty of extracting a functional white concentrate with no off-flavours.  
64 However, technological advances in extraction processes and recent studies on functionality are promoting the  
65 use of leaf protein concentrates as an ingredient in human food (Firdaous et al., 2017; Hadidi, Ibarz, Conde, &  
66 Pagan, 2019; Kiskini, Vissers, Vincken, Gruppen, & Wierenga, 2016; Martin, Castellani, de Jong, Bovetto, & Schmitt,  
67 2019; Tamayo Tenorio, Gieteling, De Jong, Boom, & Van Der Goot, 2016; Udenigwe et al., 2017). Besides its  
68 nutritional interest, rubisco has been found to have interesting functional properties (Barbeau & Kinsella, 1988;  
69 Douillard, 1985; Knuckles & Kohler, 1982). Rubisco has a foaming capacity similar to egg white proteins (Sheen &  
70 Sheen, 1985), good solubility at food pH and is able to form gels at low concentrations and low temperatures in  
71 aqueous buffers at several pH (Martin, Nieuwland, & De Jong, 2014). The thermal denaturation and gelling  
72 properties of proteins are crucial to the structure and texture of wheat-based foods. To our knowledge, the  
73 functionality of rubisco when incorporated in a dense food matrix has not yet been studied.

74 The aim of this study was thus to investigate the impact of introducing rubisco on the structure of wheat protein.  
75 Micro wheat semolina doughs were used as a model food system to investigate the interactions between proteins  
76 and other components in protein-enriched wheat matrices. In addition to rubisco concentrate, the effects of  
77 adding gluten and pea protein were also studied as a comparison. Their impact on dough structuring during heating  
78 was studied using dynamic thermo-mechanical analysis (DMTA) at several enrichment rates ranging from 0 to 33%

79 of total proteins. Protein polymerisation in doughs was studied by protein sequential extraction followed by size-  
80 exclusion chromatography both before and after dough thermal treatment. The overall structure of the protein  
81 network was visualised by confocal scanning light microscopy (CSLM).

## 82 **2. Material and methods**

### 83 *2.1. Material*

84 Wheat semolina was provided by *La Semoulerie de Bellevue* (Panzani, Marseille, France) and passed through a 200  
85  $\mu\text{m}$  sieve before use. Pea protein concentrate (Nutralys, F85F) was purchased from Roquette (Lestrem, France)  
86 and rubisco concentrate was provided by Florette (Lessay, France). Rubisco was extracted from *Cichorium endivia*  
87 leaves using the extraction conditions of the WO 2014/104880 patent but excluding the hydrophobic column  
88 adsorption step. The gluten was extracted by hand from a durum wheat semolina dough based on Auger, Morel,  
89 Dewilde, & Redl (2009) with some modifications. Briefly, doughs were prepared at 55% water content (wet basis).  
90 After a premixing step at 28 rpm during 2.5 min, the dough was mixed at 90 rpm until optimum development time.  
91 The dough was then diluted and washed under demineralized water. Gluten was recovered on 800 $\mu\text{m}$  sieve and  
92 freeze-dried. To ensure constant moisture content, at least 14 days before the experiment, wheat semolina and  
93 protein concentrates were placed in a humidity-controlled chamber with a  $\text{K}_2\text{CO}_3$  saturated salt solution at 20°C to  
94 maintain 43.16% relative humidity. The water content of powders was determined in triplicate by weighing after  
95 drying at 105°C for three hours. The protein content of all the raw materials was analysed using the Kjeldahl  
96 procedure (NF V 03-050, 1970) with a conversion factor of 5.7 for wheat semolina, pea proteins and gluten  
97 concentrates. A conversion factor of 5.8 was used for the rubisco concentrate based on the amino acid profile  
98 previously determined (data not shown). Analytical grade sodium dodecyl sulfate (SDS), sodium phosphate dibasic  
99 ( $\text{Na}_2\text{HPO}_4$ ) and monosodium phosphate ( $\text{NaH}_2\text{PO}_4$ ) were purchased from VWR international (Leuven, Belgium).  
100 Other chemicals were purchased from Sigma-Aldrich (Bornem, Belgium) and Milli-Q water was used (Millipore  
101 Systems, Guyancourt, Molsheim, France).

### 102 *2.2. Accessible free thiol content assay*

103 Free thiol contents of protein isolates were measured using Ellman's reagent, 5,5-dithiobis-2-nitrobenzoic (DNTB)  
104 (Ellman, 1959). Sodium phosphate buffer (0.1M, pH 8) containing 1mM EDTA was degassed under vacuum before  
105 use. Protein concentrates were dispersed with a solid:liquid ratio of 2.5 in sodium phosphate buffer containing  
106 DNTB (0.3 g.L<sup>-1</sup>). Tube headspaces were filled in with argon or nitrogen and the tubes were incubated on a rotary  
107 shaker at 8°C for 15 min in the dark. The tubes were then centrifuged (12000 x g, 20 min) and supernatant  
108 absorbance was measured at 412 nm. The absorbance of the buffer and of the proteins in a non-reactive buffer  
109 (free of DNTB) was subtracted from the absorbance of the samples. The concentration of the released  
110 thionitrobenzoate ion ( $\text{TNB}^{2-}$ ) was calculated using a molar absorption coefficient of 13600 M<sup>-1</sup>cm<sup>-1</sup>. Results are  
111 expressed in micromoles of accessible free thiols per gram of protein.

### 112 *2.3. Sample preparation*

#### 113 *2.3.1. Dough formulation*

114 Dough samples (3 g) were prepared using wheat semolina, water and variable amounts of protein concentrates. A  
115 standard water content of 67% was used (dry mass basis) to obtain a homogeneous cohesive dough. Protein  
116 enrichments were carried out by replacing 0 to 10% of the semolina mass with a protein concentrate. Protein  
117 enrichment is hereafter expressed as the amount of added protein as a percentage of the total protein content of  
118 the protein-enriched dough. It ranged from 0% (control dough) to 33%. When analysing protein interactions, the  
119 protein used for enrichment is called "extrinsic protein".

#### 120 *2.3.2. Raw dough production*

121 Doughs were prepared using a "2-g Mixograph" (TMCO, Lincoln, NE, USA) equipped with a homemade aluminium  
122 double-walled jacket coupled with a water-regulated bath to control the temperature of the bowl. Wheat semolina  
123 and the protein concentrate were first mixed. The water was then added. The mixture was homogenised in the  
124 mixing bowl for six seconds at 54 rpm at ambient temperature. The mixture was left to rest for 15 min at 40°C and  
125 cooled to 20°C over a period of 25 min. The dough was then mixed at 54 rpm for 220 s.

126 To study the effect of heat on protein polymerisation, a thermal treatment was applied to the mixed doughs. A  
127 hermetically sealed aluminium container (internal dimensions: 9 mm radius, 3 mm height) was made to contain  
128 1.2 g of dough. Directly after mixing, the device was filled with 1.2 g of dough and immersed in a water bath at  
129 80°C for three minutes. It was then cooled down on ice and the dough sample was removed and immersed in liquid  
130 nitrogen before freeze drying.

#### 131 *2.4. Dough mechanical properties*

132 The mechanical properties of the raw doughs were analysed immediately after mixing. Dynamic mechanical  
133 thermal analysis (DMTA) with oscillatory measurements were performed as described in (Shehzad, Chiron, Valle,  
134 Lamrini, & Lourdin, 2012). A controlled strain dynamic mechanical analyser (DMTA MK 4, Rheometric Scientific,  
135 USA) was used in compression mode at a frequency of 1Hz with a 17mm diameter plate-plate geometry. A  
136 sinusoidal strain was applied with strain amplitude of 0.10%. The behaviour of wheat dough in dynamic  
137 measurements at this strain amplitude can be considered as linear (Lefebvre, 2006). A piece of dough weighing  
138 0.90g ( $\pm 0.02$ g) was placed between the two plates and the gap was set to 3.7 mm before the experiment. The  
139 dough faces in contact with the air were covered with grease to prevent water loss during measurement. Stress  
140 was recorded during a temperature ramp of 3°C/min from ambient temperature to 140°C.

141 To relate the oven temperature to the internal temperature of the dough, the temperature of two samples was  
142 measured using a thermocouple placed in the core of the dough. Using a polynomial model, an equation was  
143 determined to obtain the dough temperature from the oven temperature for all samples. The dynamic storage  
144 modulus, or elastic modulus ( $E'$ ), was calculated using RSI Orchestrator software (Rheometric Scientific, USA).

#### 145 *2.5. Analysis of protein polymerisation and protein/protein interactions*

##### 146 *2.5.1. Extraction of the gluten-like fraction after dough mixing*

147 Samples of control and protein-enriched doughs were washed by hand under deionized water for 15 min to  
148 remove most of the starch and soluble material. The rubbery mass that remains is called the gluten-like fraction  
149 (GLF) because it is probably composed of other proteins than gluten ones when extracted from protein-enriched  
150 doughs. The GLF was then recovered on a 200 $\mu$ m sieve, immersed in liquid nitrogen and freeze-dried.

##### 151 *2.5.2. Protein analysis in denaturing conditions*

152 SE-HPLC analysis of proteins was performed on protein concentrates, ground freeze-dried doughs and ground  
153 freeze-dried GLF. Protein was extracted as described in Morel, Dehlon, Autran, Leygue, & Bar-L'Helgouac'H (2000)  
154 with some modifications. Proteins were first extracted in 0.1M sodium phosphate buffer pH 6.8 with 1% SDS. Solid  
155 to liquid ratios of 8, 0.8 and 1.5 were used for the freeze-dried dough, protein concentrates and extracted gluten,  
156 respectively. Extraction was performed on a rotary shaker set at 60 rpm at 60°C for 80 min. SDS-soluble proteins  
157 were recovered in the supernatant after centrifugation (39191 x g, 30 min, 20°C). SDS disrupts non-covalent bonds  
158 and allows small aggregates to dissolve (Khan, Huckle, & Freeman, 1994). A second extraction was performed on  
159 pellets at 60°C for 60 min, in the same buffer including 20 mM dithioerythritol (DTE), to break the disulphide bonds.  
160 The pellets were then sonicated (Vibracell 72434, Bioblock Scientific, Illkirch, France) for three minutes at 50%  
161 power setting 20 kHz. Supernatants were recovered after centrifugation as described previously and diluted twice  
162 with 0.1 M phosphate, 1% SDS buffer including 40mM of iodoacetamide (IAM), to prevent reformation of the  
163 disulphide bonds. For readability, proteins insoluble in SDS but soluble in SDS and DTE are hereafter called  
164 "SDS+DTE soluble proteins".

165 The SE-HPLC apparatus (Waters model LC Module1 plus) was equipped with an analytical column, TSK G4000-SW  
166 (7.5 x 300 mm) and a guard column, TSK G3000-SW (7.5 x 75 mm) (both from Merck, Darmstadt, Germany).  
167 Apparent molecular weights were assessed with column calibration as described in Redl, Morel, Bonicel, Vergnes,  
168 & Guilbert (1999). For the analysis, six peaks were defined on chromatograms of SDS soluble proteins: peak P1  
169 corresponds to proteins eluted in the void volume, peak P2 corresponds to proteins whose molecular weight  
170 ranged from 157 kDa to 763 kDa, peak P3 from 82 to 157 kDa, peak P4 from 50 to 82 kDa, peak P5 from 20 to 50  
171 kDa and peak P6 from 6 to 20 kDa. The signal recorded after P6 does not correspond to proteins. The sum of the  
172 areas of the elution profiles of SDS-soluble and SDS+DTE soluble proteins, from peak P1 to peak P6, corresponds

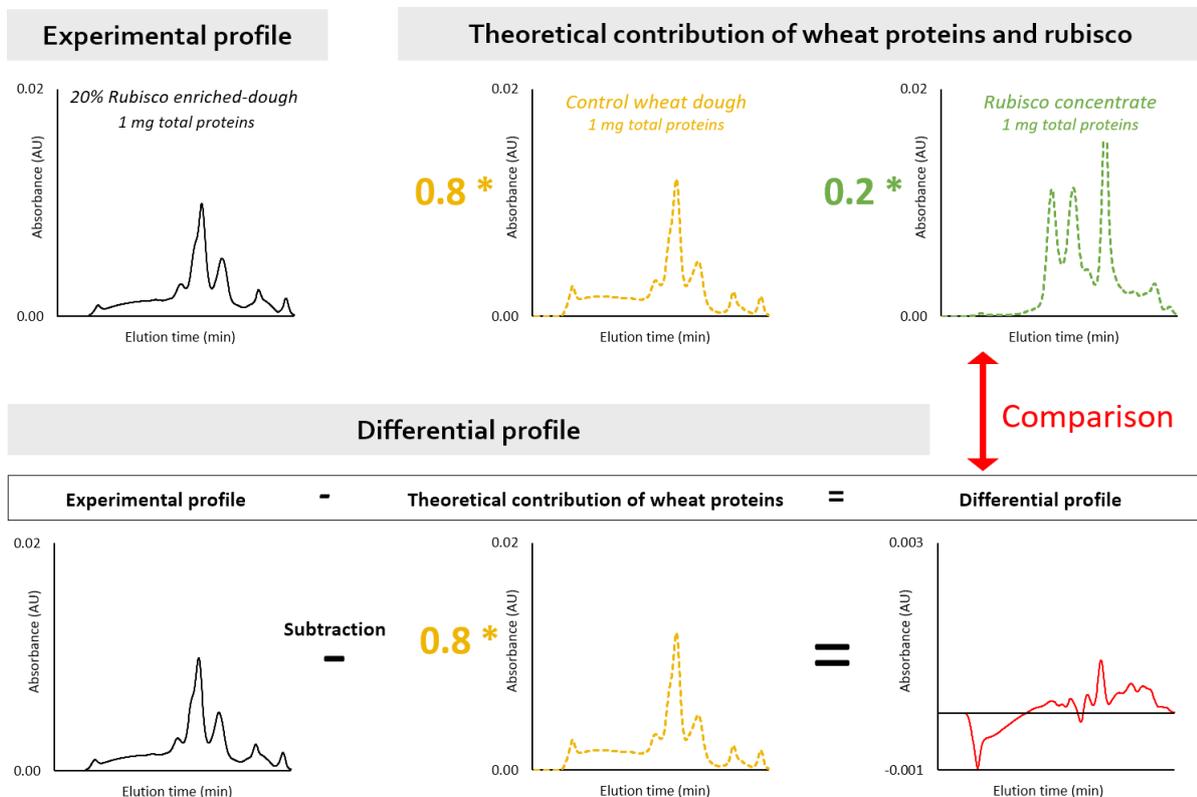
173 to the amount of total extractable proteins. The peak boundaries were defined according to the position of the  
 174 peaks in the rubisco-enriched wheat dough, so they may not exactly correspond to the peaks of the other samples  
 175 such as protein concentrates or control dough. The repeatability of dough production and protein extraction was  
 176 assessed in triplicate on several samples (**Supplementary fig. 1**). Dough fabrication and protein extraction were  
 177 then performed only once on each sample.

178 **2.5.3. Elution profile analysis**

179 Elution profiles were normalised on the basis of their total protein content before any calculation. To evaluate if  
 180 protein enrichment altered protein polymerisation in wheat dough, a differential SE-HPLC profile was calculated.  
 181 First, theoretical elution profiles of wheat proteins and extrinsic proteins of enriched dough were built. They  
 182 correspond to the elution profile of the 100% wheat control dough and to the elution profile of the extrinsic protein  
 183 concentrate, both normalised based on their respective proportion in the enriched dough studied. This is  
 184 illustrated in Diagram 1 for wheat dough enriched at 20% rubisco. If the protein enrichment of the wheat dough  
 185 does not alter the polymerization of the proteins, then its elution profile corresponds to the sum of these two  
 186 theoretical profiles.

187 Second, we subtracted the theoretical elution profile of wheat proteins from the experimental elution profile of  
 188 protein-enriched dough. The resulting profile, called the differential profile, was compared to the elution profile  
 189 of the extrinsic protein concentrate used for the enrichment. Any difference between the two latter profiles  
 190 identifies changes in protein polymerisation induced by protein enrichment of the dough.

191 A similar approach was used to analyse the gluten-like fraction extracted from rubisco-enriched dough. As it cannot  
 192 be excluded that some proteins were lixiviated during dough washing, the final proportion of rubisco and wheat  
 193 proteins in the GLF is unknown. To calculate differential profiles, we considered that the extraction yield of wheat  
 194 proteins in rubisco-enriched GLF was similar to that in the control wheat GLF. The resulting differential profile  
 195 was compared to the elution profile of the rubisco concentrate. Considering the approximation used for the calculation,  
 196 care must be taken in the interpretation of such differential profiles.



197 **Diagram 1** Method for the analysis of elution profiles of protein-enriched samples: Example for a wheat dough  
 198 enriched at 20% rubisco  
 199

200 *2.6. Microstructure of doughs observed by confocal laser scanning microscopy (CLSM)*

201 CLSM was performed on control, 29.6% gluten-enriched dough, 33% pea protein-enriched doughs and 31.9%  
202 rubisco-enriched doughs.

203 Frozen doughs were used for non-specific labelling of proteins. Samples were cut into 80 $\mu$ m thick slices at -20°C  
204 using a cryotome (Microm HM 500 OM, Microm International GmbH, Germany). They were then placed on a cold  
205 glass coverslip and proteins were stained with 0.2 $\mu$ L of Alexa Fluor™ 546 NHS Ester (Thermo Fisher Scientific,  
206 USA). The slices were then mounted on microscope slides.

207 Rubisco was specifically labelled to visualise its location compared to wheat proteins. Rubisco concentrate was  
208 first dispersed at 25 g.L<sup>-1</sup> in 0.1M sodium carbonate buffer at pH 9 for five hours at ambient temperature. The  
209 dispersion was then centrifuged (12000 x g, 20 min). The supernatant was mixed with a 1% Fluorescein  
210 isothiocyanate (FITC) solution in dimethyl sulfoxide (DMSO) at a ratio of 0.015:1. After one hour of incubation, the  
211 sample was dialysed using a 6-8 kDa molecular weight cut off tubing (Spectra/Por®, VWR, Leuven, Belgium) against  
212 water to remove free dyes for 26 hours with several changes of bath water. Specifically labelled rubisco was  
213 concentrated by osmotic compression against a 10% (wt/v) polyethylene glycol 20000 solution, until a protein  
214 concentration of 135 mg.mL<sup>-1</sup> was obtained. The protein concentration was estimated from the dry mass recovered  
215 after drying at 105°C for five hours.

216 Doughs were prepared as described in 2.3.2, using a mix of unlabelled rubisco and fluorescein isothiocyanate (FITC)  
217 specifically labelled rubisco.

218 Images were acquired at least five minutes after staining using a Nikon A1 microscope equipped with a NIS-Element  
219 AR software (Nikon, Germany). Samples were observed in confocal mode with a 561 nm and a 488 laser for Alexa  
220 Fluor™ 546 NHS Ester and FITC label respectively. Emitted light was recovered with a filter at 515/30 and 595/50  
221 nm, respectively. Images (2048 pixels) were taken at a magnification of 40 and resolution of 0.16  $\mu$ m/px in  
222 duplicate.

### 223 3. Results

#### 224 3.1. Characterisation of protein concentrate

225 Protein concentrates acquired from commercial or laboratory sources can vary considerably. Their protein content,  
226 the amount of accessible free thiols or their state of aggregation affects their functionality. For this reason, the  
227 concentrates used in this study were first characterised. Gluten, pea protein and rubisco concentrates contained  
228 respectively 67.2, 77.5 and 74.1% of protein (dry basis) as reported in **Table 1**.

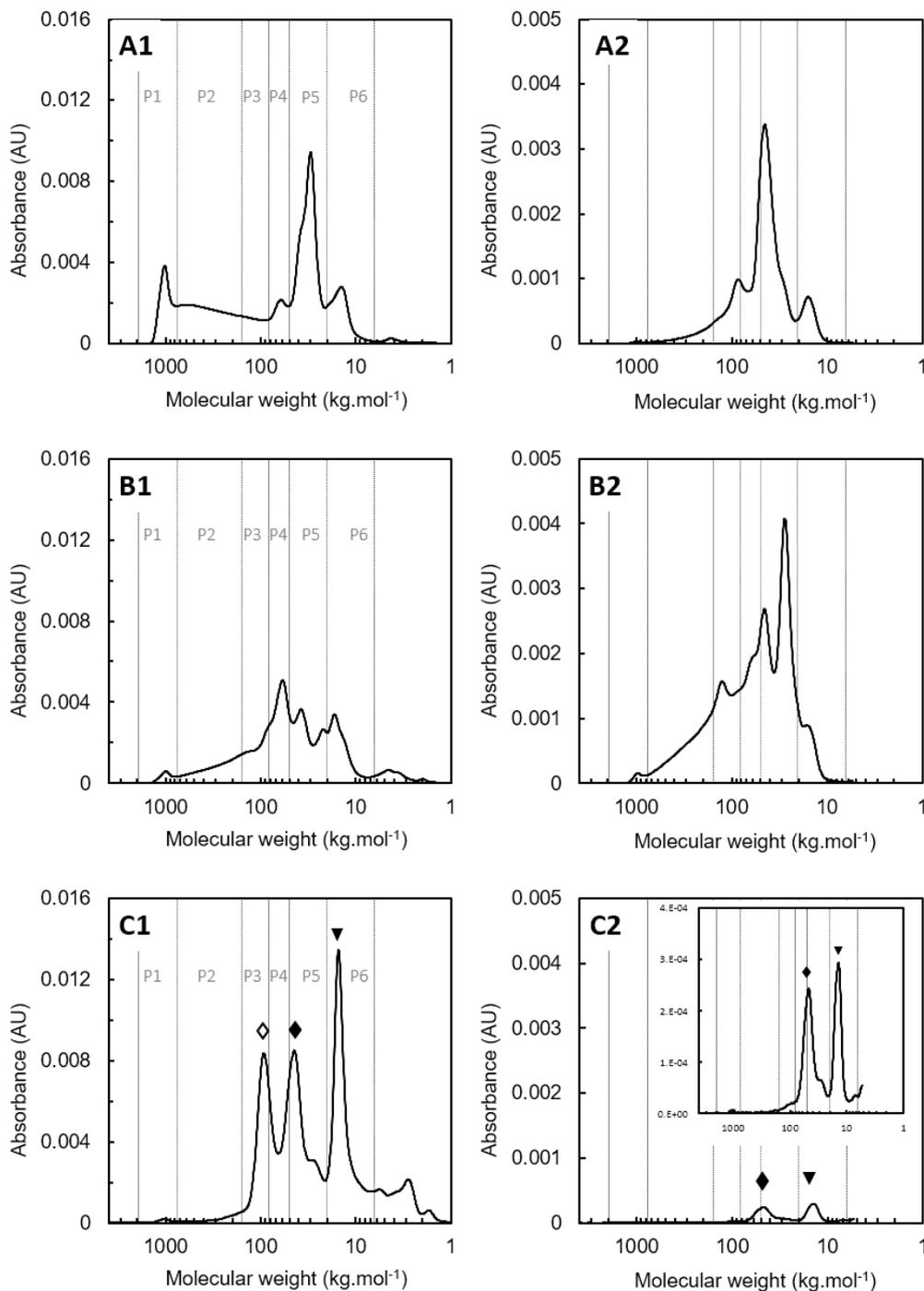
229 Gluten proteins do not contain free thiols, or an amount below the limit of detection. When propan-2-ol was used  
230 as a solvent, a low free thiol content of  $0.94 \pm 0.1 \mu\text{mol.g}^{-1}$  of vital gluten was reported (Morel, Bonicel, Micard, &  
231 Guilbert, 2000). Pea proteins contain an average of  $11.2 \mu\text{mol}$  of free thiols per gram of protein as reported  
232 previously (O’Kane, Vereijken, Gruppen, & van Boekel, 2005). Rubisco has a remarkably high free thiol content of  
233  $67.2 \pm 0.9 \mu\text{mol.g}^{-1}$ . Lower free thiol contents ( $13.66 \mu\text{mol.g}^{-1}$ ) have been reported in the literature for rubisco from  
234 alfalfa (Hood, Cheng, Koch, & Brunner, 1981). Gluten protein concentrate contains  $88.9 \pm 0.7\%$  of SDS soluble  
235 proteins as a function of total extractable proteins. In this study, pea protein concentrate contains  $76.6 \pm 0.7\%$  of  
236 SDS soluble proteins, whereas higher SDS solubility values (91.9%) have been reported for protein from pea flour  
237 (Kristiawan et al., 2018). This suggests that industrial pea protein extraction causes higher aggregation of proteins.  
238 Rubisco has the highest solubility in SDS with  $96.4 \pm 0.7\%$  of extractable proteins.

239 SE-HPLC profiles of SDS soluble and SDS+DTE soluble proteins of the three protein concentrates are shown in **Figure**  
240 **1**. SE-HPLC profile of wheat proteins is characterised by a large polydispersity comprising proteins from six to more  
241 than  $1000 \text{ kg mol}^{-1}$ . This is related to the intrinsic diversity of wheat proteins, in particular of wheat prolamins. Peaks  
242 P1, P2 and P3 of SDS soluble proteins include proteins with molecular weights higher than  $80 \text{ kg.mol}^{-1}$  (**Figure 1A**).  
243 These peaks were attributed to glutenin polymers (Morel, Dehlon, et al., 2000). Peaks P4 and P5 correspond to  
244 proteins with molecular weights ranging from 20 to  $80 \text{ kg.mol}^{-1}$  and were attributed to gliadins. Peak P6, including  
245 proteins from six to  $20 \text{ kg.mol}^{-1}$ , was attributed to wheat albumins and globulins. Three major peaks can be seen in  
246 the SDS+DTE soluble protein elution profile. The first eluting material extends from the upper column molecular  
247 exclusion size (about  $2,000 \text{ kg.mol}^{-1}$ ) to a peak centred at about  $86 \text{ kg.mol}^{-1}$ . This peak is hypothesised to correspond  
248 to the high molecular weight (HMW) glutenin subunits released from glutenin polymers after disulphide bond  
249 reduction. The main following peak could correspond to low molecular weight (LMW) glutenin subunits of about  $45$   
250  $\text{kg.mol}^{-1}$ . Finally, albumin and globulin that were initially disulphide-bonded to glutenin polymers were possibly  
251 eluted at about  $15 \text{ kg.mol}^{-1}$  (Veraverbeke & Delcour, 2002). The elution profiles of pea proteins are presented in  
252 **Figure 1B**. The SDS soluble fraction includes two major peaks at  $58\text{-}60 \text{ kg.mol}^{-1}$  (P4) and  $36\text{-}38 \text{ kg.mol}^{-1}$  (P5) which  
253 may be attributed to alpha/beta dimers of legumin and to vicilin subunits, respectively (John A. Gatehouse, Croy,  
254 Morton, Tyler, & Boulter, 1981). A shoulder is detected around  $70\text{-}80 \text{ kg mol}^{-1}$  which may be attributed to convicilin  
255 subunits (Croy, Gatehouse, Tyler, & Boulter, 1980). Smaller polypeptides at 22, 17 and  $14 \text{ kg.mol}^{-1}$  are also visible.  
256 These polypeptides cannot be attributed firmly based on molecular weights as they could correspond to alpha, beta  
257 chains of legumins (Matta, Gatehouse, & Boulter, 1981), to vicilin peptides (John A. Gatehouse et al., 1981) or pea  
258 albumins (Croy, Hoque, Gatehouse, & Boulter, 1984; J. A. Gatehouse, Gilroy, Hoque, & Croy, 1985). A fraction of  
259 proteins displayed a molecular weight higher than  $100 \text{ kg.mol}^{-1}$ , probably corresponding to aggregated proteins. The  
260 fraction of SDS-insoluble pea protein released after reduction of DTE contains several peaks, the same peaks as those  
261 in the SDS soluble protein profile, along with large species ( $> 200 \text{ kg.mol}^{-1}$ ), suggesting that some of the SDS-insoluble  
262 aggregates are stabilised by, at least, disulphide bridges. Industrial pea proteins are complex mixtures of proteins.  
263 In reduced conditions, it is difficult to attribute the nature of polypeptides based on their molecular weight. Other  
264 technics based on immunospecificity would be required. The elution profile of rubisco SDS-soluble protein fraction  
265 includes three well defined peaks (**Figure 1C1**). The main fraction was eluted in P6 (16.4 min) and was attributed to  
266 the small chain of rubisco (SC), with a molecular weight of about  $15 \text{ kg.mol}^{-1}$ . The peak eluted between P4 and P5  
267 ( $14.3 \text{ min}$ ,  $\sim 45 \text{ kg.mol}^{-1}$ ) was attributed to the rubisco large chain (LC) (Barbeau & Kinsella, 1988). Peak P3 ( $13.15$   
268  $\text{min}$ ,  $\sim 100 \text{ kg.mol}^{-1}$ ) could corresponds to large-chain dimers that may be formed during the extraction process  
269 (Rintamaki, 1989). This dimer was not detected when proteins were directly extracted in the presence of SDS and  
270 DTE (data not shown). Moreover, only two peaks attributed to the small and large subunits are present in SDS+DTE  
271 soluble fraction (**Figure 1C2, inset**), suggesting that interchain disulphide bonds would stabilise LC dimer. A shoulder

272 visible between P5 and P6 in the SDS soluble protein profile corresponds to a molecule of about 28 kg.mol<sup>-1</sup> of  
273 unknown origin. At the elution time of this peak, the ratio of intensity at 260 nm with 214 nm is much higher than  
274 for the other peaks (data not shown). This suggests that this molecule is richer in groups that absorb at 260 than  
275 other proteins of the extract. The molecular weight is too high to correspond to phenolic compound but this  
276 molecule could be a protein linked with phenolic compounds.

277 **Table 1** Characterisation of protein concentrates: protein content, accessible free thiol content and protein  
 278 sequential solubility in denaturing buffer (SDS) and reducing buffer (SDS+DTE). "N.d" stands for "not determined".  
 279 Standard deviations are expected to be similar to the standard deviations obtained on rubisco concentrate.

	Protein content <i>mg/100 g of concentrate, dry basis</i>	Free thiol content <i>μmol / g of protein</i>	SDS soluble protein <i>in % of extractable proteins</i>	SDS+DTE soluble proteins <i>in % of extractable proteins</i>
Gluten	67.2 ± 2.4	0 ± 0.1	88.9 ± n.d	11.1 ± n.d
Pea protein	77.5 ± 0.3	11.2 ± 0.1	76.6 ± n.d	23.4 ± n.d
Rubisco protein	74.1 ± 0.7	67.2 ± 0.9	96.4 ± 0.7	3.6 ± 0.7



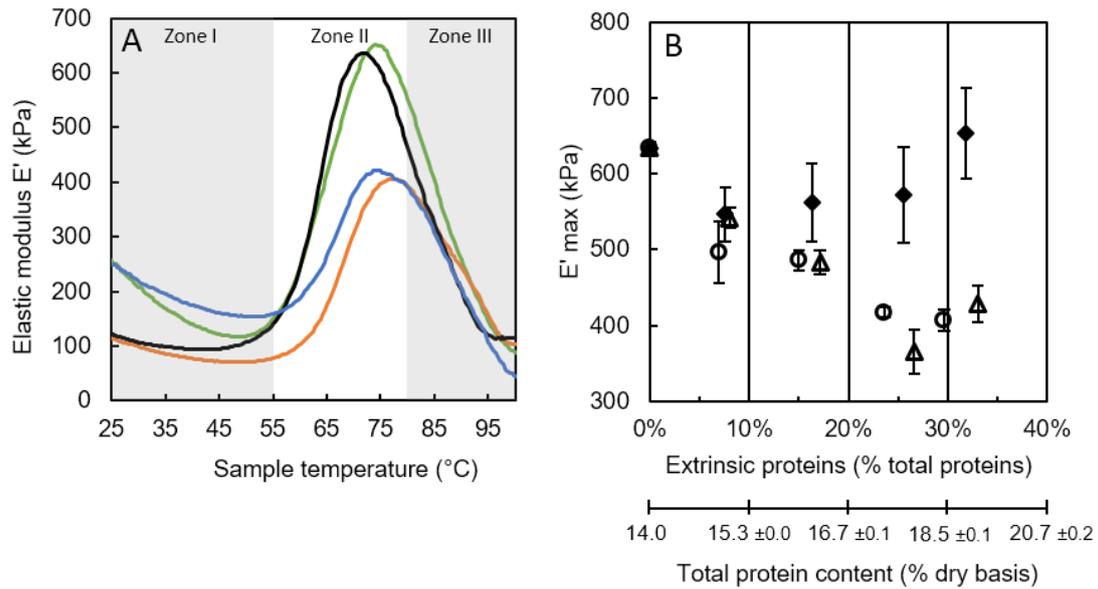
281 **Figure 1** Elution profiles of SDS soluble protein fractions (1; left) and SDS+DTE soluble protein fractions (2; right)  
282 extracted from gluten (A), pea protein (B) and rubisco (C) concentrates. The inset in C2 is a close-up of the elution  
283 profile. The symbols in figure C1 and C2 represent rubisco subunits: small chain (SC: ▼), large chain (LC: ◆) and  
284 large chain dimer (LC dimer: ◇). For the sake of readability, the two types of elution profiles are not represented  
285 at the same scale and the elution profile of SDS+DTE soluble proteins is represented only from 6 kg.mol<sup>-1</sup>. Every  
286 experimental elution profile is represented after normalisation based on 1mg of total protein in the sample.

### 287 3.2. Influence of protein enrichment on dough stiffening during heating

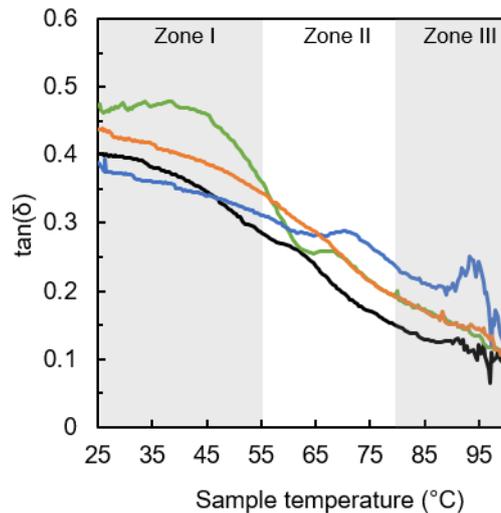
288 Temperatures above 50°C are known to trigger protein polymerisation and starch gelatinisation, and thus to affect  
289 wheat dough mechanical properties (Bloksma, 1972). Therefore, to understand the effect of protein enrichment on  
290 the structuring of dough during thermal treatment, changes in mechanical properties of doughs prepared at several  
291 enrichment rates were assessed during a temperature ramp. Representative curves of elastic modulus ( $E'$ ) as a  
292 function of the core sample temperature for control and ~32% extrinsic protein-enriched doughs are shown in **Figure**  
293 **2A**. All samples exhibited the same trend. When the temperature was increased up to 50°C,  $E'$  decreased (Zone I)  
294 due to an increase in water and polymer chain mobility (Bloksma, 1990), followed by a marked increase in  $E'$  between  
295 50°C and 75°C reflecting dough stiffening (Zone II). This increase corresponds to the cumulative effect of starch  
296 gelatinisation and protein aggregation. However, there is no clear scientific consensus on which of the two  
297 phenomena dominates the elastic modulus response in these temperature ranges. The maximum value of the elastic  
298 modulus has been shown to be positively correlated with the starch content in starch-gluten blends while gluten is  
299 assumed to have little influence on  $E'$  values (Dreese, Faubion, & Hosney, 1988; Mario Jekle, Mühlberger, & Becker,  
300 2016a; Zanoletti et al., 2017). Conversely, some studies on wheat doughs reported that changes in  $E'$  with  
301 temperature are mainly due to the polymerisation of gluten proteins (Rouillé, Chiron, Colonna, Della Valle, &  
302 Lourdin, 2010). Beyond 75°C, the elastic modulus decreased almost linearly (zone III). The mechanism related to this  
303 change is not well established in the literature as reviewed in Vanin, Michon, & Lucas, (2013). Several studies on  
304 gluten, pea or rubisco proteins system report an increase or a stabilisation of  $E'$  values at these temperatures (Felix,  
305 Perez-Puyana, Romero, & Guerrero, 2017; Martin et al., 2014; Wang et al., 2017). Therefore, elasticity drop above  
306 75°C may be associated with the softening of swollen starch granules. The peak of elastic modulus is representative  
307 of the maximal structural hardening of the dough. The change in maximum value of  $E'$  ( $E'_{max}$ ), is shown on **Figure 2B**  
308 as a function of the protein enrichment and total protein contents for the three proteins studied.  $E'_{max}$  values of  
309 doughs enriched at 8% were lower than the control dough regardless of the type of protein used. Gluten, pea  
310 proteins and rubisco alter dough thermal structuring similarly at 8% of protein enrichment. Beyond 8%, two types  
311 of behaviour were observed depending on the protein used for enrichment. Enrichment in gluten and pea proteins  
312 caused a further decrease in  $E'_{max}$  of about 36% compared to the control dough, suggesting that either starch  
313 gelatinisation or gluten polymerisation rates decrease with the incorporation of more pea and gluten proteins. In  
314 contrast, enrichment with rubisco beyond 8% resulted in  $E'_{max}$  values similar to those of the control dough,  
315 demonstrating its capacity to preserve the elastic behaviour of dough.

316 The loss factor,  $\tan(\delta)$ , represents the viscous to elastic ratio of dough and thus gives an estimate of the extent of  
317 dough structuring (M. Jekle & Becker, 2011). **Figure 3** shows how it changed with temperature in control dough,  
318 29.6% gluten-enriched dough, 33.0% pea protein-enriched dough and 31.9% rubisco-enriched dough. Its value was  
319 less than 1 and continuously decreased from 25 to 95°C in all samples. A  $\tan(\delta)$  value below 1 is typical of elastic  
320 material and a decrease indicates a gain in dough stiffness. The gluten-enriched dough displayed the same changes  
321 in  $\tan(\delta)$  as the control dough with slightly higher values, meaning that an increase in gluten content increases the  
322 liquid-like behaviour of dough. The  $\tan(\delta)$  of pea protein-enriched dough was close to that of the control dough  
323 below 47°C. It then decreased but less markedly than in the control dough, ultimately leading to a substantially  
324 higher value of  $\tan(\delta)$ . Pea proteins reduce the gain in elasticity of the semolina dough during heating. The loss factor  
325 of rubisco-enriched doughs was significantly higher than that of the control dough and remained stable from 25 to  
326 40°C. It then dropped abruptly to reach a value similar to that of the gluten-enriched dough at 65°C. This suggests  
327 that, between 40 and 65°C, rubisco contributes to dough elasticity while below 40°C, it acts as a diluent increasing  
328 the liquid behaviour of the sample. It should be noted that beyond 70°C,  $\tan(\delta)$  values of doughs enriched in either

329 rubisco (31.9%) or gluten (29.6%) were exactly the same, supporting similar involvement of both types of protein in  
 330 dough structure.



331  
 332 **Figure 2 A** Thermal variation of elastic modulus determined by DMTA in control (black) sample and 29.6% gluten  
 333 (orange line), 33.0% pea protein (blue line) and 31.9% rubisco (green line) enriched doughs. The curves are  
 334 representative of triplicate observations. **B:** Comparison of  $E'$  max values measured by DMTA on gluten-enriched  
 335 doughs (o), pea protein-enriched doughs ( $\Delta$ ) and rubisco-enriched doughs ( $\blacklozenge$ ) at several enrichment rates.



336  
 337 **Figure 3** Thermal variation of loss factor  $\tan(\delta)$  determined by DMTA in control (black) sample and 29.6% gluten  
 338 (orange line), 33.0% pea protein (blue line) and 31.9% rubisco (green line) enriched doughs. The curves are  
 339 averages of triplicate observations.

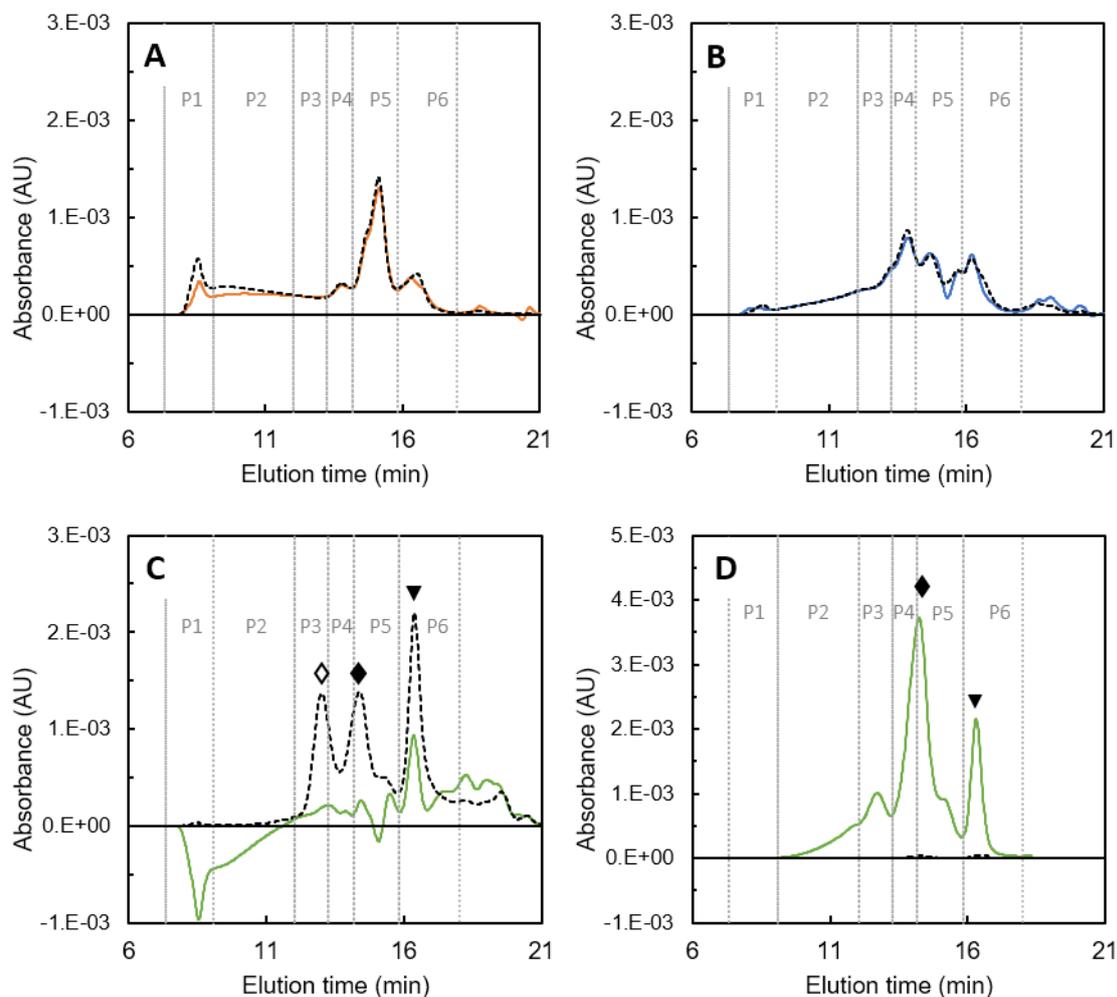
### 340 3.3. Protein polymerisation in thermally treated doughs

341 To identify heat-induced changes in protein interactions, proteins from dough samples treated at 80°C for three  
 342 minutes were successively extracted in SDS and then in SDS+DTE buffers and analysed by size-exclusion  
 343 chromatography. Experiments were performed on control and 14.9% gluten, 17.1% pea protein and 16.3% rubisco-  
 344 enriched doughs.

345 The differential SE-HPLC profiles of each protein-enriched dough were calculated and compared to the SE-HPLC  
 346 experimental profile of the extrinsic protein concentrate (as described in 2.5.3). Results concerning the SDS-soluble  
 347 protein fractions are shown in **Figure 4A, B and C**. **Figure 4D** shows the same kind of profiles for SDS+DTE soluble

348 proteins in rubisco-enriched doughs. Differences between differential and protein concentrate profiles, when  
349 present, can be linked to a change in the size distribution of the extrinsic protein or to the effect of enrichment on  
350 the overall heat-induced protein polymerisation whatever the protein considered, either extrinsic or wheat protein.  
351 The impact of protein enrichment on protein polymerisation depends on the type of protein. The differential profile  
352 corresponding to gluten-enriched dough is below the gluten concentrate profile, especially at the location of glutenin  
353 polymers (**Figure 4A**). Hence, the areas of peaks P1 and P2 are respectively 46% and 24% lower than in the gluten  
354 concentrate profile. A decrease of the same magnitude was observed between raw control doughs and thermally  
355 treated control dough (data not shown). This suggests that the differences observed between the differential profile  
356 of gluten-enriched dough and the gluten concentrate profile could reflect the effect of heating on extrinsic glutenin  
357 polymers. The differential profile of pea protein-enriched dough does not differ much from the pea protein  
358 concentrate elution profile (**Figure 4B**). Peaks P4, P5 and P6 are only about 10% lower than those of the raw pea  
359 concentrate profile

360 The differential profile of the SDS-soluble fraction obtained from the heated rubisco-enriched dough contrasted  
361 sharply with that of raw rubisco as shown in **Figure 4C**. The differential profile shows negative P1 and P2 peaks,  
362 corresponding, in the mirror image, to peaks of wheat glutenin polymers. In addition, the typical rubisco peaks, P3,  
363 P4 and P6, almost disappeared, implying that enrichment in rubisco makes these wheat and rubisco protein fractions  
364 insoluble in SDS. Moreover, the differential profile of SDS+DTE soluble proteins is well above that of the SDS+DTE  
365 soluble protein in the rubisco concentrate (**Figure 4D**). In **Figure 4D**, peak P3 may correspond to high-molecular-  
366 weight glutenin subunits as described previously, while peak P5 and P6 may partly correspond to rubisco LC and  
367 rubisco SC. The fact that addition of DTE allowed the release of these subunits from the SDS-insoluble protein pellet  
368 indicates that protein insolubility was driven by disulphide crosslinking. The same analyses performed with  
369 increasing levels of rubisco enrichment showed that the concentration of rubisco affected the formation of  
370 disulphide-linked SDS-insoluble aggregates (not shown). Whether rubisco and wheat protein interact to form mixed  
371 aggregates was not established with the present analysis. However, it is likely that rubisco increases the heat-  
372 induced aggregation of wheat glutenin polymers.



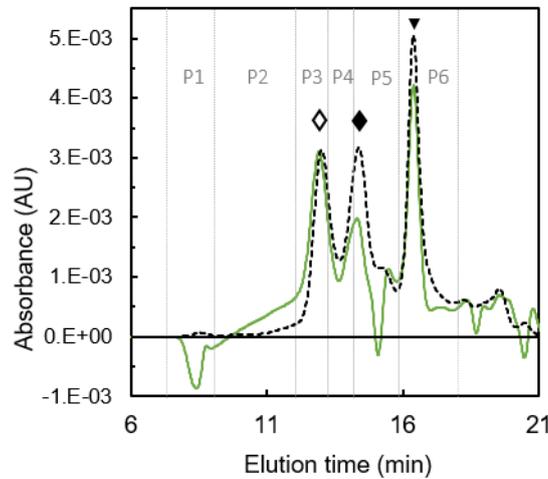
373  
 374 **Figure 4:** SDS soluble (A, B, C) and SDS+DTE soluble (D) differential profiles (solid lines) obtained from heat-treated  
 375 doughs enriched with gluten (A), pea proteins (B) and rubisco (C, D) compared with elution profile of extrinsic  
 376 protein extracts (dashed line) adjusted to the same extrinsic protein weight. Differential profiles were obtained by  
 377 subtracting the SE-HPLC elution profile of heat-treated wheat control dough from the elution profile of the heat-  
 378 treated enriched doughs (both adjusted to the same semolina weight). Doughs enrichment were 14.9% gluten (A),  
 379 17.1% pea protein (B) and 16.3% rubisco (C and D). Symbols represent rubisco subunits: small chain (SC: ▼), large  
 380 chain (LC: ◆) and large chain dimer (LC dimer: ◇). For the sake of readability, the elution profile of figure D is  
 381 represented only up to 18 min.

#### 382 3.4. Effect of incorporating rubisco on protein interaction during hydration and dough mixing

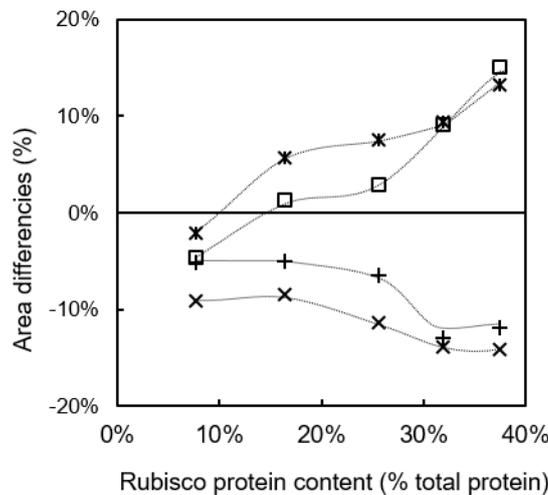
383 Incorporating rubisco enhanced wheat protein polymerisation in thermally treated doughs. Experiments were  
 384 conducted to check whether the modifications are related to heat treatment or take place during the hydration and  
 385 dough mixing steps. To this end, the differential SE-HPLC profile of SDS soluble proteins from 37.4% rubisco-enriched  
 386 raw dough was compared to the SE-HPLC profile of the rubisco concentrate (as described in 2.5.3). Noteworthy  
 387 differences were observed between the differential profile and the elution profile of the concentrate, as shown in  
 388 **Figure 5.** Like in thermally treated dough, peak P1 is negative on the differential profile, suggesting that the presence  
 389 of rubisco may have reduced wheat glutenin polymer solubility in SDS. However, part of peak P2 is positive. Taken  
 390 together, these features show that the addition of rubisco shifted the size distribution of wheat glutenin polymers  
 391 towards smaller polymers. Peak P3, corresponding to rubisco LC dimer, is slightly shifted on the left in the early  
 392 elution stage, which could be due to incorrect separation between it and peak P2. Peaks P5 and P6, corresponding  
 393 to rubisco LC and SC, respectively, are lower than in the rubisco concentrate profile. This suggests that rubisco LC  
 394 and SC aggregate during mixing. These peaks are higher than in theory in SDS+DTE soluble protein elution profile

395 **(Supplementary fig. 2).** Rubisco LC and SC may therefore form SDS-insoluble disulphide-linked complexes, between  
 396 each other or with wheat proteins, during hydration and mixing. Moreover, a negative peak can be seen after the  
 397 rubisco LC peak (P5). It means that rubisco enrichment affected the molecular weight of proteins that are supposed  
 398 to be eluted in this zone. It may correspond to a deficit in wheat gliadins and may also be due to the disappearance  
 399 of rubisco LC.

400 In contrast to thermally treated doughs, the content of SDS-insoluble polymers did not increase with increasing  
 401 doses of rubisco. However, a dose effect was observed for specific peaks (**Figure 6**). P2 and P3 areas are higher on  
 402 the differential profiles than on the rubisco concentrate profile and the difference between the two profiles  
 403 increased with increasing rubisco enrichment of the dough. The opposite was observed for P4 and P5 whose  
 404 recovery decreased with increasing rubisco enrichment. With an increase in the rubisco/wheat protein ratio, the  
 405 size of glutenin polymers decreased and the solubility of the LC and SC subunits in SDS decreased. Incorporating  
 406 rubisco modified the molecular interactions during dough mixing with an effect of dose. The size of SDS soluble  
 407 glutenin polymers decreased and rubisco LC and SC formed disulphide linked aggregates.



408  
 409 **Figure 5** Differential SE-HPLC profile (green line) of raw dough enriched at 37.4% rubisco compared to SE-HPLC  
 410 profile of rubisco protein concentrate (dashed black line) based on the same rubisco concentrate weight. The  
 411 differential profile was obtained by subtracting the SE-HPLC profile of SDS-soluble proteins of wheat control raw  
 412 dough from the profile of rubisco-enriched raw dough (both adjusted to same semolina weight). Symbols represent  
 413 rubisco subunits: large chain dimer (LC dimer; ◇), large chain (LC; ◆), small chain (SC; ▼).



414  
 415 **Figure 6** Differences of area under the curve for peaks P2 (□), P3 (×), P4 (+) and P5 (×) between experimental and  
 416 theoretical elution profiles obtained for rubisco-enriched doughs at several rubisco enrichment rates. Theoretical

417 profiles correspond to the sum of the elution profiles of the 100% wheat control dough and of the rubisco  
 418 concentrate, both normalised based on their respective proportion in the enriched dough. Differences in peak area  
 419 are expressed as a percentage of the theoretical profile. Dotted lines are included to guide the eye.

### 420 3.5. Weak bond formation during hydration and mixing

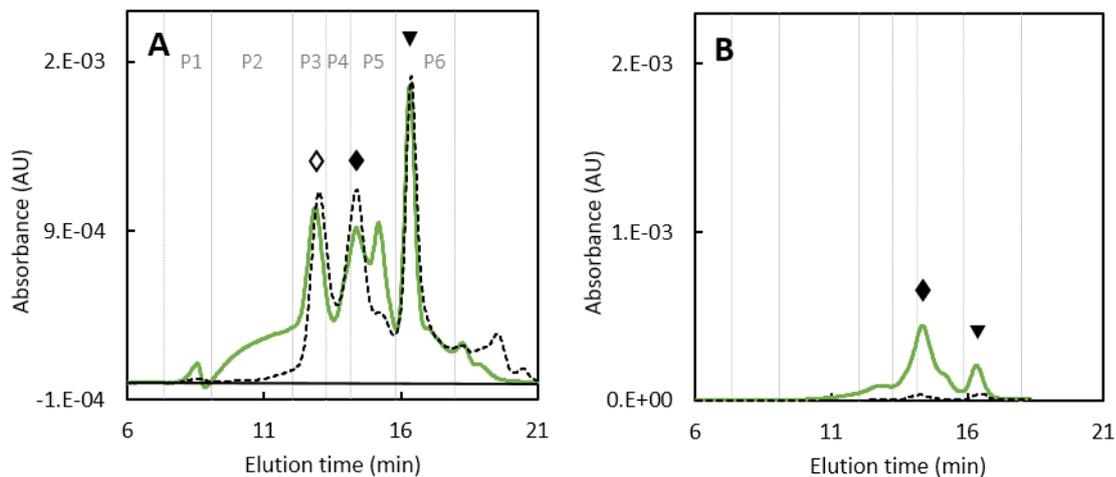
421 Previous results suggest that the introduction of rubisco affects molecular interactions in wheat dough. To highlight  
 422 a possible effect of rubisco on gluten network specifically, gluten-like fraction (GLF) were recovered from control  
 423 and rubisco-enriched raw doughs (16.3%). Extraction yields in dry mass and protein content are listed in **Table 2**. A  
 424 higher amount of GLF was recovered from rubisco-enriched dough than from control dough. Given their similar  
 425 protein content, it can be concluded that enriching the dough with rubisco increased the quantity of water-insoluble  
 426 proteins. This could be related to an increased contribution of wheat proteins, and/or of rubisco in GLF.

427 **Table 2** Extraction yield and protein content of gluten-like fractions extracted from control and rubisco-enriched  
 428 doughs.

	GLF weight			Protein content			Protein extraction yield		
	<i>g (db)</i>			<i>g/100g of GLF (db)</i>			<i>% of dough total proteins</i>		
Control wheat dough	0.225	±	0.009	75.0	±	0.1	67.1	±	2.9
16.3% rubisco-enriched dough	0.257	±	0.002	75.1	±	1.6	66.4	±	0.9

429 To assess the presence of rubisco in the GLF of rubisco-enriched dough, proteins of GLF from control and enriched  
 430 dough were sequentially extracted in SDS and SDS+DTE and analysed by SE-HPLC. Raw elution profiles of GLF from  
 431 control and rubisco-enriched doughs are given in supplementary data (**Supplementary fig. 3**). We hypothesised that  
 432 the extraction yield of wheat proteins in rubisco-enriched GLF was similar to that in the control wheat GLF (detailed  
 433 in part 2.5.3). This equates to estimating that 84.5% of the proteins of the rubisco-enriched GLF are wheat proteins  
 434 and 15.5% are rubisco. The differential profile of rubisco-enriched GLF is compared to the elution profiles of rubisco  
 435 concentrate in **Figure 7**. The SDS-soluble fraction of rubisco-enriched GLF shows a higher peak P2, meaning that  
 436 rubisco increases the concentration of medium size glutenin polymers in the GLF.

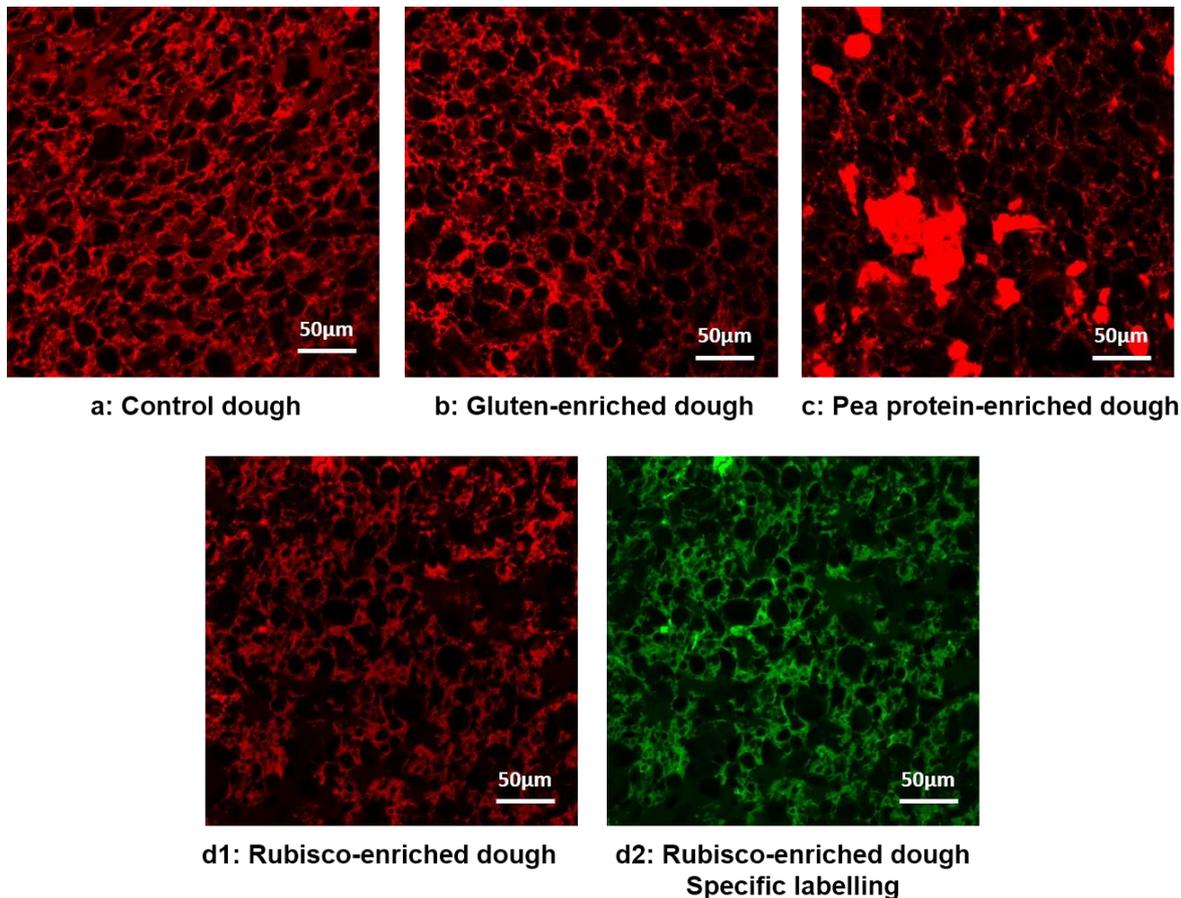
437 The presence of peaks of the rubisco subunits (P3, P5 and P6) on the differential SE-HPLC profile means they are co-  
 438 extracted with wheat proteins during dough washing. The heights of peaks P3 and P6, corresponding to rubisco LC  
 439 dimer and SC, are similar on the elution profiles of both differential and rubisco concentrate. Most LC dimers and SC  
 440 are therefore water-insoluble but SDS-Soluble. This suggests that their water-insolubility is related to the  
 441 establishment of weak interactions. In contrast, the height of the rubisco LC peak is lower on the differential profile  
 442 than on the rubisco concentrate profile but higher on the SDS+DTE profile. This suggests that rubisco LC form water-  
 443 insoluble aggregates stabilised by disulphide bounds during mixing.



444  
 445 **Figure 7:** SDS soluble (A) and SDS+DTE soluble (B) differential profiles obtained for the gluten-like fraction (GLF)  
 446 extracted from dough enriched with 16.3% rubisco (solid lines) compared with elution profile of rubisco  
 447 concentrate (dashed lines) adjusted to the same rubisco weight. Differential profiles were obtained by subtracting  
 448 the SE-HPLC elution profile of control GLF from the elution profile of the rubisco-enriched GLF. The calculation was  
 449 made considering that the semolina protein extraction yields were the same for both gluten-like fractions. Symbols  
 450 represent rubisco subunits: small chain (SC: ▼), large chain (LC: ◆) and large chain dimer (LC dimer: ◇).

### 451 3.6. Microstructure of the dough protein network

452 The microstructure of the protein network was visualized by confocal laser scanning microscopy (CLSM). The overall  
 453 arrangement of the protein network was observed by non-specific covalent labelling of proteins from control dough,  
 454 29.6% gluten-enriched dough, 33% pea protein-enriched dough and 31.9% rubisco-enriched dough. The resulting  
 455 images are shown in **Figure 8**. The protein network of the control wheat dough (**Figure 8a**) has a typical honeycomb  
 456 arrangement embedding starch granules (McCann & Day, 2013). Gluten-enriched dough has a very similar structure  
 457 (**Figure 8b**). Large protein particles are visible in the pea protein-enriched wheat dough micrograph (**Figure 8c**). Since  
 458 the emission of these structures is intense, the gluten protein network must be examined at a lower intensity to  
 459 avoid image saturation. Finally, the rubisco-enriched wheat dough has a slightly different protein network than the  
 460 control wheat dough. Indeed, the extent of the protein network seems to be less uniform with higher lacunar zones.  
 461 Further image analyses are needed to confirm this observation.  
 462 In order to reveal the segregation or co-location of rubisco and gluten, doughs were prepared using covalently-  
 463 labelled rubisco. CLSM images (**Figure 8d1 and d2**) show that rubisco does not form aggregated structures visible at  
 464 the micro-scale in contrast with pea proteins. Instead, it is co-localized with the gluten protein network.



465  
 466 **Figure 8** CLSM images of control wheat dough (a), 29.6% gluten-enriched dough (b), 33% pea protein-enriched  
 467 dough and 31.9% rubisco-enriched dough (d1, d2). Proteins were stained using Alexa Fluor™ 546 NHS Ester (in  
 468 red in the images) in all samples. Only the rubisco-enriched dough has double labelling: rubisco was specifically  
 469 labelled with FITC (in green in the images) before it was incorporated in the dough.

470 **4. Discussion**

471 Rubisco concentrate was used to improve the nutritional profile of wheat semolina dough. In comparison to gluten  
 472 and pea proteins, rubisco exhibited specific behaviour. Indeed, adding rubisco preserved the elastic potential of  
 473 wheat dough in contrast to adding gluten and pea protein. Rubisco sub-units formed both weak and covalent bonds,  
 474 with a probable interaction with wheat proteins.

475 *Unlike pea proteins or gluten, adding rubisco enhanced wheat dough stiffening during a temperature ramp*

476 Dilution of wheat semolina with increasing concentrations of extrinsic gluten reduced the rise in elastic modulus  
 477 between 55° and 80°C and shifted  $\tan(\delta)$  to higher values. However, the addition of gluten did not modify the drop  
 478 of  $\tan(\delta)$  during heating, which remained parallel to that of the standard wheat dough. Similar mechanical changes  
 479 have already been reported in wheat doughs (N. M. Edwards, Dexter, Scanlon, & Cenkowski, 1999) and in protein-  
 480 starch-water systems (Hibberd, 1970). Thus, a decrease in the starch-gluten ratio in wheat dough increases the  
 481 liquid-like behaviour of dough (rise of  $\tan(\delta)$ ) and limits the rise of the elastic modulus during heating. In addition,  
 482 SE-HPLC analysis of SDS-soluble proteins from thermally treated gluten-enriched wheat dough demonstrated that  
 483 adding gluten does not alter protein crosslinking during heating. Furthermore, extrinsic gluten and native semolina  
 484 proteins were shown to behave similarly, forming SDS insoluble aggregates under thermal treatment. Thus, the  
 485 decrease in  $E'$  values in gluten-enriched wheat doughs is not related to a change in gluten protein polymerisation  
 486 but rather to a change in starch gelatinisation. Several authors reported that adding gluten in starch-gluten blends  
 487 can affect the starch gelatinisation rate and extent due to competitive hydration or by hindering water diffusion  
 488 (Mario Jekle, Mühlberger, & Becker, 2016b). Further analysis by differential scanning calorimetry (DSC) is now  
 489 needed to determine if extrinsic gluten can act as a water trap.

490 Similarly, the enrichment of wheat dough in pea proteins reduced  $E'_{\max}$  values with no major modification of wheat  
491 protein crosslinking upon heating. It can be concluded that like gluten, pea proteins compromise starch  
492 gelatinisation. Moreover, pea protein-enriched doughs presented the highest  $\tan(\delta)$  values above a temperature of  
493  $65^{\circ}\text{C}$ , meaning that the relative viscous part of the dough increased as a result of the addition of pea protein. This  
494 could be related to the higher water-binding capacity of pea proteins compared to gluten proteins (Bravo-Núñez,  
495 Garzón, Rosell, & Gómez, 2019; Peters, Vergeldt, Boom, & van der Goot, 2017). In addition, CLSM images of enriched  
496 doughs revealed the presence of large protein particles, likely from pea proteins. They can affect the structure of  
497 the wheat gluten network due to steric hindrance. Several studies have shown that pea or faba bean enrichment  
498 reduces pasta quality by diluting the gluten network (Laleg et al., 2017; Petitot et al., 2010).  
499 Finally, rubisco enrichment of less than 8% affected the mechanical properties of dough in the same way as gluten  
500 or pea proteins, suggesting that starch gelatinisation is similarly compromised. However, above 8%, the addition of  
501 rubisco maintained a notable rise in  $E'$  during heating in a similar way to control dough. Rubisco contributes to the  
502 elastic and viscous properties of the system. Rubisco may not act as a water trap like extrinsic gluten or pea protein  
503 and may allow starch gelatinisation proceed with no hindrance. In addition, rubisco enhances the formation of  
504 disulphide-linked protein aggregates upon dough heating that may strengthen dough mechanical properties.  
505 Incorporating rubisco in dough also increases the  $\tan(\delta)$  values up to a temperature of  $65^{\circ}\text{C}$ . This increase in the  
506 liquid-like behaviour of the dough may be related to the decrease in the concentration of HMW glutenin polymers.  
507 The further decrease in  $\tan(\delta)$  values above  $70^{\circ}\text{C}$  may be related to the formation of large disulphide-linked polymers  
508 in thermally treated rubisco-enriched wheat doughs.  
509 In this study, rubisco differed from pea proteins, which do not react with semolina protein. However, other legume  
510 proteins, such as soy proteins, are able to form disulphide-bonded polymers when incorporated in wheat dough  
511 (Ribotta, León, Pérez, & Añón, 2005). But contrary to rubisco, this enrichment impairs the gluten network by  
512 reducing the overall size of the polymers (Pérez, Ribotta, Steffolani, & Le, 2008), resulting in reduced  $E'$  values during  
513 the temperature gradient (Zhou, Liu, & Tang, 2018).

514 *Rubisco sub-units participate in the network by means of both non-covalent and covalent bonds in wheat dough*  
515 Analysis of SE-HPLC profiles in control and rubisco-enriched dough led us to conclude that rubisco triggers the  
516 formation of large polymers stabilised by disulphide bonds. Rubisco subunits are involved in these large polymers  
517 by covalent bonding, sometimes stabilised by disulphide bridges.

518 The high concentration of free thiols in rubisco may partially explain rubisco's ability to form disulphide bonds when  
519 used to enrich wheat dough. Indeed, it has been suggested that free thiols enhance the formation of disulphide  
520 bond dynamics in the gluten network (Auvergne et al., 2008). It is known that an increase in temperature above  $50^{\circ}\text{C}$   
521 increases the size of glutenin polymers (Bloksma, 1972). Up to  $70^{\circ}\text{C}$ , this increase in size is explained by sulfhydryl-  
522 disulphide interchange (Schofield, Bottomley, Timms, & Booth, 1983). These interchanges are explained by radical  
523 and nucleophilic mechanisms (Auvergne et al., 2008). It has been assumed that nucleophilic mechanisms increase in  
524 the presence of thiolate groups in equilibrium with thiol functions. In that sense, incorporating rubisco could  
525 enhance the interchange of disulphide bonds by providing free thiol groups in the system. To validate this  
526 mechanism, controlled alkylation experiments should be performed on rubisco before it is incorporated in the  
527 dough. Rubisco's participation in sulfhydryl-disulphide interchanges with gluten proteins would imply it is a co-  
528 protein network.

529 Besides covalent interactions, non-covalent bonds play a significant role in the mechanical properties of the gluten  
530 network (Belton, 1999; Belton et al., 1995; Shewry, Popineau, Lafiandra, & Belton, 2001; Tkachuk & Hlynka, 1968).  
531 The balance between the amino acid residues involved in hydrogen bonds with water molecules and inter-chain  
532 hydrogen bonds would lead to the formation of a "loops and trains" structure. The deformation and relaxation  
533 capacity of these structures would provide the elasticity to gluten molecules (Belton, 1999). Moreover, recent  
534 structural models of gluten suggest that weak interactions act as junction points that initiate the formation of the  
535 gluten network during hydration and mixing. Such weak bond nodes allow gluten polymers to percolate and to form  
536 a network (Dahesh, Banc, Duri, Morel, & Ramos, 2016; Létang, Piau, & Verdier, 1999; Ng, McKinley, & Ewoldt, 2011).  
537 In our study, rubisco subunits were not leached out by water. Most were recovered in the gluten-like fraction and

538 eluted at their expected molecular weight. This suggests that rubisco subunits establish weak bonds with wheat  
539 protein during dough mixing before any thermal treatment. Electrostatic interactions are unlikely because gluten  
540 proteins are weakly charged. Hydrophobic and hydrogen interactions control rubisco heat gelation in dispersed  
541 systems (Libouga, Aguié-Béghin, & Douillard, 1996; Martin et al., 2014). The contribution of both hydrophobic and  
542 hydrogen interactions in rubisco reactivity in wheat dough during mixing can be assumed.  
543 The formation of a co-protein network between gluten proteins and rubisco is the most likely hypothesis to explain  
544 our results concerning the interactions established by rubisco during mixing and heating and the properties of the  
545 resulting dough. This hypothesis is supported by protein network microstructure visualised by CLSM since rubisco  
546 and gluten are co-located in the network before thermal treatment. However, further investigations are needed to  
547 prove that rubisco and gluten interact specifically with each other.

#### 548 **Conclusions**

549 The study of the mechanical properties and protein interactions of rubisco-enriched wheat dough clearly highlights  
550 its potential ability to increase the plant protein content of cereal-based foods. Rubisco behaviour is quite different  
551 from that of legume or even gluten proteins. Enrichment in pea proteins or gluten does not modify protein  
552 polymerisation even after thermal treatment. The thermo-mechanical properties of these pea or gluten-enriched  
553 doughs appear to be affected probably due to a modification of the distribution of water in the system, thereby  
554 limiting starch gelatinisation. The behaviour of rubisco is very different. Interestingly, rubisco protein is able to  
555 preserve the increase in elasticity of the dough during heating thanks to its own reactivity and to possible low  
556 competition with starch for water. Remarkably, hydrated and mixed with wheat semolina, rubisco formed both weak  
557 and disulphide bridges. It then joined the water-insoluble protein network. The concentration of large covalently  
558 linked polymers increased considerably during dough thermal treatment, because of the ability of rubisco to form  
559 new aggregates in these conditions. The colocalisation of gluten and rubisco proteins on micrographs supports the  
560 hypothesis that they even formed a co-protein network. To confirm the benefits of using rubisco to enrich cereal  
561 based food, it would be useful to test the mechanical properties of rubisco-wheat matrices at high deformations to  
562 better investigate the effect of protein enrichment on the rheological properties of the new food system in  
563 comparison to the better-known pea protein-enriched wheat matrices.

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#### 574 **Competing interest statement**

575 None

576 **References**

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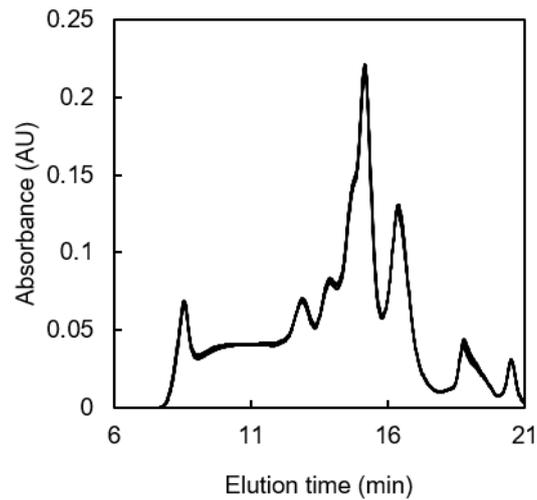
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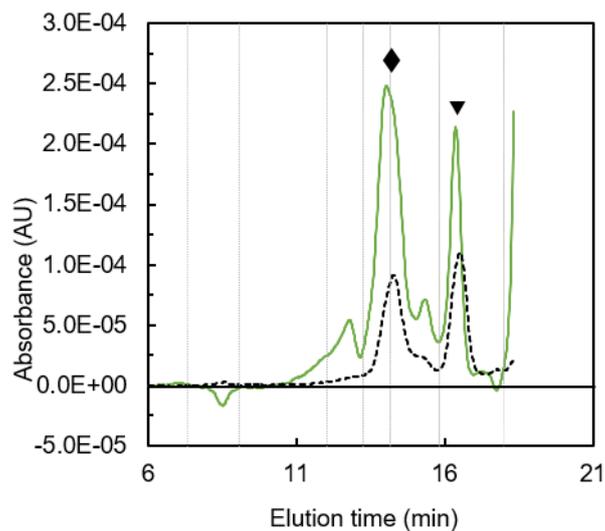
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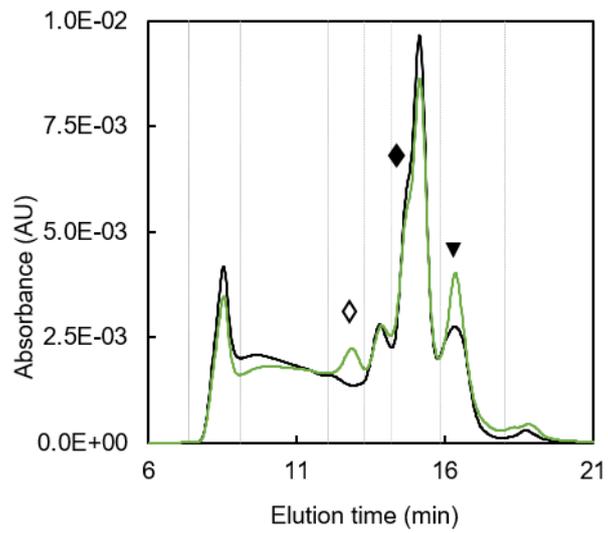
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**Supplementary material**

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 805 **Supplementary fig. 1** Comparison of three SE-HPLC elution profiles of SDS soluble proteins extracted from the  
 806 same sample and two chromatograms extracted from another sample in exactly same conditions (16.3% rubisco-  
 807 enriched dough, 48.2% water wb).  
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 810 **Supplementary fig. 2** Differential SE-HPLC profile (solid line) of SDS+DTE soluble proteins from a 37.4% rubisco-  
 811 enriched raw dough compared to the profile of rubisco protein concentrate (dotted line) adjusted to the same  
 812 rubisco weight. Differential profiles were obtained by subtracting the SE-HPLC profiles of SDS+DTE soluble proteins  
 813 of wheat control raw dough from the profile of rubisco-enriched raw dough (both adjusted to same semolina  
 814 weight). For the sake of readability, the elution profile is represented only up to 18 minutes. Symbols represent  
 815 rubisco subunits: small chain (SC: ▼) and large chain (LC: ◆).



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 817 **Supplementary fig. 3** Experimental SE-HPLC profiles (solid lines) of SDS soluble proteins of gluten-like fraction  
 818 extracted from raw control doughs (black line) and 37.4% rubisco-enriched dough (green line). Symbols represent  
 819 rubisco subunits: small chain (SC; ▼), large chain (LC; ◆) and large chain dimer (LC dimer; ◇). Elution profiles after  
 820 normalisation on the basis of 1mg of total protein in the sample.  
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