1 1 **Acidification of protein-enriched rice starch doughs: effects on breadmaking**

2 2 Marina Villanueva¹, Raul R.Mauro¹, Concha Collar², Felicidad Ronda^{1*}

3

4 4 (1) Department of Agriculture and Forestry Engineering, Food Technology, College of
5 5 Agricultural and Forestry Engineering, University of Valladolid, Av. Madrid, 44, 34004
6 6 Palencia, Spain.

7 7 (2) Food Science Department, Instituto de Agroquímica y Tecnología de Alimentos,
8 8 IATA-CSIC, Avda. Catedrático Agustín Escardino 7, 46980 Paterna, Spain.

9 9 *Corresponding author: Felicidad Ronda. College of Agricultural and Forestry
10 10 Engineering, University of Valladolid, Av. Madrid, 44, 34004 Palencia, Spain.

11 11 Tel: +34979108339; Fax: +34 979108200. E-mail: fronda@iaf.uva.es

Abstract

The impact of acid incorporation (acetic+lactic, 0.5%) into rice starch-based doughs enriched with different proteins (egg albumin, calcium caseinate, pea and soy protein isolates) at different doses (0, 5 and 10%) was investigated on dough proofing and thermal properties, and bread quality. Proteins from vegetal sources led to breads with lower specific volume and harder crumb, effects being magnified with protein dose and reduced with acid addition. Incorporation of proteins from animal source resulted in different behaviours according to the protein type, dosage and acidification. Protein addition increased the dough pH and total titratable acidity, and reduced the impact of acid addition on dough acidity. Albumin-added doughs had significantly higher temperature of gelatinization than most of other supplemented doughs, while vegetal proteins led to significantly lower gelatinization enthalpy than the control dough. Acid addition improved significantly the volume and texture of protein-enriched breads without detriment of either odour or taste.

Keywords: Acetic acid; Gluten-Free Doughs; Lactic acid; Proteins; Gluten-free bread

1
2
3 29 **1. Introduction**
4

5 30 Legume- and cereal-based foods such as bread are rich in complex carbohydrates and
6
7 31 other essential nutrients, representing staple foods in many countries worldwide. The
8
9 32 digestion of the gluten-forming storage proteins present in cereals such as wheat and
10
11 33 related grains like barley, rye and triticale, release a peptide from the α -gliadin fraction
12
13 34 which induces a systemic immunemediated disorder, called coeliac disease, in
14
15 35 genetically susceptible persons (Fasano and Catassi, 2012). Rising demands for gluten
16
17 36 free (GF) products parallels the apparent or real increase in coeliac disease, or other
18
19 37 allergic reactions/intolerances to gluten consumption (Lazaridou, Duta, Papageorgiou,
20
21 38 Belc and Biliaderis, 2007). Therefore, the target group of GF products is currently
22
23 39 expanding to adhere, in addition to coeliac patients, people looking for nonallergenic
24
25 40 ingredients, leading to a new market that welcomes a variety of products. Also, GF
26
27 41 products can act as templates for the development of other products addressed to
28
29 42 specific vulnerable groups of population with special nutritional needs (Kelly, Moore &
30
31 43 Arendt, 2008). One of the main issues of the bakery industry is still to improve the
32
33 44 quality of GF breads currently available in the market.
34
35
36 45 Starch is the main component of dry substance of bread and plays an important role in
37
38 46 establishing its structure and mechanical properties. The role is even greater in the case
39
40 47 of GF bakery, where the visco-elastic wheat protein network is replaced by blends of
41
42 48 different structuring agents (Gallagher, 2009; Ziobro, Korus, Witczak & Juszczak,
43
44 49 2012). Rice starch is one of the more preferred starches for GF bread production
45
46 50 (Onyango, Mutungi, Unbehend and Lindhauer, 2011; Kittisuban, Ritthiruangdej and
47
48 51 Supphantharika, 2014). Rice flour has several significant properties such as natural,
49
50 52 hypoallergenic, colorless, and bland taste. It has also very low level of protein, sodium,
51
52 53 fat, fiber and high amount of easily digested carbohydrates. Hydroxy propyl methyl
53
54
55
56
57
58
59
60

cellulose (HPMC) is one of the most suitable hydrocolloids to enhance the optimum volume expansion, texture and a general improvement of rice-based GF bread quality (Marco and Rosell, 2008a; Nikolić, Dodić, Mitrović and Lazić, 2011; Ronda, Pérez-Quirce, Angioloni and Collar, 2013). Proteins from different sources (soybean, pea, egg albumen and casein) can be added to GF basic formulations resulting in nutritional benefits and, in some cases, improved volume and appearance of GF breads (Marco and Rosell, 2008b; Ziobro, Witczak, Juszczak and Korus, 2013). Proteins are added to increase elastic modulus by cross linking, to improve perceived quality by enhancing Maillard browning and flavour, to improve structure with gelation and to aid in foaming (Ronda, Villanueva and Collar, 2014b). Protein addition results in breads with increased loaf volume, improved crumb regularity and enhanced sensory characteristics (Crocket, Le and Vodovotz, 2011). Starch-protein interactions can affect starch gelatinization and further retrogradation, bread quality and shelf life.

Acidification through lactic acid and acetic acid addition confers suitable properties to final breads in terms of both odour and taste either when produced by the exogenous microflora or added into breadmaking matrices, increasing protease and amylase activities that lead to a retarded staling during storage (Moore, Dal Bello and Arendt, 2008). This has been attributed to an increase in net protein charge, which leads to promoted solvent interaction and easier protein unfolding, but which could eventually prevent strong network formation (Jayaram, Cuyvers, Verstrepen, Delcour and Courtin, 2014). Despite dough acidification by acetic acid and lactic acid addition is known to impact the properties of dough (Ohishi, Kasai, Shimada and Hatae, 2007; Jeckle and Becker, 2012; Ronda et al., 2014b;) and, therefore, also to affect the quality of the final baked product (Jayaram et al., 2014), the impact of acidification in protein enriched GF breads has not been reported so far.

It is assumed that inter e intra-molecular interactions established between exogenous proteins and starch molecules that are the main responsible for dough structurization and bread characteristics, certainly dependent on dough pH. This study aims to determine the combined effect of acid addition and protein supplementation on dough proofing, thermal properties and quality attributes of GF breads.

2. Material and methods

2.1. Materials

Rice starch (9.9 % moisture, 0.2 % ash and 0.5 % protein) from Ferrer Alimentación S.A. (Barcelona, Spain), and salt, sugar and sunflower oil purchased from the local market, were used to make GF doughs. Hydroxypropylmethylcellulose (HPMC, Methocel K4M Food Grade) was provided by Dow Chemical (Midland,USA). Proteins used in GF formulations were: soybean protein isolate (SPI) Supro 500-E IP from Proveedora hispano-holandesa S.A. (Barcelona, Spain), calcium caseinate (CA) from Armor proteins (Saint-Brice-en-Coglès, France), egg albumin (EA) in dry powder from Eurovo (Valladolid, Spain) and pea protein isolate (PPI) branded Pisane C9, from Cosucra (Warcoing, Belgium). Acetic acid and lactic acid (analytical grade; Panreac, Barcelona) were used as a source of hydrogen ions.

2.2. Methods

2.2.1 Dough preparation and breadmaking

A straight dough process was performed using the following formula on a 100 g rice starch (or rice starch+protein) basis: 6 % oil, 5 % sucrose, 1.5 % salt, 2 % HPMC, 3% dried yeast and 80 % water. All proteins were added at 0 %, 5 % and 10 % w/w (starch+protein basis) levels. Doughs were supplemented with 0.1 % + 0.4 % (w/w starch+protein basis) of acetic and lactic acid, respectively, when acid-treatment was

1
2
3 104 applied. The experimental design is shown in Table 1. GF dough-making was achieved
4
5 105 by blending first solid ingredients and oil in a kitchen-aid professional mixer (KPM5).
6
7 106 Then water was added and hand mixed. Finally the dough was mixed with dough hook
8
9 107 at a speed 4 for 8 min. Acid blend, when added, was diluted in a small part of water (7
10
11 108 % of total) and adjusted to the dough before the mixer was powered on. The dough, 200
12
13 109 g, was placed into an aluminium pan and was proofed at 27°C and (85 ± 5) % relative
14
15
16 110 moisture for 50 min. Subsequently, baking was carried out in a Salva oven (Lezo,
17
18 111 Spain) at 190°C for 40 min. After baking, breads were left for one hour at room
19
20 112 temperature before analysis. To study the effect on staling, breads were stored for 2
21
22 113 days at (4 ± 2) °C.

23 114 2.2.2 Dough measurements

24
25 115 Gas production along fermentation of formulated GF doughs was continuously
26
27 116 registered in the rheofermentometer (Chopin Rheofermentometer F3, Chopin
28
29 117 Technologies, Villeneuve-La-Garenne Cedex, France). In contrast to the traditional
30
31 118 method, the weight of dough was reduced to 200 g and the weights were removed
32
33 119 adapted to dough softness.. Fermentation was carried out at 37 °C for 4 h. The
34
35 120 parameters registered included: H_m , height of dough at maximum development time
36
37 121 (mm); h , height of dough at the end of the test (mm); $(H_m - h)/H_m$ that is inversely related
38
39 122 to dough stability; T_1 time corresponding to H_m (min); T_2 : Time of stabilisation, where
40
41 123 the dough height is above 90% H_m (min); H'_m , maximum height of CO₂ production
42
43 124 (mm); T'_1 , time of the maximum gas formation (min); V_T , total volume of CO₂ (mL)
44
45 125 produced during 4 h of fermentation; V_r , total volume of the CO₂ (mL) retained by the
46
47 126 dough; R_C , the CO₂ retention coefficient V_r/V_T , which measures the amount of CO₂
48
49 127 liberated and retained from the dough and therefore is related to the porosity of the
50
51 128 dough; T_X , the time (min) when the porosity of the dough develops.
52
53
54
55
56
57
58
59
60

129 pH measurements of dough samples were made in a Oakton-Eutech Instruments PH6
130 pH-meter at regular intervals of 5 min up to 60 min during proofing.
131 Total titratable acidity (TTA) was measured on ten grams of dough blended with 100
132 mL acetone/water (5/95, v/v) under constant stirring. The titration was carried out
133 against 0.1N NaOH until a final pH of 8.5. The results were expressed as
134 milliequivalents of lactic acid/g of dough. This measurement was carried in triplicate on
135 unyeasted doughs.
136 Thermal characteristics of doughs were determined using a differential scanning
137 calorimeter (DSC-822e, Mettler Toledo, SAE). Freeze-dried hydrated doughs (≈ 6 mg
138 dry matter) were weighed into aluminium pans of 40 μ L (ME-29990, Mettler Toledo,
139 SAE) and distilled water was added using a micropipette to make 70% moisture
140 content. The samples were scanned from 20 to 110°C at 5 °C/min using an empty pan as
141 reference. Starch retrogradation was evaluated in the samples previously gelatinized in
142 the DSC oven stored in the pans at (4 $\pm$ 2) °C for 2 days. These samples were scanned
143 from 0 to 110 °C at a heating rate of 5 °C/min. The enthalpy (ΔH), left temperature
144 (T_{left}), peak temperature (T_p) and the difference $T_{\text{right}} - T_{\text{left}}$ (ΔT), as a measurement of the
145 width of the endotherm peaks, were measured in both scans, at 0 and 2 days of storage.
146 Reported values are the mean of duplicate measurements.

147 2.2.3 Evaluation of bread quality

148 The volume, height and width of bread were determined from four replicates using a
149 Volscan profiler 300 (Stable Microsystems, Surrey, UK) analyser. The breads were
150 weighed immediately after removal from the pan once cooled.

151 Crumb texture was determined in quadruplicate with a TA-XT2 texture analyser (Stable
152 Microsystems, Surrey, UK) provided with the software “Texture Expert”. An
153 Aluminium 20 mm diameter cylindrical probe was used in a compression test (TPA) to

penetrate to 50% depth, at 1 mm/s speed test. Hardness (N) was the force at the maximum deformation. Analysis were carried out at (20 ± 2) °C for two bread slices of 20 mm thickness taken from the centre of the loaf. Two loafs were measured.

Colour was measured with a Minolta spectrophotometer CN-508i (Minolta, Co.LTD, Japan). Results were obtained in the CIE L*a*b and CIE L*C*h coordinates using the D65 standard illuminant, and the 2° standard observer. Colour determinations were made 5x5 times: bread crumb and crust colours were checked at five different points on each bread and every point was measured five times. The colour of proteins was also measured.

Sensory analysis was performed by a panel of ten trained judges (two males and eight females aged 25–53) from the baking laboratory. Eight training sessions were carried out; five of them in the specific attributes tested in breads. An intensity non-structured scale from 1 to 10 was used. The attributes tested were cell regularity (1 = very large and inhomogeneous cells; 10 = very small and homogeneous cells), acid taste and acid odour (1: very little; 10: very much) taste and odour (different than acid) intensity (1: very little; 10: very much) and aftertaste persistency (1: very little; 10: very much).

3 Results and discussion

3.1 Dough properties

3.1.1 pH and total titratable acidity of doughs

Table 2 compiles values for dough properties according to the presence, type and dose of the different proteins. All doughs were made with and without acid addition in order to study the effect of acid presence and its possible interaction with the type or dose of protein. Protein presence increased significantly ($p < 0.01$) dough pH between 7 % (5% EA) and 12 % (10% CA, PPI or SPI) with respect to control dough. The lowest increase was obtained with EA. The dose of protein also affected significantly ($p < 0.01$) dough

pH. Acidification of protein-enriched doughs resulted in pH values 15 % – 34 % higher than the acid-added control dough. Proteins exerted a buffering effect on doughs, also confirmed by the higher TTA of the protein-enriched doughs in spite of its higher pH. TTA also increased significantly ($p<0.01$) as the protein dose increased. The highest effect on TTA was exerted by CA. As expected, acid addition increased dough TTA. In general, acidification of protein-enriched doughs led to significantly higher TTA than the acid-added control dough. However the increase was not very relevant except for CA-added doughs. The decrease of pH during proofing, ΔpH , in unacidified doughs, with or without proteins, was 0.21-0.36. However, acidified doughs had a stable pH that varied less than 0.1 units in spite of acid generation during proofing, showing the highest buffering capacity around $pH=4.5$.

3.1.2 Fermentative properties of doughs

Figure 1 shows the gassing power (production/retention) and dough development of protein-added doughs in presence/absence of acid. Acid addition to control dough delayed significantly both gas production and maximum dough development along proofing. T_1' and T_1 passed from 93 and 189 min to 162 and 236 min, respectively. Low pH of acid-added control doughs, 3.9, could explain the effect. Similar results were observed in acidified GF doughs, and related to yeast inhibition (Blanco, Ronda, Pérez and Pando, 2011). The undissociated forms of acetic acid can pass across the membrane into the cell by simple diffusion (Stratford, 1999; Piper, Calderon, Hatzixanthi and Mallapour, 2001). Once inside the cytoplasm, the undissociated form of the acid dissociates, liberating protons. The lower internal pH prevents normal yeast growth (Krebs, Wiggins, Stubbs, Sols and Bedoya, 1983) and leads to other physiological alterations, affecting fermentative activity, yeast cell viability (Peres, Tininis, Souza, Walker and Lalue, 2005) and effective dough development (Blanco et al., 2011).

204 Acidification of protein-enriched doughs showed a much more smoothing effect on the
205 time of maximum gas production except for EA-added doughs that increased by 20%
206 and 50% with respect to unacidified doughs for 5 and 10% dosages, and for 5% SPI-
207 and 5% PPI-added doughs that showed a delay in gas production of 27% and 168%,
208 respectively. A significant negative correlation was obtained between dough pH and
209 time of the maximum gas production, T_1' ($p < 0.001$; $r = -0.80$) in agreement with the
210 effect of acetic acid on yeast activity explained previously.

211 The maximum height of gas production (H'_m) declined with the presence of protein in
212 doughs. However, except for 10% PPI, where no effect was observed, the acidification
213 of the protein-enriched doughs improved the H'_m value, opposite to the effect observed
214 when the control dough was acidified. In general, little or no permeability was observed
215 in rice starch doughs. More than 90% of the gas produced during fermentation was
216 retained in all doughs. The lowest retention coefficient was achieved for 10% PPI- or
217 SPI-added doughs. Again, vegetal and animal proteins led to different behaviour. The
218 effect of protein and acid addition on dough development depended significantly
219 ($p < 0.001$) on the type and dose of protein. The maximum height of dough development,
220 H_m , was dependent not only on the volume of gas produced or retained by dough during
221 proofing, but also on the capacity of expanding under the action of the gas produced.
222 Positive correlations were obtained between H_m and V_T and V_r ($p < 0.01$ $r = 0.87$ and
223 0.95 respectively). As well, H_m was negatively correlated with all dough rheological
224 properties related to consistency: G' and G'' modulus ($p < 0.001$; $r = -0.72$ and $p < 0.05$;
225 $r = -0.55$ respectively), the maximum stress in the linear viscoelastic region, τ_{max} ,
226 ($p < 0.01$; $r = -0.66$) and the steady state viscosity, μ_o , ($p < 0.05$; $r = -0.54$), and positively
227 correlated with the properties that relate to the elastic or viscoelastic deformation under

228 the application of a constant stress, J_0 or J_1 from a creep test ($p<0.05$; $r=+0.51$ in both
229 cases) (Ronda et al., 2014b).

230 *3.1.3 Thermal properties of doughs*

231 Dough thermal properties, studied by DSC, are summarized in Table 2. A significant
232 effect of protein presence on starch gelatinization enthalpy (ΔH_{gel}) was observed. The
233 enthalpy, expressed on starch basis, is independent on the sample starch content, that
234 obviously was lower in protein-enriched doughs. The average ΔH_{gel} of the control
235 dough, 11.4 J/g starch, was similar to that of doughs enriched with animal proteins but
236 superior to that of doughs with vegetal ones with an average value of 10.8 J/g starch, for
237 both SPI and PPI. The increase in protein dose did not exert a significant decrease on
238 this transition enthalpy. Noisuwan, Bronlund, Wilkinson and Hemar (2008), who
239 studied the effect of milk proteins on thermal properties of rice starch, found a decrease
240 in ΔH_{gel} when the concentration of sodium caseinate passed from 5% to 10% in the
241 mixture casein/rice starch. Similar tendency was observed in the current study although
242 the difference was very small and not significant. In this case, the different counter-ion,
243 calcium instead of sodium, and the application of mechanical energy during mixing for
244 dough making of protein/starch, could account for the observed differences. The
245 acidification of the dough did not affect ΔH_{gel} significantly as was reported by others
246 (Ohishi et al., 2007).

247 The gelatinization temperature was significantly affected by the type and dose of protein
248 and by the interaction (protein type*dose). Acid also affected rice starch gelatinization
249 temperature in presence of vegetal proteins. When gluten was added to wheat starch, the
250 gelatinization temperature of the starch increased. However, the influence of proteins
251 other than gluten on DSC parameters were not very deeply determined (Eliasson &
252 Larsson, 1993). Both CA and SPI decreased T_{onset} and T_{peak} by $\sim 1.4^{\circ}\text{C}$ and $\sim 1.1^{\circ}\text{C}$

respectively with respect to the control dough. Doughs with EA, showed a higher gelatinization temperature than the remaining protein-enriched doughs, although values were not significantly different than that of control dough. With vegetal proteins, the higher the dose the lower the temperature of gelatinization, although only in PPI presence the effect was significant. No effect of casein dose was observed on T_{onset} and T_{peak} as reported Noisuwan et al., (2008). The acidification of the dough did not affect T_{onset} and T_{peak} of the control dough and doughs enriched with animal proteins; other authors found endothermic peak temperatures of rice starch lower in presence of acetic acid (Hibi, 2002; Ohishi et al., 2007). However, the acetic acid concentration added to rice starch was 6 – 10 times the amount of acetic+lactic used in the current study, where the addition was only the acid concentration usually provided by sourdoughs. The limited acid concentration was probably unable to promote starch hydration and further enhancement of starch gelatinization, as stated by Ohishi et al., (2007). Conversely, T_{onset} and T_{peak} increased significantly $\sim 1.5^{\circ}\text{C}$ and $\sim 1.0^{\circ}\text{C}$, respectively with the acidification of 5%/10% PPI- or 5% SPI-enriched doughs. The nature of proteins and their physico-chemical properties (size, shape, composition, structure, net charge and charge distribution, ability to interact or to repel other components, etc.) affect the gelatinization of studied doughs. The higher temperatures obtained with EA might be due to its lower water absorption (Damodaran, 2008).

The second scan from 0°C to 110°C applied to gelatinized samples stored in the DSC pans at $(4\pm 2)^{\circ}\text{C}$ for 2 days (retrogradation scan) led to two visible peaks. The first one, very wide, at a peak temperature that ranged $49\text{--}50^{\circ}\text{C}$ was related to the melting of the recrystallized amylopectin, and the second one at temperatures of $97\text{--}102^{\circ}\text{C}$ probably related to the amylose-lipid complex dissociation (Eliasson, 1994). This transition, not observed in the gelatinization scan, took place at an usual temperature for this

phenomenon when the water content of the starch suspension is high enough (> 60-70%) as it was the case. Eliasson (1994) reported that the increased values during the second scan, also observed by other authors, are probably due to better conditions for complex formation after the first heating. In the case of starch it has relation to the leaking of amylose from granules that occurs at temperatures above gelatinization temperature range. The average enthalpy quantified in tested samples for amylose-lipid complex dissociation was 1.2 J/g starch ranging between 0.8 y 1.8 J/g starch, without significant differences based on acid or protein presence. Similar enthalpy for this endotherm was previously reported (1.3 J/g starch) for wheat starch in excess water where the lipid compound of the complex was almost exclusively attributed to lysolecithin (Eliasson & Larsson, 1993). The melting enthalpy of the recrystallized amylopectin (ΔH_{ret}) in two days ranged 0.5 – 1.3 J/g starch. The large width of these small peaks was responsible for the low accuracy of the enthalpy and peak temperature values making it difficult obtaining significant differences. It seems that, in protein presence the retrograded amylopectin melted at higher temperatures meaning higher stability of crystal structure. It is still pending to measure retrogradation after long storage periods to assess the effect of proteins on retrogradation extension. At short storage times different retrogradation seems no justify the differences observed in bread hardening, as could be seen below.

3.2 Bread quality properties

3.2.1 Loss of weight during baking

The loss of weight during baking varied between 15% (no acid 10% EA) and 21% (acidified 5% EA) (Table 3). Protein addition affected loss of weight, effects being dependent on the protein type and dose, and on the absence/presence of acid. In general,

except for CA, the presence of protein led to a decrease in the loss of weight during baking that was more marked at higher protein concentration, with a reduction of 21% when 10% of EA, PPI and SPI were added. The well established water binding capacity of proteins would explain this effect (Zayas, 1997). Acidification effect depended on protein presence. Acidification of control dough reduced 10% the losses during baking, while significant increases in presence of protein —7% and 15% for 5 and 10% of SPI and 7% for 5% EA— were observed. Probably the increase of protons concentration in dough stabilised some high electronic density functional groups, decreasing interactions with water molecules by hydrogen bridges and, hence water binding capacity. This must be dependent on dough pH and protein nature. The correlation study of the loss of weight with dough rheological properties previously reported (Ronda et al., 2014b) denoted a strongly correlation when the study was confined only to vegetal proteins. There was a negative correlation with the elastic, G' , and viscous, G'' , moduli ($p<0.01$, $r=0.99$ and $p<0.05$, $r=0.97$ respectively) and a positive correlation with the instant elastic compliance, J_0 , ($p<0.01$ $r=0.98$). Probably the same factor that improved dough consistency or reduced the capacity of deformation under a stress increased the loss of weight. The loss of weight showed a general significant positive correlation with H_m and H_m' ($p<0.01$; $r=0.69$ and $p<0.05$; $r=0.63$ respectively) and with the specific volume ($p<0.001$; $r=0.86$). The same factor (probably the presence of protein) that decreased the dough development during proofing and led to lower bread volumes, improved the water binding capacity causing a decrease in baking loss.

3.2.2 Specific volume and height/width of breads

Loaf-specific volume, which varied from 1.36 mL/g (no acid 10% PPI) to 4.59 mL/g (acidified 5% EA), and the loaf height/width, which varied from 0.37 to 0.91, exhibited similar trends (Table 3). Both properties were strongly correlated ($p<0.001$, $r=0.82$) as

could be expected in pan breads. A slight lack of symmetry in breads could explain a Pearson coefficient different than 1. The presence of protein affected bread specific volume differently depending on the type and dose and on the absence/presence of acid. In acid-free doughs, both CA and PPI decreased the specific volume by 13% and 65% respectively, irrespective of the dose. However the effect of the addition of EA or SPI was strongly dependent on the dose. The addition of 5% SPI only reduced 18% the volume while the highest dose led to a decrease of 64%. In gluten-free breads, a negative effect of SPI (Ribotta, Ausar, Morcillo, Pérez, Bertramo and León, 2004; Marco and Rosell, 2008a; Crockett et al., 2011) and PPI and other proteins, except EA, (Ziobro et al., 2013) on final bread quality has been reported. 5% EA was the only protein addition with no negative effects on bread volume as observed by others (Ziobro et al., 2013). These breads were significantly bigger than the rest of supplemented breads. This effect could be attributed to EA foaming capacity (Zayas, 1997). EA exhibits relatively low molar masses, and contains mostly acidic amino acids. Their ability to bind carbon dioxide could have an important influence on dough structure during baking (Ziobro et al., 2013). The EA addition at 10% level reduced the volume dramatically. Ziobro et al., (2013) still found an important positive effect on GF bread specific volume at 10% dosage. In this paper, the control bread had a notably higher specific volume than that reported by Ziobro et al., (2013), probably due to the use of different starch source and different hydrocolloid to mimic the gluten action. This could explain the different effect of the dose of EA on the final volume of breads. The acid addition exerted a varying effect depending on the presence, type and dose of protein. The acidification of the control dough reduced the bread specific volume in ~34%. However, when applied to protein-supplemented doughs always increased the bread volume, except with 5% EA, 10% CA and 10% PPI, where no significant effect

was obtained. Specific volume showed a significant correlation with H_m ($p < 0.001$; $r = 0.80$). The different expansion of doughs during proofing and baking could be due to several factors: i) the lower gas production as consequence of a lower substrates concentration for yeast as took place in our case in protein presence (Witczak, Korus, Ziobro and Juszczak, 2010; Ziobro et al., 2013); ii) the higher dough consistency that can restrict dough expansion under the action of the stress produced by the gas formed during proofing and expanded during baking (Perez-Quirce, Collar and Ronda, 2014) until the bread crumb structure is formed, which is mainly dependent on starch gelatinization and protein denaturation (Eliasson & Larson, 1993). A proper consistency of doughs is necessary. A too low dough consistency, as those with 10% EA (Ronda et al., 2014b) could explain the low specific volume of breads by its inability to retain the produced and expanded gas.

3.2.3 Colour of breads

The crust colour, an important parameter for consumer acceptance (Ziobro et al., 2013), is mainly determined by Maillard reaction. It depends directly on the available water, the concentration of carbonyl groups from reducing sugars, the amount of amine groups mainly proceeding from the proteins added (Purlis, 2010), and the pH (Pyler, 2000). It varied significantly with the presence, type and dose of protein, and the presence/absence of acid (Figure 2). It was also influenced by 2nd order interactions of all the factors studied. The crust lightness, L_c^* , ranged 66 – 45 (Fig. 2a). As could be expected the protein presence always decreased it, in greater extent when the dose increased. The lightest crust corresponded to the control bread and the darkest one to that with 10% EA. The acid addition led to lighter bread crusts, with L^* values 6% (5% SPI) to 25% (10% SPI) higher than the unacidified doughs. The hue of the crust (Fig 2b) ranged from 69 (control bread) to 50 (10% EA) degrees and followed a parallel

evolution to the lightness. Protein concentration decreased the hue significantly, leading to more reddish crust. At 5% level the hue reduction ranged 6% - 16% for SPI and EA while it decreased to 16% -28% for the same proteins at 10% level. Acid addition also increased the crust hue. So that, acid blend counteracted the protein effect. The crust of acidified 5% protein-added breads had similar hue to that of the control bread whereas acidified 10% protein-added breads still showed a smaller crust hue than the control. The crust chroma (Fig. 2b) ranged from 26 to 37, for no acid 10% EA- and acid/5% EA-enriched bread respectively. The latter showed the most vivid colour. Proteins, except EA and SPI, at 5% level decreased the crust chroma between 6% and 8%, while breads with 10% SPI or EA showed a crust chroma 22% lower than the control bread. Colours of the proteins used for the enrichment of breads were measured in order to explain the trends observed in crumbs. The L^*C^*h coordinates were: CA (95.8; 5.5; 98.0), EA (96.3; 11.9; 91), PPI (87.3; 17.8; 80.2) and SPI (85.7; 17.5; 82.6). Consequently, both, EA and CA were practically white, with a very slight pure yellow hue with a very little tendency to red in the case of EA and to green in the case of CA. PPI and SPI were hardly darker than the two former, as indicated by smaller L^* coordinate. The hue of these vegetal proteins was also mainly yellow although they had a small reddish component (Fig.2c). PPI and SPI colours are represented in the first quadrant of the chromatic a^*-b^* diagram while CA and EA are depicted in the second quadrant, very near the y-axis. The crumb of breads enriched with EA or CA showed a^*-b^* coordinates that fell in the same quadrant than the pure proteins (Fig. 2c), showing similar hues. The crumb lightness varied from 66 to 80%. The highest lightness was from 10% EA- and 5% CA-enriched breads, with an increase of 14% and 9% with respect to the control crumb lightness. 10% SPI-added breads had the lowest lightness, 7% lower than the control.

3.2.4 Texture of breads

1
2
3 403 Fresh bread crumb firmness varied from 0.7 N (5% CA with acid) to 11.8 N (no acid
4
5 404 10% SPI) (Table 3). In this work, protein addition affected bread hardness, the extent of
6
7 405 the changes being dependent on the type and the dose of protein and on acid addition.
8
9 406 At 5% level only PPI increased the crumb firmness significantly with respect to the
10
11 407 control bread (360%). Increased dosage from 5 to 10% promoted dramatically the
12
13 408 crumb hardening of breads enriched with vegetal proteins, by two fold and eleven fold
14
15 409 the value of the control for PPI and SPI, respectively, while only breads containing EA
16
17 410 animal protein underwent a significant hardening promotion (+145%). Current results in
18
19 411 agreement with previous studies (Ahlborn, Pike, Hendrix, Hess and Huber, 2005; Marco
20
21 412 and Rosell, 2008a; Crockett et al., 2011; Ziobro et al., 2013) are compatible with the
22
23 413 creation of a robust cross linked structure by added proteins, especially supported in the
24
25 414 case of SPI by glicinin and a high water retention ability (Crockett et al., 2011). CA did
26
27 415 not promote any crumb hardening with respect to the control bread regardless either the
28
29 416 protein level or acid addition. Acid blend addition encompassed a great decrease in
30
31 417 vegetal-supplemented bread firmness, (-92% for 10% SPI and -79% and -37% for 5%
32
33 418 and 10% PPI respectively), as a result of the higher amount of air entrapped in the
34
35 419 crumb of more developed breads. Acidification of 10% EA-added breads also reduced
36
37 420 significantly ($p<0.05$) crumb firmness (- 46%). However, the acidification of the control
38
39 421 bread led to a 70% firmness increase. A significant correlation was obtained between
40
41 422 the dough elastic modulus at 1 Hz G_1' and the bread crumb firmness ($p<0.001$; $r=0.70$)
42
43 423 (dough rheological data from Ronda et al., 2014b). A neutral charge causes less
44
45 424 repulsion forces and less space for water molecules between the proteins. This repulsion
46
47 425 forces increase with increasing charge and more water molecules can be attached to the
48
49 426 protein strands whereby less mobile water is available in the dough system (Jekle &
50
51
52
53
54
55
56
57
58
59
60

427 Becker, 2012). As expected, a strong negative correlation was obtained between the
428 crumb firmness and the bread specific volume ($p<0.001$; $r=-0.85$).

429 The effect of acid on crumb firmness could also be related to a reduction of the extent of
430 amylose retrogradation, as it was previously reported (Ronda et al., 2014b). The setback
431 from RVA tests was strongly correlated with acid presence ($p<0.001$; $r=-0.81$). This
432 means that the strong tendency of amylose toward association by intramolecular
433 hydrogen bonds is reduced in a higher proton concentration resulting a lower tendency
434 toward crystallization. Amylopectin retrogradation is related to bread staling while
435 amylose recrystallization can be partially responsible for the initial firmness of fresh
436 breads (Zobel & Kulp, 1996).

437 *3.2.5 Bread staling*

438 Bread staling was assessed by means of crumb firmness evolution after two days of
439 storage at $(4 \pm 2)^{\circ}\text{C}$. (Table 3). It has been evidenced that amylopectin recrystallization
440 occurs faster in GF bread, than in traditional bakery products, where the structure is
441 determined by the presence of gluten (Eliasson & Larsson, 1993). Ziobro et al., (2012)
442 observed that bread staling was accelerated by vegetal protein supplementation. The
443 hardening of no acid PPI-, both at 5% or 10% level, and 10% SPI-enriched bread
444 crumbs was ten and three times higher respectively than that of the control bread while
445 animal proteins hardly affected bread staling. Dough acidification counteracted the
446 protein effect on bread ageing except for 10% PPI, which effect was kept despite the
447 addition of the acid blend. The hardening of the crumb is a complex phenomenon in
448 which multiple mechanisms operate. All involve starch recrystallization and water
449 migration (Ronda, Caballero, Quilez and Roos, 2011; Ronda, Quilez, Pando and Roos,
450 2014a). Factors affecting crumb bread staling have been extensively investigated (Zobel
451 and Kulp, 1996; Chinachoti and Vodovotz, 2001; Osella, Sánchez, Carrara, de la Torre

1
2
3 452 & Buera, 2005; Ziobro et al., 2012). In the current study differences among 2-day-
4
5 453 retrograded amylopectin extent were not detectable. The averaged melting enthalpy of
6
7 454 the recrystallized amylopectin was 0.8 J/g starch. Probably, molecular mobility and
8
9
10 455 water restriction in the real system played an important role in bread staling that could
11
12 456 not be detected in the DSC pan-baked doughs where starch was in an excess of water in
13
14 457 order to no restricting starch gelatinization.

15 16 458 *3.2.6 Sensory evaluation*

17
18 459 The results of sensory analysis of rice bread samples are given in Table 4. The presence
19
20 460 of protein notably improved cell regularity of bread. The lowest scores were obtained
21
22 461 for the control breads, with larger and inhomogeneous cells and the highest for 10%
23
24 462 EA, CA or SPI and 5% PPI. Acidification did not show a significant effect on cell
25
26 463 regularity of protein enriched breads, except when SPI was added that promoted a score
27
28 464 reduction of 42% and 24% for 5% and 10% respectively. The use of acetic and lactic
29
30 465 acid tried to simulate the mildly acid taste of conventional white bread that comes from
31
32 466 water-soluble organics acids formed by yeast and bacterial fermentation (Pyler, 2000).
33
34 467 What stood out from the sensory analysis was protein addition neutralised the acid
35
36 468 odour and taste of acidified breads. Unsupplemented (control) bread increased markedly
37
38 469 acid odour and taste scores (57% and 175% respectively) when the acid blend was
39
40 470 added whereas any significant effect was observed in protein-enriched breads except in
41
42 471 breads supplemented with 10% SPI whose acidification was detected by panellist who
43
44 472 gave a significantly higher acid taste score to the acidified bread. Acidified 10% CA-
45
46 473 enriched breads had the highest acid taste score among protein-supplemented breads,
47
48 474 95% higher than no acid control bread. The highest taste intensity and persistency
49
50 475 scores were obtained by 10% PPI-enriched breads either with or without acid addition.
51
52
53
54
55
56
57
58
59
60

1
2
3 476 In general, bakery products based on starch with proteins of different sources are
4
5 477 characterized by typical odour and taste of such ingredients (Ziobro et al., 2013). EA or
6
7 478 PPI odour and taste intensity and persistency could adversely affect overall acceptability
8
9 479 as happened to other authors (Crockett et al., 2011). Although the use of proteins
10
11 480 significantly improves the nutritional value of gluten-free breads because of its
12
13 481 enrichment in lysine and methionine (Marco & Rosell, 2008a), the addition of these
14
15 482 proteins usually reduced its sensory acceptability (Ziobro et al., 2013). Acid addition
16
17 483 improved significantly the volume and instrumental texture of protein-enriched breads
18
19 484 without detriment of its odour and taste.
20
21
22

23 485 **4 Conclusions**

24
25 486 Gluten-free formulations based on rice starch in association with different proteins (egg
26
27 487 albumin, calcium caseinate, pea protein and soy protein isolates) and acid addition led
28
29 488 to dough matrices with variable acidification, fermentative, and thermal profiles, and
30
31 489 subsequent different fresh and stored bread quality. Proteins exhibited a dosage
32
33 490 dependent buffering capacity on doughs, particularly for caseinate, and counteracted, in
34
35 491 general, the delay and decline of gas production and the inhibition of dough
36
37 492 development in acidified doughs. Supplementation of GF doughs with vegetal proteins,
38
39 493 that modified starch gelatinization transitions, led to lower volume and harder breads,
40
41 494 effects being magnified with protein dose and reduced with acid addition. Incorporation
42
43 495 of animal proteins gave different functional impact depending on the protein type,
44
45 496 dosage and acidification. Albumin-added doughs provided significantly higher
46
47 497 temperature and enthalpy of gelatinization than most of the other supplemented breads.
48
49 498 Acid addition improved significantly the colour, volume and texture (instrumentally
50
51 499 measured) of protein-enriched breads without impairment of sensory scores.
52
53
54
55
56
57
58
59
60

500

Acknowledgements

The research was supported by the Spanish Institutions Ministerio de Economía y Competitividad and the European Regional Development Fund (FEDER) (Projects AGL2012-35088 and AGL2011-22669), and Comunidad de Castilla y León (Project VA252A12-2). Special thanks to Sandra Pérez-Quirce for her help in baking and other laboratory tasks.

REFERENCIAS

- Ahlborn, G.J., Pike, O.A., Hendrix, S.B., Hess, W.M., & Huber C.S. (2005). Sensory, mechanical and microscopic evaluation of staling in low-protein and gluten-free breads. *Cereal Chemistry*, 82, 328-335.
- Blanco, C. A., Ronda, F., Pérez, B., & Pando, V. (2011). Improving gluten-free bread quality by enrichment with acidic food additives. *Food Chemistry*, 127, 1204-1209.
- Chinachoti, P., & Vodovotz, V. (2011). Bread Staling. CRC, Boca Ratón, Florida.
- Crockett, R., Ie, P., & Vodovotz, Y. (2011). Effects of soy protein isolate and egg white solids on the physicochemical properties of gluten-free bread. *Food Chemistry*, 129, 84-91.
- Damodaran, S. (2008). Aminoácidos, Péptidos y Proteínas. In: Fenemma. Química de los Alimentos. Ed. Damodaran, S., Parkin, K.L., Fennema, O.R. Editorial ACRIBIA S.A. Zaragoza (España).
- Eliasson, A. C., & Larsson, K. (1993). Interactions between components. In: Cereals in Breadmaking: A molecular Colloidal Approach. Marcel Dekker: New York.
- Eliasson, A. (1994). Interactions between starch and lipids studied by DSC. *Thermochimica Acta*, 246, 343-356.
- Fasano, A., & Catassi, C. (2012). Celiac disease. *New England Journal of Medicine*, 367, 2419-2426.
- Gallagher, E. (2009). Gluten-free. Food Science and Technology. John Wiley and Sons.
- Hibi, Y. (2002). Effects of salt, sugar and vinegar on melting of the amylose-lipid complex in rice starch. *Journal of Home Economics of Japan*, 53, 805-810.
- Jayaram, V. B., Cuyvers, S., Verstrepen, K. J., Delcour, J. A., & Courtin, C. M. (2014). Succinic acid in levels produced by yeast (*Saccharomyces cerevisiae*) during fermentation strongly impacts wheat bread dough properties. *Food Chemistry*, 151, 421-428.

534 Jekle, M., & Becker, T. (2012). Effects of Acidification, Sodium Chloride, and
535 Moisture Levels on Wheat Dough: I. Modeling of Rheological and Microstructural
536 Properties. *Food Biophysics*, 7, 190-199.

537 Kelly, A. L., Moore, M. M., & Arendt, E. K. (2008). 18 - New product development:
538 The case of gluten-free food products. In E. K. Arendt, & F. D. Bello (Eds.), *Gluten-*
539 *Free Cereal Products and Beverages* (pp. 413-431). San Diego: Academic Press.

540 Kittisuban, P., Ritthiruangdej, P., & Supphantharika, M. (2014). Optimization of
541 hydroxypropylmethylcellulose, yeast β -glucan, and whey protein levels based on
542 physical properties of gluten-free rice bread using response surface methodology. *LWT -*
543 *Food Science and Technology*, 57, 738-748.

544 Krebs, H.A., Wiggins, D., Stubbs, M., Sols, A., & Bedoya, F. (1983). Studies on the
545 mechanism of the antifungal action of benzoate. *Biochemical Journal*, 214, 657-663.

546 Lazaridou, A., Duta, D., Papageorgiou, M., Belc, N., & Biliaderis, C. G. (2007). Effects
547 of hydrocolloids on dough rheology and bread quality parameters in gluten-free
548 formulations. *Journal of Food Engineering*, 79, 1033-1047.

549 Marco, C. & Rosell, C.M. (2008a). Breadmaking performance of protein enriched,
550 gluten-free breads. *European Food Research and Technology*, 227, 1205-1213.

551 Marco, C. & Rosell, C. M. (2008b). Effect of Different Protein Isolates and
552 Transglutaminase on Rice Flour Properties. *Journal of Food Engineering*, 84, 132-139.

553 Moore, M., Dal Bello, F., & Arendt, E. K. (2008). Sourdough fermented by
554 *Lactobacillus plantarum* FST 1.7 improves the quality and shelf life of gluten-free
555 bread. *European Food Research and Technology*, 226, 1309-1316.

556 Nikolić, N., Dodić, J., Mitrović, M., & Lazić, M. (2011). Rheological properties and the
557 energetic value of wheat flour substituted by different shares of white and brown rice
558 flour. *Chemical Industry and Chemical Engineering Quarterly*, 17, 349-357.

559 Noisuwan, A., Bronlund, J., Wilkinson, B., & Hemar, Y. (2008). Effect of milk protein
560 products on the rheological and thermal (DSC) properties of normal rice starch and
561 waxy rice starch. *Food Hydrocolloids*, 22, 174-183.

562 Ohishi, K., Kasai, M., Shimada, A., & Hatae, K. (2007). Effects of Acetic Acid on the
563 Rice Gelatinization and Pasting Properties of Rice Starch during Cooking. *Food*
564 *Research International*, 40, 224-231.

565 Onyango, C., Mutungi, C., Unbehend, G., & Lindhauer, M. G. (2011). Modification of
566 gluten-free sorghum batter and bread using maize, potato, cassava or rice starch. *LWT -*
567 *Food Science and Technology*, 44, 681-686.

568 Osella, C.A., Sánchez, H.D., Carrara, C.R., de la Torre, M.A., & Buera, M.P. (2005).
569 Water Redistribution and Structural Changes of Starch During Storage of a Gluten-free
570 Bread. *Starch*, 57, 208-216.

- 571 Peres, M. F. S., Tininis, C. R. C. S., Souza, C. S., Walker, G. M., & Lalue, C. (2005).
572 Physiological responses of pressed baker's yeast cells pre-treated with citric, malic and
573 succinic acids. *World Journal of Microbiology and Biotechnology*, 21, 537-543.
- 574 Pérez-Quirce, S., Collar, C., & Ronda, F. (2014). Significance of healthy viscous dietary
575 fibres on the performance of gluten-free rice-based formulated breads. *International*
576 *Journal of Food Science & Technology*, 49, 1375-1382.
- 577 Piper, P., Calderon, C. O., Hatzixanthis, K., & Mollapour, M. (2001). Weak Acid
578 Adaptation: The Stress Response that Confers Yeasts with Resistance to Organic Acid
579 Food Preservatives. *Microbiology*, 147, 2635-2642.
- 580 Purlis, E. (2010). Browning development in bakery products – A review. *Journal of*
581 *Food Engineering*, 99, 239-249.
- 582 Pyler, E.J., & Gorton L.A. (2000). Fundamental Bakery Dough Processes. In Baking
583 Science & Technology, Volume II: Formulation and Production. Ed SOSLAND.
- 584 Ribotta, P. D., Ausar, S. F., Morcillo, M. H., Pérez, G. T., Beltramo, D. M., & León, A.
585 E. (2004). Production of gluten-free bread using soybean flour. *Journal of the science of*
586 *food and agriculture*, 84, 1969-1974.
- 587 Ronda, F., Caballero, P. A., Quilez, J., & Roos, Y. H. (2011). Staling of frozen partly
588 and fully baked breads. Study of the combined effect of amylopectin recrystallization
589 and water content on bread firmness. *Journal of cereal science*, 53, 97-103.
- 590 Ronda, F., Pérez-Quirce, S., Angioloni, A., & Collar, C. (2013). Impact of viscous
591 dietary fibres on the viscoelastic behaviour of gluten-free formulated rice doughs: A
592 fundamental and empirical rheological approach. *Food Hydrocolloids*, 32, 252-262.
- 593 Ronda, F., Quilez, J., Pando, V., & Roos, Y. H. (2014a). Fermentation time and fiber
594 effects on recrystallization of starch components and staling of bread from frozen part-
595 baked bread. *Journal of Food Engineering*, 131, 116-123.
- 596 Ronda, F., Villanueva, M., & Collar, C. (2014b). Influence of acidification on dough
597 viscoelasticity of gluten-free rice starch-based dough matrices enriched with exogenous
598 protein. *LWT - Food Science and Technology*, 59, 12-20.
- 599 Stratford, M. (1999). Traditional preservatives – Organic acids. In R. K. Robinson, C.
600 A. Batt, & P. D. Patel (Eds.), *Encyclopedia of Food Microbiology*. New York:
601 Academic Press.
- 602 Witzak, M., Korus, J., Ziobro, R., & Juszczak, L. (2010). The effects of maltodextrins
603 on gluten-free dough and quality of bread. *Journal of Food Engineering*, 96, 258-265.
- 604 Zayas, J.F. (1997). *Functionality of proteins in food*. Ed: Springer-Verlag Berlin
605 Heidelberg. Germany.
- 606 Ziobro, R., Korus, J., Witzak, M., & Juszczak, L. (2012). Influence of modified
607 starches on properties of gluten-free dough and bread. Part II: Quality and staling of
608 gluten-free bread. *Food Hydrocolloids*, 29, 68-74.

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

609 Ziobro, R., Witczak, T., Juszczak, L., & Korus, J. Supplementation of gluten-free bread
610 with non-gluten proteins. Effect on dough rheological properties and bread
611 characteristic. *Food Hydrocolloids*, 32, 213-220.

612 Zobel, H.F., & Kulp, K. (1996). The Staling Mechanism. In: Hebeda, R.E., Zobel, H.F.
613 (Eds.), *Baked Goods Freshness: Technology, Evaluation and Inhibition of Staling*.
614 Marcel Dekker, New York.

615

616

For Peer Review

Table 1. Experimental design

Fórmula	Proteín				Acetic/Lactic acid
	CA	EA	SPI	PPI	
1	0	10	0	0	0.1/0.4
2	0	5	0	0	0
3	0	0	10	0	0
4	0	0	5	0	0
5	0	10	0	0	0
6	0	5	0	0	0.1/0.4
7	0	0	0	0	0
8	0	0	5	0	0.1/0.4
9	0	0	0	5	0
10	0	0	0	10	0.1/0.4
11	0	0	0	0	0.1/0.4
12	10	0	0	0	0.1/0.4
13	5	0	0	0	0
14	5	0	0	0	0.1/0.4
15	0	0	0	5	0.1/0.4
16	0	0	10	0	0.1/0.4
17	0	0	0	10	0
18	10	0	0	0	0

CA: Calcium Caseinate; EA: Egg Albumin;

SPI: Soya Protein Isolate; PPI: Pea Protein Isolate

Amounts are in % w/w, starch +protein basis

Table 2: Dough properties: Total Titrable Acidity (TTA), initial pH (pH₀), change of dough pH during proofing (ΔpH) and thermal properties: ΔH_{gel}: Enthalpy associated to gelatinization; ΔH_{ret}: Melting enthalpy of the recrystallized amylopectin after storage of the gelatinized sample at 4°C for 2 days. ΔT: T_{peak}-T_{onset} of the peak; T_{peak ret}: amylopectin recrystallization peak temperature

	TTA (meq/g)	pH ₀	ΔpH	ΔH _{gel} (J/g starch)	T _{peak} (°C)	T _{onset} (°C)	ΔT (°C)	ΔH _{ret} (J/g starch)	T _{peak ret} (°C)
Control	0.0048 a	5.21 f	0.36 f	11,5 efgh	70.86 cde	63.98 efgh	6.88 abc	0.52 a	48.7 a
Control-A	0.0281 g	3.88 a	0.04 a	11,4 defg	70.82 cde	63.97 efg	6.85 abc	1.35 a	49.4 a
Egg Albumin									
5%	0.0070 b	5.56 g	0.29 ef	11,4 cdefgh	71.38 de	64.44 efgh	6.94 abcd	0.79 a	55.0 abc
5%-A	0.0304 i	4.46 b	0.01 a	11,8 gh	71.14 cde	64.23 efgh	6.91 abcd	1.32 a	54.3 abc
10%	0.0081 cd	5.73 hij	0.21 bcde	11,8 fgh	71.39 de	64.51 fgh	6.88 abcd	0.82 a	53.9 abc
10%-A	0.0279 g	4.80 d	0.08 ab	12,2 h	71.56 e	64.76 h	6.80 abc	0.74 a	56.2 abc
Calcium caseinate									
5%	0.0097 e	5.71 hi	0.29 ef	11,3 cdefg	69.65 a	62.34 ab	7.31 cd	0.58 a	53.3 abc
5%-A	0.0321 j	4.73 cd	0.09 a	10,7 abcd	69.60 a	62.28 ab	7.32 cd	0.54 a	52,5 abc
10%	0.0134 f	5.84 ij	0.24 def	11,0 abcdefg	69.65 a	62.37 ab	7.28 cd	1.21 a	54.5 abc
10%-A	0.0436 k	5.10 ef	0.12 abcd	10,7 abc	69.58 a	62.92 bc	6.67 a	1.08 a	58.7 c
Isolated pea proteína									
5%	0.0075 bc	5.73 hij	0.30 ef	10,8 abcd	70.41 bc	63.68 de	6.73 ab	1.17 a	56.5 abc
5%-A	0.0298 hi	4.72 cd	0.07 a	10,6 ab	71.19 de	64.58 gh	6.61 a	0.96 a	58.5 bc
10%	0.0085 d	5.85 j	0.21 bcde	10,9 abcde	69.89 ab	62.45 ab	7.44 d	1.21 a	54.1 abc
10%-A	0.0289 h	5.20 f	0.10 abc	10,9 abcde	70.46 bc	63.25 cd	7.22 bcd	0.80 a	53.8 abc
Isolated soya protein									
5%	0.0075 bc	5.68 gh	0.27 ef	10,6 abc	69.64 a	62.41 ab	7.23 cd	0.73 a	51.3 bc
5%-A	0.0296 hi	4.64 c	0.03 a	11,0 bcdefg	70.59 bcd	63.88 def	6.93 abc	0.92 a	53.1 abc
10%	0.0097 e	5.82 hij	0.23 cdef	10,3 a	69.52 a	62.09 a	7.43 c	0.72 a	53.5 abc
10%-A	0.0314 j	5.03 e	0.13 abcd	11,0 abcdef	69.51 a	62.18 a	7.33 cd	0.54 a	52.1 bc
SD	0.0003	0.04	0.03	0.10	0.09	0.09	0.05	0.12	6,3

-A: Acid addition
Each data is the average of duplicates. The standard deviation (SD) was established from MANOVA study

Table 3: Textural and morphogeometrical properties of bread

	Baking Loss (%)		Specific Volume (mL/g)		Height/Width		Firmness (N)		ΔFirmness (2days) (N)	
Control	19.42	f	4.44	g	0.81	c	1.02	abc	8.9	abcd
Control-A	17.19	bc	2.90	c	0.65	bc	1.73	d	7.4	abc
Egg albumin										
5%	19.43	ef	4.39	g	0.79	bc	1.21	abcd	10.1	abcd
5%-A	20.89	g	4.59	g	0.79	bc	1.34	bcd	8.7	abcd
10%	15.16	a	2.19	b	0.40	a	2.97	e	16.8	e
10%-A	15.48	a	3.38	d	0.70	bc	1.61	cd	13.0	de
Calcium caseinate										
5%	19.00	ef	3.77	def	0.73	bc	0.98	ab	10.3	bcd
5%-A	18.88	ef	4.34	g	0.83	c	0.69	a	6.2	a
10%	18.64	def	3.95	ef	0.77	bc	0.94	ab	9.9	abcd
10%-A	18.44	de	3.96	ef	0.81	c	0.79	ab	11.3	cd
Isolated pea protein										
5%	16.68	b	1.69	a	0.40	a	4.72	f	81.3	g
5%-A	16.67	b	3.74	de	0.91	c	1.00	ab	7.3	ab
10%	15.28	a	1.36	a	0.36	a	11.36	h	90.3	h
10%-A	15.60	a	1.48	a	0.37	a	7.20	g	93.1	h
Isolated soya protein										
5%	17.94	cd	3.62	de	0.75	bc	1.04	abc	9.4	abcd
5%-A	19.28	f	4.00	f	0.77	c	1.07	ab	7.9	abc
10%	15.42	a	1.60	a	0.56	ab	11.75	h	31.1	f
10%-A	17.69	cd	3.54	d	0.71	bc	0.90	ab	6.7	ab
SD	0.30		0.12		0.09		0.22		1.5	

Values with a common letter in the same column are not significantly different ($p>0.05$);

SD: Standard deviation obtained from variance analysis

Table 4: Sensory evaluation of breads

	Cell Regularity		Odor Intensity	Acid Odour		Taste Intensity		Acid Taste		Taste Persistency			
Control	2,07	ab	6,79	a	2,43	bcd	5,30	ab	1,95	abcd	3,82	ab	
Control-A	1,21	a	6,89	a	5,63	e	5,66	abc	5,38	f		3,64	ab
Egg Albumin													
Albumin													
5%	3,61	cd	6,46	a	2,08	abc	5,41	ab	1,40	ab		3,71	ab
5%-A	4,47	de	7,13	a	2,64	abcd	5,48	abc	1,13	abc		3,13	ab
10%	6,81	gh	6,03	a	1,51	ab	4,96	a	1,43	abc		3,30	ab
10%-A	6,81	gh	5,32	a	0,98	ab	6,00	abcd	2,10	abcde		3,94	abcd
Calcium caseinate													
Casein													
5%	5,11	ef	6,53	a	1,36	ab	5,90	abcd	1,48	abc		3,23	ab
5%-A	4,84	ef	6,70	a	3,48	cd	5,93	abcd	2,51	bcde		3,63	ab
10%	7,71	h	6,43	a	2,26	abcd	5,97	abcd	2,79	cde		2,63	a
10%-A	7,52	h	6,74	a	3,76	d	6,06	abcd	3,80	e		3,36	ab
Isolated pea protein													
Pea													
5%	7,51	gh	5,83	a	1,62	abc	6,42	bcd	1,38	abc		4,80	bcd
5%-A	6,82	gh	6,65	a	1,54	ab	6,80	cd	1,29	ab		4,49	bcd
10%	6,04	fg	6,19	a	1,00	ab	7,04	cd	1,34	abc		5,86	cd
10%-A	7,19	gh	6,70	a	1,49	ab	6,84	d	2,62	cde		5,70	d
Isolated soya protein													
Soya													
5%	4,96	ef	6,72	a	3,28	cd	6,05	bcd	1,54	abc		3,55	ab
5%-A	2,87	bc	6,60	a	2,54	abcd	6,36	bcd	2,18	abcde		3,84	abc
10%	7,09	gh	6,30	a	0,97	a	5,81	abc	1,29	a		4,07	b
10%-A	5,35	ef	6,74	a	2,86	bcd	6,11	abcd	3,09	de		3,97	ab
SD	0.5		0.7		0.8		0.6		0.7			0.75	

Value with a common letter in the same column are not significantly different (p>0.05);

SD: Standard deviation obtained from variance analysis

Figure captions

Figure 1: Gas production and dough development obtained from rheofermentometer of doughs with egg albumin (A) calcium caseinate (B) isolated pea protein (C) isolated soya protein (D); Control doughs are included as references in all graphics. Control (without protein) (●○); 5% protein (■ □); 10% protein (▲△); without acid (solid points) or with acid (void points). The same symbol is used for gas produced and retained.

Figure 2: Crust lightness (a) crust chromaticity (b) and crumb chromaticity (c) depending on protein presence type and dose and acid blend addition. EA: Egg albumin; CA: Calcium caseinate; PPI: Isolated pea protein; SPI: Isolated soya protein; A- With acid addition; P-EA, P-CA, P-PPI; P-SPI are referred to the colour of pure protein ingredients

Figure 1

Figure 2