Effect of rice, pea, egg white and whey proteins on crust quality of rice flour-corn starch based gluten-free breads

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Abstract

Gluten-free bread crusts are known for their crumbly texture, light colour, poor nutritional quality and weak aroma. The objective of this research was to improve crust quality of gluten-free breads by the addition of rice, pea, egg white and whey proteins to the bread formulation in two levels (5% and 10%). Moisture, water activity, thickness, microstructure, texture, colour and volatile compounds were measured. A 99% negative significant correlation between spatial frequency of structural ruptures and crust moisture content was found. Results from texture analysis indicated that animal and 10% pea protein crusts were less crispy than control. Moreover, crust microstructure of animal protein bread was very different from control and vegetal protein crusts. Animal and vegetal protein crusts showed darker colour than control and the darkest was obtained from whey protein inclusion. With respect to the volatile profiles, rice protein crusts had the highest content of 2-acetyl-1-pyrroline while whey protein crusts had high level of pyrazines, which was in concordance with their dark colour. However, whey protein and, above all, rice protein also increased the content of volatile compounds from lipids oxidation. Thus, the suitable proportion between rice and whey protein should be found to achieve the most pleasant aroma.

Keywords: gluten-free bread; protein; crust; colour; volatile compounds; textural properties

1. Introduction

The market for gluten-free products has been expanding in recent years. Bread is the most complex gluten-free product due to the importance of wheat gluten proteins in the bread matrix, which form a network necessary to retain the carbon dioxide during bread fermentation and facilitate expansion. Due to this, gluten-free bread presents a lack of quality in comparison to wheat bread. Arendt et al. (2008) suggested the use of a range of gluten-free flours rather than just one to achieve gluten-free breads with good sensory and textural properties. Moreover, they reported that the addition of a certain percentage of starch improved the overall quality of the gluten-free bread. Thus, mixtures of gluten-free flours and starches have been commonly used, usually maize starch and rice flours (Masure, Fierens & Delcour, 2016). However, these flours and starches present low protein content, which leads to breads with lower protein but higher carbohydrates content than wheat breads (Segura & Rosell, 2011). As a consequence, breads with poor nutritional quality, weak aroma and light crust are obtained, since proteins are necessary for Maillard reactions, which are responsible for crust colour (Purlis & Salvadori, 2009) and generation of volatile compounds (Pico, Bernal & Gómez, 2015). Therefore, to improve nutritional and organoleptic qualities of gluten-free bread the incorporation of proteins has been proposed (Gallagher, Gormley, & Arendt, 2003; Marco & Rosell, 2008; Mezaize, Chevallier, Le Bail, & De Lamballerie, 2009; Krupa-Kozak, Baczek, & Rosell, 2013; Ziobro, Witczak, Juszczak & Korus, 2013; Aguilar, Albanell, Miñarro, & Capellas, 2015). The majority of these studies have carried out the evaluation of bread volume or crumb texture, but few have evaluated crust colour (Gallagher et al., 2003; Krupa-Kozak, et al., 2013) and none of them the volatile profile. Moreover, there are no studies evaluating crust texture of gluten-free breads supplemented with proteins of different origins. Regarding crust colour, in most cases the presence of protein increased the darkness of the crust, and the type of protein contributed to this effect. Gallagher et al. (2003) were the only that analysed crust texture of breads enriched with proteins, concretely of dairy origin, and they observed a decrease in crust texture hardness.

Finally, concerning the aroma of wheat bread crust, it has been characterised by volatile compounds from Maillard reactions, caramelisation and thermal degradation (Poinot et al., 2008; Zehentbauer & Grosch, 1998) , with 2-acetyl-1-pyrroline (2-ACPY) constituting the key aroma of wheat flour bread crust (Zehentbauer & Grosch, 1998). There can be also volatile

compounds from lipid oxidation, although in smaller proportions (Moskowitz, Bin, Elias, & Peterson, 2012), such as 2(E)-nonenal and 2,4-(E,E)-decadienal (Zehentbauer & Grosch, 1998). However, there is little knowledge regarding the aroma of gluten-free bread crusts (Pico, Antolín, Román, Gómez & Bernal, 2018). To our knowledge, only Pacyński, Wojtasiak, and Mildner-Szkudlarz (2015) studied the addition of amino acid – sugar pairs with the aim of promoting the generation of Maillard compounds and improving the aroma of gluten-free breads crust. However, we reason that the impact of the addition of amino acids should not been the same as that of the whole protein. This is the first time that the effect of animal and vegetal protein inclusion on the aroma of gluten-free bread crust has been studied.

The main aim of the present research was to evaluate, for the first time, the changes in the colour, physical parameters as well as the impact on the corresponding volatile profiles of gluten-free bread crusts due to the incorporation of proteins of different origins at different levels. For that purpose, two vegetal proteins (pea and rice) and two animal proteins (egg white and whey) were chosen and two percentages of substitution were proposed (5 and 10%). Moisture, water activity, thickness, microstructure, texture, colour and volatile compounds of crust gluten-free breads were evaluated.

2. Materials and methods

2.1 Materials, standards and solvents

Rice flour (8.21% moisture, 8.01% protein and 74.35% starch) was provided by Molendum Ingredients (Zamora, Spain) and Miwon corn starch (8.07% moisture, non-detected protein) (Daesang Co., Seoul, Korea) was purchased from the local market. Four different proteins (two vegetal and two animal) were used: Remypro N80+G rice protein (79% protein) by Beneo (Mannheim, Germany), Nutralys BF pea protein (78.13% protein) by Roquette (Leutrem, France), egg white powder (81.66% protein) by EPS S.P.A (Occhiobello, Italy) and Provon 295 IP whey protein (92% protein) by Glanbia (Kilkenny, Ireland) with a solubility of 0.01, 0.38, 1.11 and 0.54 mg soluble protein/ mg total protein, respectively, evaluated with the Quick Start™ Bradford Protein Assay (Bio Rad, Hercules, California, United States). Water binding capacity of proteins were 2.47, 5.40, 0 and 0 grams of retained water per grams of dry sample, respectively, measured as described by the method 56.30 (AACC 2012).

Bread formulation also included: white sugar (AB Azucarera Iberica, Valladolid, Spain), refined sunflower oil (Langosta, F. Faiges, S.L., Daimiel, Ciudad Real, Spain), salt (Disal, Unión Salinera de España S.A, Madrid, Spain), instant dry yeast (Dosu Maya Mayacilik A.Ş, Istanbul, Turkey) and Hydroxypropyl Methylcellulose (VIVAPUR 4KM HPMC, JRS, Rosenberg, Germany).

For the volatile profiles characterisation, 2-acetyl-1-pyrroline (2-ACPY) was purchased from Eptes (Vevey, Switzerland) and the other pure standards labelled from 1 to 17 and from 19 to 44 in Table 1 were obtained from Sigma-Aldrich (Steinheim, Germany). Dichloromethane was obtained from Scharlab (Barcelona, Spain) and methanol from VWR International (Fontenay-sous-Bois, France). Argon, nitrogen and helium were acquired from Carburos Metálicos (Barcelona, Spain).

2.2 Methods

2.2.1 Preparation of standard solutions

2-ACPY solutions were prepared in dichloromethane, as 2-ACPY is only stable in dichloromethane and ethyl acetate. It was necessary to work under inert atmosphere of argon at all times due to the lack of stability of the compound to oxygen and moisture. For this reason, dichloromethane was dried in a SDS PS-MD-5 purification system from Düperthal Sicherheitstechnik (Karlstein am Main, Germany).

For the other 43 volatile compounds marked from 1 to 17 and from 19 to 44 in Table 1, working solutions of each volatile compound were prepared in methanol. All the solutions were stored in a freezer at -21°C.

2.2.2 Bread formulation and bread making process

Both the recipe and the bread making process have been based on a previous study of the research group for optimization of gluten free bread quality (Mancebo, Merino, Martínez, & Gómez, 2015). A mixture of rice flour and maize starch (50%-50%) was used as the control sample. In protein-enriched bread formulations, the flour-starch mixture was replaced by 5% and 10% of protein. The follow samples were obtained: R5, R10 (5% and 10% rice protein

substitution), P5, P10 (5% and 10% pea protein substitution), E5, E10 (5% and 10% egg protein substitution) and W5, W10 (5% and 10% whey substitution).

The other ingredients used in dough preparation were (expressed as g/100 g of flour-starch mixture): salt (1.8 g/100 g), sunflower oil (6 g/100 g), sugar (5 g/100 g), instant yeast (3 g/100 g), HPMC (2 g/100 g) and water at 20-22ºC (90 g/100 g). Firstly, the yeast was rehydrated in water. Then, dry ingredients were mixed using a Kitchen-Aid Heavy Duty mixer (KitchenAid, St. Joseph, Michigan, USA) with a dough hook (K45DH) at speed 1 for 1 minute. Later, the water with the yeast was added and mixed for 8 min at speed 2. 150 g of the dough were put into aluminum pans of 159 x 119 x 35 mm. Fermentation of the dough took place at 30ºC at 60 min and a 90% relative humidity in an FC-K proofer (Salva, Lezo, Spain). After proofing, the breads were baked in the central part of an electric modular oven (Salva ST02/E20) for 40 min at 190 ºC. During baking steam was not applied. After baking, breads were unmolded, cooled for 60 min at room temperature and packed into polyethylene sealed bags to prevent dehydration. The water activity, moisture content, crust thickness, specific volume, texture and colour of the breads (sub-sections 2.2.3, 2.2.5, and 2.2.6) were analyzed the day of elaboration. Breads for microstructure evaluation (sub-section 2.2.4) were frozen at -18ºC until their observation. In the case of the breads used for volatile compounds analyses (sub-section 2.2.7), each bread was wrapped in heavy duty aluminum foil and placed in plastic bags in order to prevent migration of volatile compounds from the plastic material to the bread. Breads were frozen at -18°C until their analysis during the same week as preparation. Each bread was prepared in duplicate.

2.2.3 Water activity, moisture content, crust thickness and specific volume

Crust and crumb were separated using a knife based on white versus brown colour. The measurement of water activity (aw) of crust samples was carried out with a precision multi-function measuring instrument Testo 650 (Testo SE & Co, Lenzkirch, Germany). Crust moisture content of the breads was determined following the method 44-15.02 (AACC 2012). Thickness of crust was evaluated using a digital calliper. The mean thickness was calculated from measurements taken at 6 different locations on the crust sample from each bread. Bread volume was measured by a Volscan Profiler 300 volume analyser (Stable Mycrosystems, Surrey, UK) and expressed as specific volume. The bread specific volume was calculated as the ratio between

the volume of the bread and its weight. All measurements were performed in two breads for each elaboration.

2.2.4 Microstructural analysis of bread crust

Surface and cross-section photomicrographs of bread crusts were taken with a Quanta 200FEI (Hillsboro, Oregon, USA) environmental scanning electron microscope (ESEM). Photomicrographs were taken in beam deceleration mode (BDM) at 1.5 keV in high vacuum mode with a backscattered electron detector (BSED). Bread samples were unfrozen 1 hour before evaluation and crust was separated from crumb using a cutter.

2.2.5 Crust texture

Texture of the crust was measured with a TA-XT2 texture analyser with a 5-kg load (Stable Microsystems, Surrey, UK) fitted with the "Texture Expert" software. Fresh breads after 2 hours of cooling were puncture tested at a deformation speed of 0.5mm/s using a 2 mm diameter cylindrical probe (punching area = 3 mm^2) and a distance of penetration fixed at 4 mm. Five puncture tests were carried out at different locations in each bread: at the midpoint of the crust area and 2 cm from that point at 12, 3, 6, and 9 o'clock. Three different breads were evaluated on each elaboration.

From force-deformation curves obtained, the following parameters were calculated using the method proposed by Van Hecke, Allaf, & Bouvier (1998):

Average puncturing force F_m (N) = $\frac{A}{d}$

Spatial frequency of structural ruptures N_{wr} (mm^{-1}) = $\frac{No}{d}$

Average specific force of structural ruptures f_{wr} (N) = $\Sigma \frac{\Delta F}{No}$

Crispness work W_c (N.mm) = $\frac{Fm}{\mathrm{Nwr}}$

Where N_o: is the total number of peaks, d is the distance of penetration (mm), ΔF is the individual force drops for each peak (N) and A is the area under the force-deformation curve.

2.2.6 Colour of bread crust

Bread crust colour was determined using a Minolta CN-508i spectrophotometer (Minolta, Co., Ltd. Tokyo, Japan) with the D65 standard illuminant and the 2° standard observer. Results were expressed in the CIE L*a*b* colour space. Colour measurements were made in 4 points on the surface of the breads. Two breads of each elaboration were evaluated.

2.2.7 Volatile compounds analysis by SPME-GC/QTOF

After thawing, each loaf of bread was cut into slices of 5 cm width, including the ends. The crust was scratched with a knife, taking care not to remove pieces of crumb. Then the crust was frozen with liquid nitrogen and ground in an Ika grinder model M20 (Staufen, Germany) for 10 seconds.

The SPME conditions were previously optimised and validated by the research group for the analysis of volatile compounds in bread crust (Pico et al., 2018). 0.75 g (± 0.0050 g) of each bread crust powder was weighed into a 20 mL vial. The selected fibre was 50/30 µm DVB/CAR/PDMS (Sigma Aldrich, Gillingham, UK). The sample was incubated for 5 min at 60°C (without the fibre) and then extracted for 51 min at 60°C, without agitation. After that, the fibre was inserted into the GC injector port for thermal desorption for 5 min at 270°C, with an injection volume of 1 µL. Finally, the fibre was conditioned for 30 min at 270°C after each analysis. Each sample was analysed in triplicate.

GC/QTOF analysis conditions were the same as used in Pico et al. (2018). All the volatile compounds labeled from 1 to 44 in Table 1 were identified by comparison of their retention times and accurate mass spectra (with four decimal places) with standards as well as using their Kovats Index (KI) and their Mass Spectra Library (NIST MS Search 2.2 & MS Interpreter). Those labeled from 45 to 61 were identified using their KI and their Mass Spectra Library.

2.3 Statistical analysis

Differences between the different parameters evaluated for the samples were examined by analysis of variance (ANOVA). Fisher's least significant difference (LSD) was used to describe means with 95% confidence intervals. The statistical analysis was performed with the

Statgraphics Centurion XVII (StatPoint Technologies Inc, Warrenton, USA). Correlations were obtained using the same program. For assessing the variation of the volatile profiles from the Maillard reaction (compounds indicated in Table 1 with the superscript "M"), a PCA was conducted using the average peak area for each bread crust sample prepared in duplicate and analysed in triplicate (n=6). The PCA was performed with the software LatentiX version 2.00 (Latent5, Copenhagen, Denmark), with data standardised prior to the analysis.

3. Results and Discussion

3.1 Crust moisture, water activity and thickness

In some countries, like in south of Europe, fresh bread is associated with crispy and crunchy crust by consumers. It is known that water content predetermines textural properties of the product (Altamirano-Fortoul, Hernández-Muñoz, Hernando, & Rosell, 2015). As noted in Table 2, no clear relationship emerged regarding the effect of protein inclusion on bread crust moisture. While P10, W10 and E5 breads had higher moisture content than the control bread, the rice protein breads and W5 showed drier crusts. Results for water activity were similar to moisture content results, in fact, a significant correlation at 99% between both parameters (r=-0.79) was found. However, only P10 breads showed significant differences in comparison with the control sample, showing higher water activity, which was in concordance with the high moisture of their crust. Castro-Prada, Primo-Martin, Meinders, Hamer, and Van Vliet (2009) established a range of 0.68–0.69 in aw crust content as the point where mechanical transition occurs, yielding unfavorably tough breads. Regarding crust thickness, only P10 and W10 breads showed significant differences in comparison with the control, having thinner crusts. Moreover, these breads also had the smallest specific volume. Therefore, due to their lack of size increase, the mold likely protected them from desiccation. In this way, differences in water activity, moisture content and also thickness of bread crust could be justified by the protective character of the mold instead of the protein effect. When the higher protein level was added, lower specific volume was obtained in all cases and this is in agreement with previous reports about gluten-free breads enriched with proteins (Mezaize et al., 2009; Ziobro et al., 2013). Solely, P5 breads showed similar specific volume to the control bread and no significant differences in other parameters such as crust moisture and thickness. However, it must be noted that it was not

possible to analyse W5 breads because they broke when they were unmolded. In general, it could be expected that proteins with higher water absorption capacity exhibited less loss during baking and in consequence show a larger crust moisture content. In this study, vegetal proteins presented high water absorption capacity, but in general our vegetal protein breads had low moisture content except P10 having one of the highest moisture content. Likely, this may be due to the fact that water absorption capacity tests and the baking process were carried out at different temperatures, the first at room temperature and the second one above 100ºC, which might have affected the water absorption properties of proteins. Another possible explanation to the low moisture content for vegetal proteins could be due to a slower migration of water from crumb to crust during cooling. The exception found in high moisture content for P10 could be related to its low specific volume. On the other hand, animal proteins used in this study are more soluble than vegetal proteins, and they dissolved in the water-hydrocolloid mixture, as it can be noted from the images from microphotographic analysis (Figure 1a). Taking that into consideration, egg white protein and whey protein should interact more with water and retain it, increasing moisture content, but this effect was not found in all our animal protein breads (only for W10 and E5). Moreover, egg white protein is capable of coagulating with heat, due to its denaturalization, letting it form gels with greater water absorption capacity (O´Brien, Baker, Hood & Liboff, 1982). In this way, it may be thought that egg white protein gels might retain water in bread crust and, as a consequence, increase moisture content, but this effect was not observed in E10 breads.

3.2 Microstructural analysis

In order to understand the influence that the addition of proteins used in this study had on crust structure, the microstructure of the surface (Figure 1a) and cross-section (Figure 1b) were analysed by ESEM. Both surface and cross-section photographs showed starch granules of different sizes, both of which were structurally intact. This is because during baking a rapid evaporation of water takes place in the crust, which minimizes starch gelatinization and thereby allowing starch granules to maintain their granular structure (Martínez & Gómez, 2017). It is known that maize contains the largest granules and rice contains the smallest granules; the rice starch granules detached from flour particles and can be considered of polyhedral shape. It can also be observed that vegetal protein crusts had a similar structure to the control bread, while a

film covering starch granules could be observed in animal protein crusts. This film may be a mixture of hydrocolloid and animal protein since whey and egg white proteins are soluble in water and water is evaporating rapidly in the crust during baking. However, when comparing animal protein surface microphotographs, it can be appreciated that egg white protein builds a uniform and continuous film on the crust, while for whey crust there are holes in the crust surface. In the case of vegetal proteins, cross-sectional photographs show small filaments that are connected to starch granules, especially for rice protein crust, which are likely to be protein molecules.

3.3 Crust texture

Crust texture is a very important parameter used to evaluate bread quality, since it is well known that consumers demand crispy and crunchy bread crust. Crispness is perceived as the rupture of a product during a bite, so it is associated with rapid drop in force, which is based on fracture propagation during the mastication process (Vincent, 1998). To measure crispness, a puncture test has been previously proposed by researchers (Van Hecke et al., 1998; Altamirano-Fortoul, Hernando, & Rosell, 2013), since it simulates the teeth mastication and has been correlated with sensory criteria. The mean values obtained for the textural parameters for each sample are shown in Table 3, except for 5% whey protein breads which were not able to unmold and thus their texture was not evaluated. Regarding average puncturing force (F_m) values, which represent the mechanical resistance that a product exhibits when a force is applied, no clear trends were observed, and only E5 and W10 breads showed values significantly different from control sample ($P < 0.05$), with egg white bread as the lowest and whey bread the highest. Results for spatial frequency of structural ruptures (N_{wr}), which is related to the number of peaks caused during the fracture of the sample, and average specific force of structural ruptures (f_{wr}) were clearer. Crispy products showed a highly jagged curve with a large number of peaks due to a multitude of fracture events taking place during the test (Vincent, 1998). Accordingly, a high value of N_{wr} indicates that the sample is crispier (Altamirano-Fortoul et al., 2013). So, it was observed that all crusts with animal proteins and P10 crusts had lower N_{wr} value than control sample and vegetal proteins, being were less crispy. Moreover, they also presented higher f_{wr} values, with only P10 bread showing significant differences from control bread. Therefore, breads with high values of moisture content and water activity were the less crispy, with a

significant correlation at 99% between N_{wr} and moisture content (r=-0.81) and aw (r=-0.86). This is in agreement with numerous researchers who have reported that the increase in moisture content or water activity reduces jaggedness of the deformation curve, which will be evident through the frequency distribution of the number of fractures (Van Hecke et al., 1998; Jakubczyk, Marzec, & Lewicki, 2008; Castro-Prada et al., 2009). In wheat bread studies, it was concluded that the presence of water induced plasticization and softening of the starch-protein matrix structure (Jakubczyk et al., 2008), and the high-water activity values caused a transition from the glassy to rubbery state (Castro-Prada et al., 2009), which coincides with our observations.

In the case of crispness work (Wc), indicating the difficulty to break the structure, the higher the crispness work, the more resistant and the harder the product structure (Van Hecke et al., 1998). Regarding this parameter, the highest levels of pea and whey protein crusts (P10 and W10) were the unique samples with significant differences from the control, having the greatest values of crispness work. Likely, these differences are due to the lower N_{wr} values in these types of breads and in the case of whey protein crust are also conditioned by higher F_m value. Therefore, the inclusion of these proteins presents disadvantages regarding crust texture. However, as it was said previously, these results should not only be attributed to the direct effect of protein on crust texture but also the lowest specific volume of P10 and W10 breads in which the mold could reduce surface desiccation.

3.4 Crust colour

Crust colour results are showed in Table 4 and reveal that inclusion of protein had a great effect on the colour of the bread crusts. In fact, previous studies evidenced that larger increment of protein level in wheat breads induced darker crusts, due to Maillard reactions that take place between amino acids and reducing sugars (Aguilar et al., 2015: Smak, 1972). In agreement with that, the incorporation of proteins also gave darker crusts in our study (lower values of L*), and crusts were yet darker when higher protein levels were added. These results are in agreement with previous researches about gluten-free breads with dairy proteins incorporation (Gallagher et al., 2003; Krupa-Kozak et al., 2013). Among the proteins studied, the animal proteins had a more pronounced effect on crust colour than the vegetal ones, and among the first, whey protein

had a greater effect than the egg white. Regarding vegetal proteins, rice crust showed lighter color than pea crust. In this study, differences between animal and vegetal proteins could be attributed to the higher solubility that animal proteins have, which may induce their contact and reactivity with reducing sugars, as they are also in solution. With respect to the tone, the addition of protein resulted in an increase of a* value in all breads, showing red tone; furthermore, the more protein added, the higher value of a* obtained. Vegetal protein crusts had lower values of a*, while the highest value corresponded to the egg white crust. The protein addition increased the values of b* (yellow tones) in all cases except for whey bread that did not modify this parameter. However, the amount of protein incorporates did not modify this effect. The highest values of b* were obtained with egg white protein addition. Likely, differences found in crust colour might result from the different amino acid composition of protein since it is known that the type of amino acids that participate in Maillard reaction influence the compounds generated through it and, in consequence, condition final bread colour (Lund & Ray, 2017).

3.5 Volatile profile

It is well known that they type of sugar affects the rate of the Maillard reaction and the amount of carbonyl compounds generated, while the type of amino acid controls the volatile compounds generated (Kiely, Nowlin & Moriarty, 1960). As the amino acid profiles of the added proteins are different, it is expected to have interesting differences in the volatile compounds from Maillard reaction. Moreover, the differences in the amino acids profile also impact the Strecker degradation and Ehrlich pathway and volatile compounds like 3-methyl-1-butanol comes from leucine, 2-methyl-1-propanol from valine, 2-methyl-1-butanol from isoleucine, 2-phenylethanol from phenylalanine and methional from methionine (Pico et al., 2015). Nevertheless, predict the volatile compounds through the higher concentration of a specific amino acid is not easy, since usually volatile compounds are generated by different reactions (Birch et al., 2014; Pico et al., 2015). SPME-GC/QTOF semi-quantitative analyses (Table 1, results given in peak areas) showed that the control sample presented low proportion of almost all the volatile compounds compared to the breads enriched with proteins, with the exception of 2-ethyl-1-hexanol, likely from lipid oxidation (Pico et al., 2015), and phenylethyl alcohol, purportedly from fermentation (Birch, Petersen, & Hansen, 2014). The control sample also presented high content of 3-methyl-1-butanol and acetoin from fermentation, 2-(E)-nonenal from lipid oxidation and

phenylacetaldehyde from fermentation and Maillard reaction (Birch et al., 2014). The contents of 3-methyl-1-butanol and acetoin were higher in the crust W10 and E10, respectively, and the content of phenylethyl alcohol was higher in W10 following the control sample. This suggests that the content of important volatile compounds from fermentation was increased by the addition of animal proteins, which can be due to a higher binding effect of the volatile compounds release by vegetal proteins (Thanh, Thibeaudeau, Thibaut and Voilley, 1991). Other possible explanation for the higher content in volatile compounds from fermentation in breads with animal proteins is the interaction between the sugars added to the bread and the proteins of polar characteristics, which lead to a lower availability of sugars for yeast fermentation. The occurrence of volatile compounds from fermentation in the crust may be due to transferences from the crumb to the crust. The content of 2-(E)-nonenal was higher in R10 crust, which also presented the highest content of heptanal, 1-pentanol, 1-hexanol, 1-octen-3-ol and similar content of 2-ethyl-1-hexanol to the control sample, all of which were likely from lipid oxidation (Birch et al., 2014). The contents of benzaldehyde, benzyl alcohol and hexanoic acid, which can be some of the products from lipid oxidation (Birch et al., 2014), were also the highest in R10. Only the contents of hexanal, nonanal and 2,4-decadienal were the highest for crusts with animal proteins added (W5). This suggests that the content of a great number of volatile compounds from lipid oxidation is increased by relatively large quantities of rice protein. The high amount of lipoxygenases in rice (Wongdechsarekul & Kongkiattikajorn, 2010) as well as the low concentration of antioxidants (Inglett, Chen, & Liu, 2015), such as flavonoids and vitamin E, encourage the lipid oxidation reaction. As the protein is the only ingredient changed between the breads, and the substrates (lipids) and enzymes (lipoxygenases) should be present if the lipids oxidation reaction is taking place, the rice protein should have been contaminated with lipids, lipoxygenases and antioxidants when the protein was isolated, justifying the highest contents in volatile compounds from lipid oxidation. All of these have been reported to correlate negatively with the aroma of bread due to their fatty-rancid notes (Table S1). Heptanal, 1-octen-3-ol, hexanal, nonanal and 2,4-decadienal show low odour threshold (OT) (Table S1), thus the effect of the off-flavour is expected to be significant.

Taking into consideration the heterocyclic compounds from Maillard reactions, the content of 1-methylpyrrol, 2,6-dimethylpyrazine, 2-ethylpyrazine and 2-acetylpyrroline did not change with the increase in the addition of protein between E5 and E10. The same occurred with the increase

between R5 and R10, as the content of n-methyl-m-ethylpyrazines (more than one possible isomer) and 3-ethyl-2,5-dimethylpyrazine remained unchanged. Maillard compounds have been reported to correlate positively with the crust colour (Cho & Peterson, 2010) and, effectively, the crusts with the highest substitution of rice protein (R10) and egg protein (E10) were darker than their corresponding 5% substitution. Therefore, the fact that these volatile compounds from the Maillard reaction remained constant from 5% to 10% substitution levels suggested that they were not involved in the development of the colour of the rice and egg crusts.

The PCA (Figure 2) was performed with the aim of evaluating the changes in the volatile profiles of the 23 Maillard compounds (see Table 1), which are the volatiles that are expected to be more affected by the addition of proteins. As can be seen in the scores plot, the P5, E5 and E10 crusts were not characterised by high contents of any of the volatile compounds from the Maillard reaction. The separation of R5 and R10 in the negative PC1 was due mainly to their high content in 2-ACPY as well as high content in volatile compounds with lipid oxidation as a secondary origin (benzaldehyde, benzyl alcohol). Indeed, 2-ACPY has been described as a key contributor to the aroma of cooked rice (Grimm, Bergman, Delgado & Bryant, 2001), which justifies the higher content in the crusts enriched with rice protein. Finally, all the pyrazines were located in the positive component of the PC1, which explained the separation of W5, W10 and P10. Specifically, most of them were in higher concentration in W5, which moreover presented 11 of the 23 volatile compounds from the Maillard reaction. Only 2-ethylpyrazine was highlighted in W10 and 2,6-dimethylpyrazine in P10. This is in accordance with the fact that the crusts enriched with whey proteins were also darkest.

Therefore, a combination of rice protein and whey protein, would seem to be the most suitable for a pleasant aroma due to the high content of 2-ACPY, a key aroma of the wheat bread crust (Zehentbauer & Grosch, 1998), for R10 and the high content of pyrazines with pleasant nutty aromas for whey protein breads, preferably W5 due to the highest content in 11 of the 23 volatile compounds from Maillard reaction in the corresponding bread. The selection of W5 is also related to the crispness, since the increase in the percentage of whey protein led to lower crispness, indeed W10 presented the highest value of Wc and higher moisture content than W5. Higher crispness is associated to lower moisture content, as it was explained in sub-section 3.3, and consequently higher water evaporations during baking. Thus, volatile compounds with low

boiling points or high affinity to water are going to be evaporated during baking and they are usually volatile compounds from fermentation (Pico, Martinez, Bernal & Gomez, 2017). However, loss of water also leads to a major extension of the Maillard reaction during crust formation (Peterson, Tong, Ho & Welt, 1994), justifying the higher amount of these compounds in W5 related to W10. In the case of R5 and R10, there are no significant differences in the Wc values and there are no significant differences with the moisture content regarding W5 neither; thus, the use of the highest substitution of rice protein should not affect negatively to the crispness. In fact, the addition of R10 instead of R5 gives rise to darker crusts. Finally, it should be also taken into consideration that, in general, the inclusion of whey proteins and, above all, the inclusion of rice proteins increased the content of volatile compounds from lipid oxidation. Therefore, sensory analyses of different proportions of rice and whey should be done in order to ensure the most pleasant aroma as possible as a result of 2-ACPY, pyrazines and volatile compounds from lipids oxidation.

4. Conclusion

Protein addition is an adequate technique to produce gluten-free breads with darker crusts and to improve their aroma, even though other crust properties such as thickness or texture may be affected. However, it is necessary to consider how the protein introduced could potentially affect bread volume, since volume changes can indirectly cause changes in moisture and texture of crust. Concretely, the addition of animal proteins tested in this study decreases the crispness in the crust of gluten-free breads. Moreover, crust colour and volatile composition present in crusts are dependent on the type of protein added, with whey protein yielding darker crust and generating higher pyrazines levels with pleasant aroma notes. Regarding vegetal proteins, rice protein generates darker crust than pea protein, and rice protein also causes a considerable increase of the pleasant 2-ACPY concentration, which is key to the aroma of crust. In spite of the high contents of pleasant volatile compounds from fermentation, whey proteins and, above all, rice proteins showed high contents of volatile compounds from lipids oxidation that give fatty rancid notes to the bread. Thereupon, the most suitable proportion of rice and whey proteins should be found in order to improve the final aroma. Moreover, the combination of whey and rice proteins results thinner and crispier crusts due to the whey protein and higher specific volumes due to the rice protein. Nevertheless, it would be important to adjust the

hydration of the doughs regarding the final selected formula. Future studies would also be necessary to verify how other factors influence the quality of protein-enriched bread crusts, such as temperature and time of baking or the kind of flour base employed (for the study of the possible interaction of the flour with the added proteins).

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6. Conflict of interest statement

The authors declare no competing financial interest.

7. References

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Figure Captions

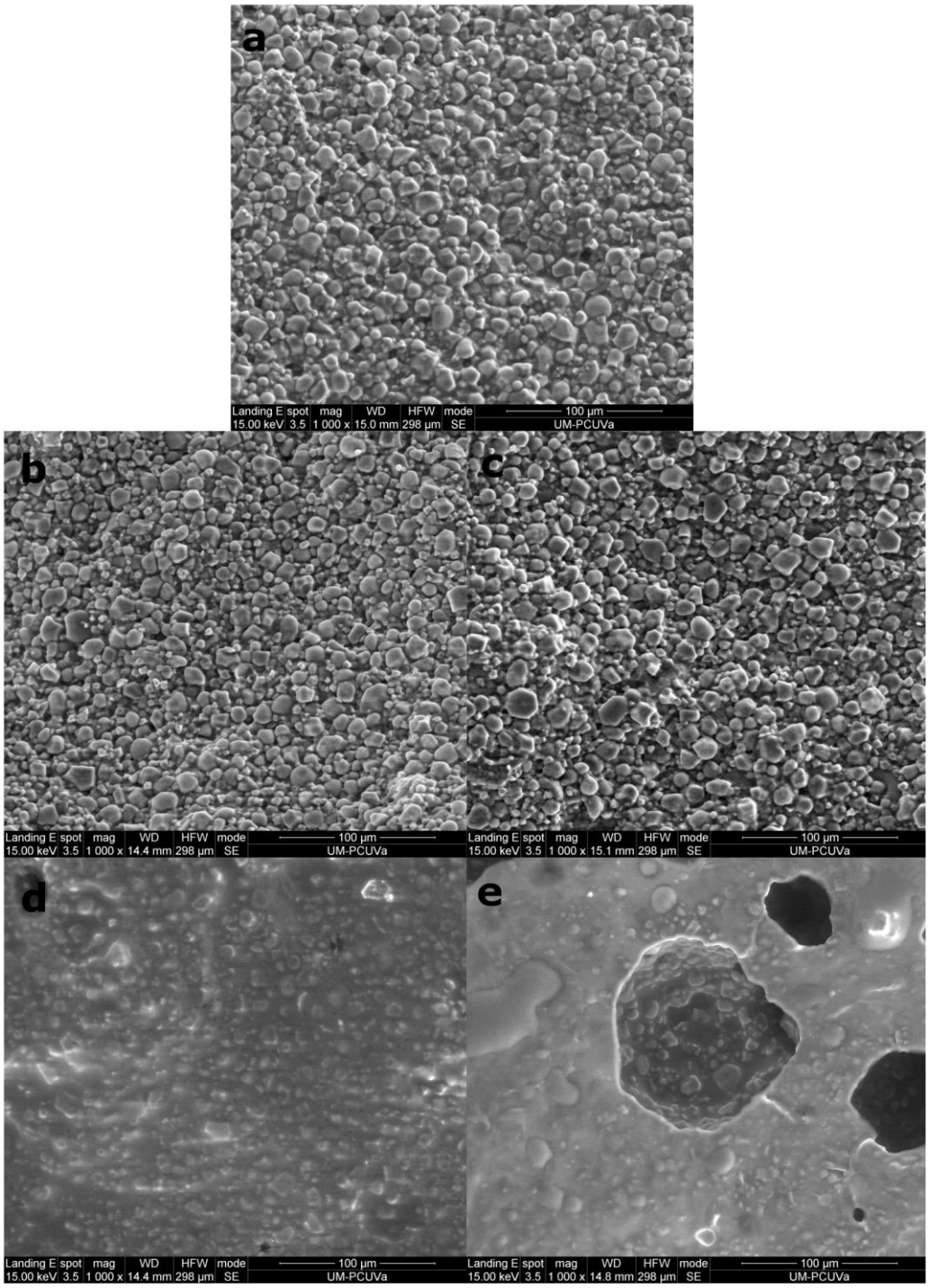

Figure 1a. Micrographs of surface of crust bread at 1000x magnification. Images correspond to breads supplemented with 10% protein and control sample: Control (a), R10 (b), P10 (c), E10 (d), W10 (e).

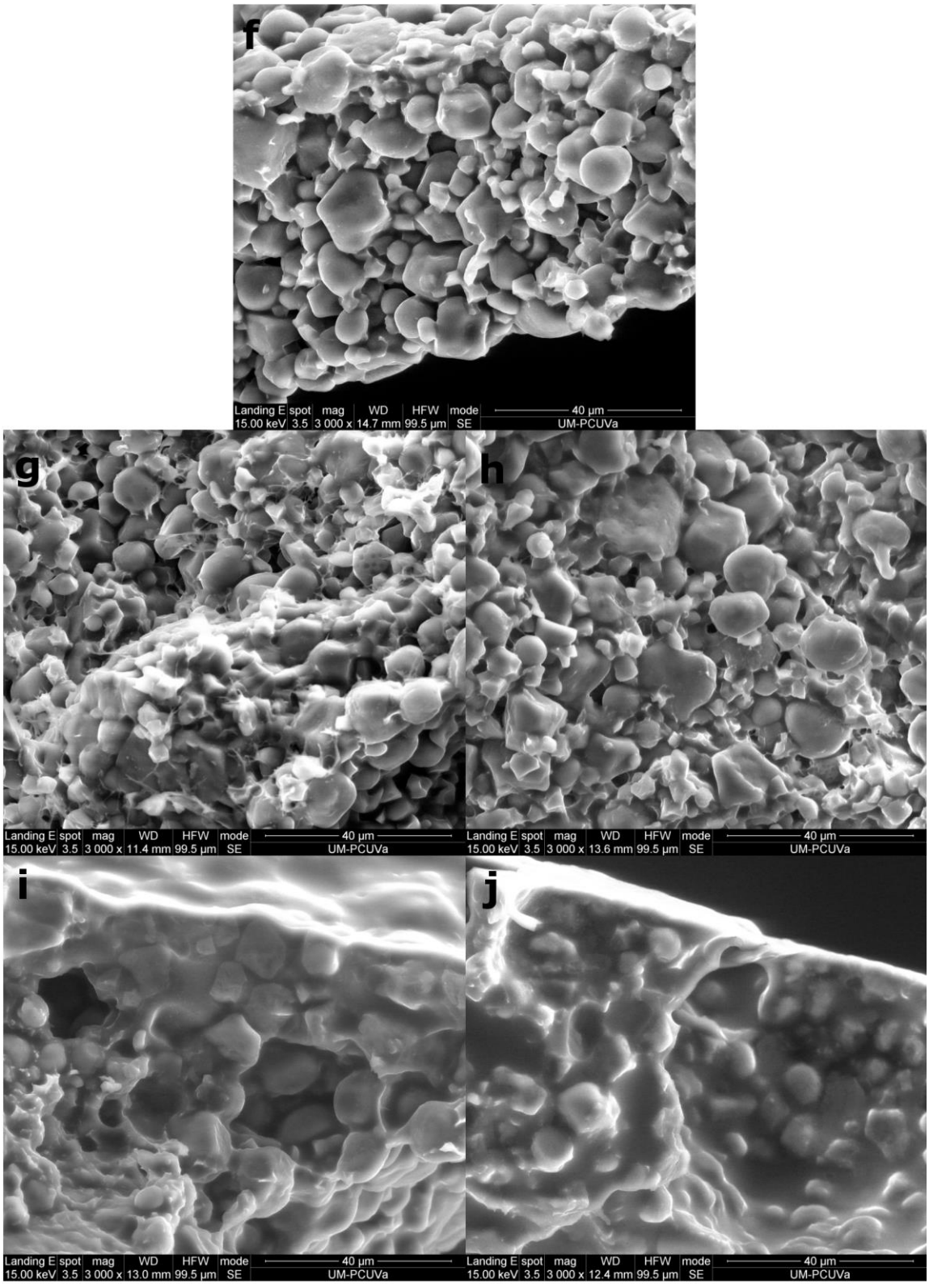

Figure 1b. Micrographs of crust cross-section at 3000x magnification. Images correspond to breads supplemented with 10% protein and control sample: Control (a), R10 (b), P10 (c), E10 (d), W10 (e).

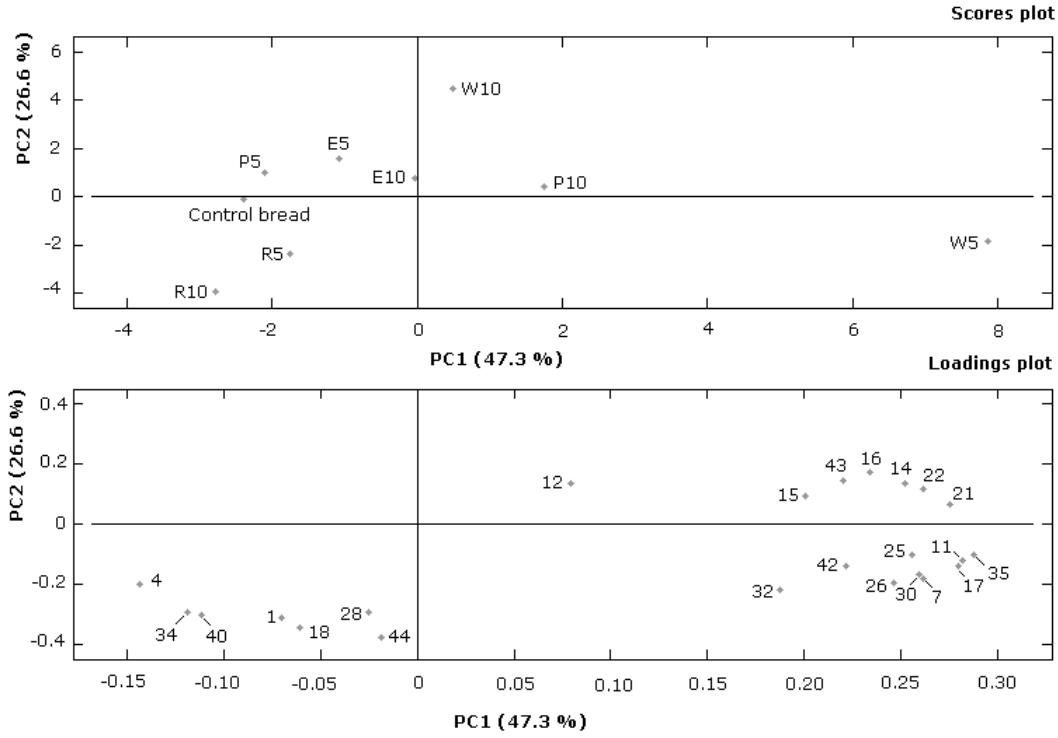

Figure 2. PCA of the 8 gluten-free bread crusts with proteins added (R5, R10 (5% and 10% rice protein substitution), P5, P10 (5% and 10% pea protein substitution), E5, E10 (5% and 10% egg protein substitution) and W5, W10 (5% and 10% whey substitution)) as well as the control sample (50% corn starch, 50% rice flour) analysed semi-quantitatively by SPME-GC/QTOF (peak areas represented). The scores plot represents the 9 samples and the loadings plot for the 23 volatile compounds from Maillard. The numbers corresponding to each volatile compound are indicated in Table 1.

Table 1. Peak areas, divided by 10^4, of the 61 volatile compounds found in the crusts of the 8 gluten-free breads enriched with proteins as well as the control sample. The numeration given in parentheses of each volatile compound corresponds to the numbers assigned in the corresponding PCA (Figure 2) and the superscript "M" indicates those volatile compounds that come from Maillard reactions.

	Control sample	**R5**	**R10**	**P5**	**P10**	**E5**	**E10**	**W5**	**W10**
2,3-Butanedione [M] (1)	129d ± 3.96	295f ± 1.83	396g ± 12.0	85.4ab ± 4.40	111c ± 8.73	72.6a ± 0.543	97.6bc ± 3.17	160e ± 3.32	129d ± 10.8
Hexanal (2)	114d ± 1.39	89.6bc ± 1.24	93.0c ± 2.39	115d ± 1.95	140e ± 3.18	84.6b ± 1.90	151f ± 1.68	77.8a ± 2.26	187g ± 3.81
2-Methyl-1-propanol (3)	86.7d ± 2.39	82.0c ± 1.20	89.1d ± 2.51	90.7d ± 2.34	109e ± 0.764	63.9a ± 2.26	118f ± 0.503	72.7b ± 1.82	170g ± 0.777
1-Methylpyrrol [M] (4)	2.90b ± 0.0995	6.26d ± 0.197	13.2f ± 0.00278	11.1e ± 0.0185	6.56d ± 0.0891	3.33c ± 0.121	3.63c ± 0.318	2.47a ± 0.0678	2.62ab ± 0.163
Heptanal (5)	216a ± 8.98	387e ± 7.49	466f ± 12.5	245b ± 6.77	276c ± 18.4	198a ± 5.27	200a ± 8.06	273c ± 9.50	202a ± 7.51
R-Limonene (6)	89.6b ± 2.11	171f ± 0.913	146e ± 1.08	167f ± 0.0218	421g ± 0.319	111c ± 0.0335	80.4a ± 2.25	94.4b ± 1.95	128d ± 6.30
Pyrazine [M] (7)	11.9b ± 0.345	20.3d ± 0.117	17.0c ± 1.22	11.3b ± 0.344	16.2c ± 0.984	8.98a ± 0.0939	17.0c ± 0.364	52.7e ± 1.73	11.2b ± 0.959
2-Methyl-1-butanol (8)	28.9e ± 1.04	24.5c ± 0.455	30.0e ± 0.542	24.3c ± 0.0586	26.9d ± 0.637	19.8b ± 0.518	31.8f ± 0.590	17.0a ± 0.286	49.7g ± 0.470
3-Methyl-1-butanol (9)	56.7f ± 1.29	43.4d ± 0.124	51.7e ± 1.02	37.3c ± 0.604	41.3d ± 1.26	31.3b ± 0.490	49.8e ± 0.430	29.0a ± 0.775	74.4g ± 1.60
1-Pentanol (10)	6.36c ± 0.0254	8.22f ± 0.199	15.6h ± 0.431	2.79a ± 0.175	7.33de ± 0.0174	6.98d ± 0.103	7.55e ± 0.176	13.0g ± 0.221	3.29b ± 0.0264
2-Methylpyrazine [M] (11)	119b ± 1.96	176e ± 1.99	137c ± 2.61	134c ± 1.64	222f ± 2.94	111a ± 2.19	232g ± 2.31	530h ± 1.46	160d ± 1.49
Acetoin [M] (12)	35.1f ± 0.234	31.3d ± 0.0219	25.9b ± 0.598	24.2a ± 0.184	30.2c ± 0.359	34.1e ± 0.112	39.1g ± 0.299	33.2e ± 0.778	33.7e ± 0.256
2-Octanone (13)	22.3bc ± 0.219	24.5de ± 0.458	29.3f ± 2.53	26.4e ± 1.07	35.7g ± 0.240	21.0b ± 0.0190	23.4cd ± 0.427	47.5h ± 0.0771	14.8a ± 0.108
2,5-Dimethylpyrazine [M] (14)	114a ± 4.39	182b ± 2.05	195c ± 1.84	258d ± 3.32	467f ± 4.18	188bc ± 0.998	350e ± 0.744	867h ± 5.56	812g ± 0.147

Table 1. (continued)

	Control sample	R5	R10	P5	P10	E5	E10	W5	W10
2,6-Dimethylpyrazine [M] (15)	56.4c ± 1.88	53.9bc ± 2.26	43.2a ± 0.545	112d ± 2.93	188f ± 2.44	49.4b ± 1.18	52.8bc ± 2.97	148e ± 4.44	111d ± 0.937
2-Ethylpyrazine [M] (16)	70.4a ± 0.538	99.2a ± 0.357	85.9a ± 0.178	96.9a ± 1.64	157abc ± 1.10	117ab ± 55.3	111ab ± 45.3	223bc ± 111	240c ± 91.9
2,3-Dimethylpyrazine [M] (17)	12.8b ± 0.283	20.8f ± 0.199	15.5c ± 0.0260	16.7d ± 0.340	30.6g ± 0.207	11.4a ± 0.105	18.8e ± 0.278	67.3h ± 0.0981	11.7a ± 0.0150
2ACPY [M] (18)	665d ± 4.69	930f ± 17.2	1234g ± 26.8	564c ± 5.42	624d ± 11.8	443a ± 1.10	423a ± 11.5	713e ± 15.7	493b ± 43.3
1-Hexanol (19)	8.81c ± 0.0996	21.2f ± 0.977	30.1g ± 0.0336	10.7d ± 0.484	11.6e ± 0.228	7.48ab ± 0.129	8.31bc ± 0.0487	6.73a ± 0.125	8.54c ± 0.103
Nonanal (20)	721e ± 2.51	985g ± 4.66	888f ± 6.87	417b ± 23.5	695d ± 4.86	367a ± 10.7	578c ± 11.2	1050h ± 3.81	700de ± 1.94
2,3,5-trimethylpyrazine [M] (21)	19.7a ± 0.256	26.5b ± 0.197	35.5d ± 0.616	31.7c ± 0.00943	75.7f ± 0.603	24.4b ± 1.96	51.7e ± 1.36	157h ± 2.32	113g ± 1.13
2-Ethyl-3-methylpyrazine [M] (22)	88.0a ± 1.06	116c ± 2.02	119c ± 1.55	140d ± 1.07	293f ± 8.18	103b ± 2.27	204e ± 2.14	499h ± 2.48	436g ± 15.2
Ethyl octanoate (23)	672e ± 6.22	777g ± 8.97	1022h ± 23.6	321b ± 11.9	283a ± 5.17	309ab ± 8.60	406c ± 2.27	449d ± 17.4	750f ± 1.88
1-Octen-3-ol (24)	25.5d ± 1.54	30.8e ± 0.877	50.4f ± 1.01	18.9a ± 1.32	24.5d ± 0.277	20.7ab ± 0.665	22.0bc ± 0.542	31.2e ± 0.477	23.7cd ± 0.187
Acetic acid [M] (25)	227b ± 0.543	246c ± 2.55	243c ± 4.48	184a ± 4.30	259d ± 8.36	286e ± 2.36	295e ± 2.27	397f ± 10.3	227b ± 2.95
Furfural [M] (26)	41.2e ± 0.844	55.2g ± 0.625	36.1d ± 1.11	16.7b ± 1.71	35.6d ± 0.836	31.1c ± 0.124	44.9f ± 0.506	144h ± 0.289	12.1a ± 0.625
2-Ethyl-1-hexanol (27)	191f ± 0.808	194f ± 0.433	191f ± 0.937	123d ± 0.276	128e ± 1.70	104c ± 0.780	97.7b ± 1.47	123d ± 2.85	71.4a ± 1.95
Benzaldehyde [M] (28)	122b ± 1.91	164d ± 2.78	212g ± 1.39	138c ± 4.55	201f ± 4.77	121b ± 0.767	173e ± 0.211	141c ± 1.25	70.1a ± 0.304
2-(E)-Nonenal (29)	319e ± 0.0423	303d ± 15.4	358f ± 9.20	150b ± 2.41	165b ± 2.88	117a ± 4.79	154b ± 6.89	317de ± 0.295	286c ± 0.702

Table 1. (continued)

	Control sample	R5	R10	P5	P10	E5	E10	W5	W10
5-Methyl-2-furaldehyde [M] (30)	19.3def ± 0.750	20.6f ± 0.586	18.4d ± 0.288	13.0b ± 0.0292	19.8ef ± 0.484	18.9de ± 0.0947	16.6c ± 0.272	63.4g ± 1.34	10.1a ± 0.00334
Butyrolactone (31)	243b ± 4.54	346d ± 3.61	305c ± 3.25	306c ± 19.4	418e ± 5.05	303c ± 3.39	354d ± 1.18	572f ± 10.7	188a ± 12.1
2-Acetylpyrazine [M] (32)	51.2c ± 0.407	98.9e ± 0.597	117g ± 1.42	102f ± 1.28	179h ± 0.880	34.7b ± 0.282	77.8d ± 0.202	188i ± 1.89	30.8a ± 0.304
Butyric acid (33)	12.3de ± 0.0313	13.2f ± 0.138	14.0g ± 0.177	9.09a ± 0.0289	10.3c ± 0.118	12.1de ± 0.151	12.4e ± 0.144	12.1d ± 0.00948	9.82b ± 0.0217
Phenylacetaldehyde [M] (34)	287h ± 1.88	296i ± 1.37	233g ± 3.50	126e ± 3.55	78.0b ± 1.53	106c ± 5.29	118d ± 1.44	143f ± 0.154	38.8a ± 0.401
Furfuryl alcohol (35)	94.9d ± 0.791	94.4d ± 0.0401	80.9c ± 0.433	75.4b ± 0.245	133g ± 1.02	98.5e ± 2.11	113f ± 0.976	295h ± 1.93	69.6a ± 1.63
2-Methylbutanoic acid (36)	16.5e ± 0.0853	15.3c ± 0.000815	14.6b ± 0.350	13.0a ± 0.108	17.8e ± 0.0231	16.0d ± 0.130	16.1d ± 0.0772	17.7e ± 0.0529	14.9b ± 0.0395
3-Methylbutanoic acid (37)	16.6d ± 0.256	15.4b ± 0.0119	15.0a ± 0.00879	29.2g ± 0.00896	44.2h ± 0.365	19.0f ± 0.0549	18.0e ± 0.124	19.2f ± 0.130	16.2c ± 0.101
2,4-(E,E)-Decadienal (38)	534e ± 1.60	723f ± 4.92	862g ± 1.57	203a ± 1.76	267bc ± 14.9	285cd ± 18.8	266b ± 1.39	1182h ± 4.01	295d ± 0.486
Hexanoic acid (39)	26.4d ± 1.24	53.6f ± 1.98	102g ± 2.21	13.6a ± 0.0986	22.0c ± 0.682	22.9c ± 0.845	17.7b ± 0.819	43.9e ± 0.569	12.3a ± 0.403
Benzyl alcohol (40)	6.98e ± 0.0771	10.0g ± 0.0206	11.3h ± 0.0314	5.73b ± 0.174	6.01c ± 0.139	6.77e ± 0.0480	9.48f ± 0.0883	6.46d ± 0.170	4.98a ± 0.0876
Phenylethyl alcohol (41)	347f ± 8.63	223ab ± 0.0626	235bcd ± 1.19	235bcd ± 11.4	242cd ± 8.69	215a ± 5.55	229abc ± 5.90	251d ± 7.61	269e ± 10.3
2-Acetylpyrrol [M] (42)	19.0b ± 0.0529	22.6d ± 0.0483	19.3b ± 0.237	19.9c ± 0.205	34.9g ± 0.0287	28.9f ± 0.184	26.2e ± 0.248	42.3h ± 0.245	9.71a ± 0.237
4-Hydroxy-2,5-dimethyl-3(2H)-furanone [M] (43)	7.30a ± 0.0890	10.7b ± 0.105	9.83b ± 0.101	9.96b ± 0.0160	26.0e ± 1.43	22.5d ± 0.486	19.7c ± 0.295	24.4de ± 1.62	20.0c ± 1.43
4-Vinylguaiacol (44)	24.7de ± 1.13	25.8ef ± 0.694	28.0f ± 1.33	19.6b ± 1.62	22.0c ± 0.571	22.5cd ± 0.0366	20.6bc ± 0.377	24.8de ± 1.48	16.7a ± 0.602

Table 1. (continued)

	Control sample	R5	R10	P5	P10	E5	E10	W5	W10
2,3-Pentanedione (45)	316b ± 0.608	386c ± 11.0	220a ± 19.3	462d ± 4.64	444d ± 2.72	455d ± 1.40	504e ± 12.1	465d ± 1.17	388c ± 25.6
Octanal (46)	226abc ± 18.3	457d ± 23.5	472d ± 3.18	238abc ± 11.5	294bc ± 21.5	185ab ± 12.3	141a ± 11.1	311c ± 27.3	278bc ± 9.17
1-Hydroxy-2-propanone (47)	290b ± 2.66	395d ± 4.88	332c ± 9.73	380d ± 30.4	497f ± 3.34	387d ± 2.01	458e ± 5.03	635g ± 4.87	255a ± 10.0
Ethyl heptanoate (48)	32.8d ± 0.858	157e ± 1.86	277f ± 10.9	14.7ab ± 0.179	13.7a ± 0.180	11.6a ± 0.111	19.6abc ± 0.348	23.3bc ± 0.00444	26.6cd ± 3.69
6-Methyl-5-hepten-2-one (49)	117a ± 3.54	172b ± 3.73	190b ± 1.97	224c ± 13.8	449e ± 8.34	181b ± 3.02	351d ± 2.09	846g ± 21.6	793f ± 22.1
2-Heptanol (50)	304bc ± 4.60	482f ± 20.0	535f ± 31.2	366de ± 4.91	384e ± 12.3	261ab ± 18.8	317cd ± 11.1	204a ± 56.8	222a ± 17.6
n-Methyl-m-ethyl-pyrazine (51)	18.8a ± 0.234	31.8b ± 2.64	29.5b ± 0.145	28.0ab ± 1.02	61.6d ± 0.0769	23.1ab ± 2.18	44.0c ± 1.14	123f ± 4.35	96.0e ± 11.4
n-Methyl-m-ethyl-pyrazine (52)	83.1a ± 2.71	103a ± 0.891	101a ± 0.134	132b ± 12.4	263d ± 8.16	102a ± 1.24	193c ± 4.36	444f ± 20.2	388e ± 18.1
2-(E)-Octenal (53)	280c ± 1.64	430d ± 8.68	482d ± 8.16	145ab ± 6.31	193abc ± 15.4	109a ± 7.47	146ab ± 0.265	632e ± 6.92	244bc ± 1.46
3-Ethyl-2,5-dimethylpyrazine (54)	408a ± 14.6	465b ± 4.51	473b ± 10.2	624d ± 26.5	580c ± 12.6	448b ± 0.989	608d ± 12.9	571c ± 5.37	625d ± 0.0136
2-Methyl-5-propyl-pyrazine (55)	96.4a ± 4.67	118a ± 9.30	119a ± 2.82	207c ± 24.1	342d ± 4.36	114a ± 11.6	182b ± 14.9	345d ± 1.88	417e ± 5.43
2,3-Butanediol (56)	510abc ± 7.02	584d ± 0.353	550bcd ± 29.9	494ab ± 68.4	483a ± 15.2	550bcd ± 7.92	558cd ± 0.663	601d ± 5.82	507abc ± 3.81
Ethyl decanoate (57)	97.7c ± 0.480	68.0a ± 3.17	86.7bc ± 8.67	59.9a ± 5.91	59.4a ± 5.70	63.1a ± 3.76	72.9ab ± 2.37	119d ± 5.09	280e ± 16.0

Table 1. (continued)

	Control sample	R5	R10	P5	P10	E5	E10	W5	W10
2-Butyl-2-octenal (58)	nd *	81.2a ± 2.16	167b ± 3.53	nd *	nd *	nd *	nd *	nd *	nd *
5-Methyl-2-furanmethanol (59)	264b ± 5.99	399e ± 5.07	330c ± 7.29	384d ± 5.84	594h ± 1.35	541g ± 2.51	496f ± 5.98	497f ± 1.58	240a ± 12.4
2-Methyl-benzenemethanol (60)	642e ± 12.0	505bc ± 1.35	526c ± 22.3	526c ± 0.320	513c ± 6.51	464a ± 5.61	471a ± 4.90	485ab ± 8.05	555d ± 1.84
5-Hydroxymethyl-furfural (61)	151d ± 12.2	121bcd ± 28.5	114bc ± 20.2	87.8b ± 4.81	90.0b ± 13.6	133cd ± 17.4	117bc ± 3.66	217e ± 10.6	37.7a ± 8.11

Values are means of three determinations ± SD. Different letters in the same row indicate significant differences.
Control sample (50% corn starch, 50% rice flour), R5, R10 (5% and 10% rice protein substitution), P5, P10 (5% and 10% pea protein substitution), H5, H10 (5% and 10% egg protein substitution) and W5, W10 (5% and 10% whey substitution). * nd = not identified (not present or under the limits of detection).

Table 2. Effect of protein inclusion on aw, moisture content, thickness and specific volume

	Specific volume (ml/g)	Crust moisture (%)	aw crust	Crust thickness (mm)
C	6.92cd ± 0.75	8.47c ± 0.01	0.52abc ± 0.01	4.05cde ± 0.03
R5	7.58d ± 0.17	7.27ab ± 0.01	0.47a ± 0.05	3.99cd ± 0.16
R10	6.29bc ± 0.54	7.57ab ± 0.55	0.54abc ± 0.01	4.31e ± 0.30
P5	6.89cd ± 0.52	8.06bc ± 0.03	0.54abc ± 0.05	4.00cd ± 0.15
P10	2.71a ± 0.22	10.97de ± 0.01	0.71d ± 0.04	3.22b ± 0.02
E5	6.03bc ± 0.78	11.74e ± 1.03	0.64cd ± 0.02	4.24de ± 0.04
E10	5.51b ± 0.78	8.09bc ± 0.07	0.55abc ± 0.09	3.85c ± 0.01
W5	NA	7.07a ± 0.04	0.48ab ± 0.14	NA
W10	1.91a ± 0.04	10.18d ± 0.06	0.62bcd ± 0.02	1.78a ± 0.04

Values with different letters for the same parameter are significantly different ($P < 0.05$), ($n = 2$).
C, Control sample (50% corn starch, 50% rice flour), R5, R10 (5% and 10% rice protein substitution), P5, P10 (5% and 10% pea protein substitution), H5, H10 (5% and 10% egg protein substitution) and W5, W10 (5% and 10% whey substitution). NA not available

Table 3. Crust mechanical properties determined by puncture test for gluten-free breads supplemented with protein

	F_m(N)	N_{wr}(mm^{-1})	f_{wr}(N)	W_c(N*mm)
C	2.82bcd ± 0.04	2.02b ± 0.21	0.15ab ± 0.02	1.63a ± 0.33
R5	2.69abc ± 0.37	2.22b ± 0.45	0.13ab ± 0.03	1.44a ± 0.47
R10	2.82bcd ± 0.28	2.21b ± 0.37	0.17ab ± 0.04	1.61a ± 0.06
P5	3.47d ± 0.04	2.08b ± 0.08	0.09a ± 0.00	1.68a ± 0.06
P10	2.44ab ± 0.19	0.68a ± 0.14	0.43c ± 0.22	4.30b ± 1.80
E5	2.03a ± 0.23	0.89a ± 0.23	0.32bc ± 0.08	2.63ab ± 0.83
E10	3.34cd ± 0.30	1.17a ± 0.28	0.30bc ± 0.02	3.31ab ± 0.01
W5	NA	NA	NA	NA
W10	4.34e ± 0.58	1.08a ± 0.07	0.23abc ± 0.06	4.59b ± 1.29

Values with different letters for the same parameter are significantly different ($P < 0.05$), ($n = 2$).
C, Control sample (50% corn starch, 50% rice flour), R5, R10 (5% and 10% rice protein substitution), P5, P10 (5% and 10% pea protein substitution), H5, H10 (5% and 10% egg protein substitution) and W5, W10 (5% and 10% whey substitution). NA not available
F_m, Average puncturing force; N_{wr}, Spatial frequency of structural ruptures; f_{wr}, Average specific force of structural ruptures; W_c, Crispness work

Table 4. Effect of protein inclusion on crust colour

	L*	a*	b*
C	73.48h ± 0.03	0.24a ± 0.16	15.83a ± 0.73
R5	69.79g ± 0.62	3.08b ± 0.35	19.34d ± 0.76
R10	62.40e ± 0.54	5.13c ± 0.12	18.27cd ± 0.01
P5	67.26f ± 0.54	3.72b ± 0.21	18.04cd ± 0.83
P10	59.88d ± 1.39	6.06d ± 0.61	17.37bc ± 0.98
E5	59.63d ± 0.21	8.97e ± 0.44	22.92e ± 0.88
E10	54.14c ± 1.06	10.37f ± 0.42	21.58e ± 0.05
W5	49.64b ± 1.01	6.01cd ± 0.05	16.36ab ± 0.35
W10	46.79a ± 0.52	9.26e ± 0.74	15.75a ± 0.36

Values with different letters for the same parameter are significantly different ($P < 0.05$), ($n = 2$).
C, Control sample (50% corn starch, 50% rice flour), R5, R10 (5% and 10% rice protein substitution), P5, P10 (5% and 10% pea protein substitution), H5, H10 (5% and 10% egg protein substitution) and W5, W10 (5% and 10% whey substitution).

Supplementary material

Table S1. Kovats index (KI), odour thresholds (OT) and organoleptics characteristics of the 61 volatile compounds studied among the four gluten-free breads as well as the wheat bread) The type of identification of each volatile compound (superscript 1 and 2) is also indicated.

Volatile compounds	KI calculated	KI literature [a,e]	OT [a,d] (µg Kg^{-1})	Organoleptic [a,b,c,d] characteristics
2,3-Butanedione [1]	1004	984	6.5	Buttery
Hexanal [1]	1060	1080	4.5	Green grass
2-Methyl-1-propanol [1]	1073	1052	3200	Wine, malty
1-Methylpyrrol [1]	1046	1140	37	Toasted
Heptanal [1]	1170	1168	3	Fatty, pungent
R-Limonene [1]	1185	1202	10	Citrus
Pyrazine [1]	1205	1216	100	Nutty
2-Methyl-1-butanol [1]	1212	1218	40000	Sweet
3-Methyl-1-butanol [1]	1213	1218	250	Balsamic, alcohol
1-Pentanol [1]	1254	1257	4000	Fusel-like
2-Methylpyrazine [1]	1263	1268	105	Green, nutty, cocoa
Acetoin [1]	1281	1286	800	Buttery
2-Octanone [1]	1284	1283	50	Cheesy, musty
2,5-Dimethylpyrazine [1]	1316	1316	800	Chocolate,earthy
2,6-Dimethylpyrazine [1]	1322	1319	200	Fried potato
2-Ethylpyrazine [1]	1327	1323	6000	Musty,nutty,peanut
2,3-Dimethylpyrazine [1]	1326	1325	2500	Green, nutty, cocoa
2-ACPY [1]	1339	1330	0.053	Roasted, popcorn
1-Hexanol [1]	1354	1359	2500	Sweet alcohol
Nonanal [1]	1390	1396	1	Waxy, green, fatty
2,3,5-trimethylpyrazine [1]	1395	1396	400	Nutty, baked potato
2-Ethyl-3-methylpyrazine [1]	1395	1400	0.4	Potato, burnt nutty
Ethyl octanoate [1]	1433	1437	92	Fruity, floral
1-Octen-3-ol [1]	1451	1456	1	Mushroom
Acetic acid [1]	1453	1465	32300	Vinegar-like
Furfural [1]	1461	1467	3000	Woody, almond
2-Ethyl-1-hexanol [1]	1489	1489	138	Sweet, floral
Benzaldehyde [1]	1510	1521	350	Bitter almond
2-(E)-Nonenal [1]	1528	1546	0.08	Green,tallow
5-Methyl-2-furaldehyde [1]	1565	1574	16000	Sweet, caramellic
Butyrolactone [1]	1609	1622	20000	Sweet, caramel
2-Acetylpyrazine [1]	1612	1614	62	Creamy
Butyric acid [1]	1622	1636	240	Rancid, sweaty
Phenylacetaldehyde [1]	1627	1642	4	Honey-like
Furfuryl alcohol [1,M]	1652	1666	8	Coffee
2-Methylbutanoic acid [1]	1662	1674	1600	Cheesy, rancid

Volatile compounds	KI calculated	KI literature [a,e]	OT [a,d] (µg Kg^{-1})	Organoleptic [a,b,c,d] characteristics
3-Methylbutanoic acid [1]	1662	1679	120	Rancid, sweaty
2,4-(E,E)-Decadienal [1]	1797	1797	0.1	Fatty, deep-fried
Hexanoic acid [1]	1900	1880	3000	Fatty
Benzyl alcohol [1,M]	1951	1893	10000	Fruity, balsamic
Phenylethyl alcohol [1]	2029	1942	1100	Rose-like
2-Acetylpyrrol [1]	2164	1950	170000	Nutty, musty
4-Hydroxy-2,5-dimethyl-3(2H)-furanone [1]	2203	2020	30	Caramel-like
4-Vinylguaiacol [1,M]	2253	2230	3	Amber, cedar
2,3-Pentanedione [2]	905	1035	20	Buttery
Octanal [2]	1280	1278	0.7	Citrus
1-Hydroxy-2-propanone [2]	1289	1284	nf [h]	Caramellic
Ethyl heptanoate [2]	1329	1328	2.2	Cognac-like
6-Methyl-5-hepten-2-one [2]	1330	1330	50	Herbaceous, green
2-Heptanol [2]	1350	1344	3	Citrus
n-Methyl-m-ethyl-pyrazine [2]	1372	1371	ini [g]	ini [g]
n-Methyl-m-ethyl-pyrazine [2]	1377	1377	ini [g]	ini [g]
2-(E)-Octenal [2]	1419	1419	3	Fatty, nutty
3-Ethyl-2,5-dimethylpyrazine [2]	1433	1455	nf [h]	Nutty
2-Methyl-5-propyl-pyrazine [2]	1434	1458	nf [h]	Roasted
2,3-Butanediol [2]	1534	1532	nf [h]	Creamy
Ethyl decanoate [2]	1634	1634	510	Waxy
2-Butyl-2-octenal [2]	1655	1697	nf [h]	Pineapple
5-Methyl-2-furanmethanol [2]	1714	1722	nf [h]	Sweet caramellic
2-Methyl-benzenemethanol [2]	2021	1996	nf [h]	nf [h]
5-Hydroxymethyl-furfural [2]	2318	2440	nf [h]	Camomile flower

[a] https://pubchem.ncbi.nlm.nih.gov/compound/

[b] http://www.pherobase.com

[c] http://www.thegoodscentscompany.com

[d] Birch, Petersen & Hansen (2013).

[e] http://www.chemspider.com/

[f] http:// *www.leffingwell.com*/

[g] "ini" means "isomer not identified".

[h] "nf" means "not found".

[1] Volatile compounds that were identified by comparison with pure standards, KI and spectral library.

[2] Volatile compounds that were identified by comparison with KI and spectral library.

[M] Volatile compounds from Maillard reaction