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Analytical Methods

Improvement of gluten-free bread properties by the incorporation of bovine plasma proteins and different saccharides into the matrix

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ABSTRACT

The aim of this work was to improve the quality of gluten-free bread, incorporating plasma bovine proteins concentrated by ultrafiltration and freeze-dried with saccharides (inulin and sucrose). The influence of these compounds on textural properties and final bread quality was assessed. The textural studies revealed that with the addition of proteins and inulin, homogeneous and smaller air cells were achieved improving the textural properties while the bread hardness was comparable with breads with gluten. The volume of gluten-free breads increased with increasing proteins and inulin concentrations, reaching a maximum at a protein concentration of 3.5% (w/w). The addition of the enhancers improved moisture retention of the loaves after cooking and an increase of lightness of crumb with respect to the control was observed. The sensory analysis found no statistically significant difference in sensory attributes evaluated with respect to the control, so these ingredients do not negatively affect the organoleptic properties of bread.

1. Introduction

In the production of breads, gluten is essential to form the strong protein network required for retention of gas produced during fermentation, and the desired volume and structure of the breads (Demirkesen, Mert, Sumnu, & Sahin, 2010). However, there is an increasing interest in gluten-free products with an increase in numbers of celiac patients. Celiac disease is a disorder of the intestine caused by the intake of gluten as reviewed by Marsh (1992) and Fasano and Catassi (2001). Gluten ingestion causes inflammation of the small intestine, leading to the mal-absorption of important nutrients including iron, folate, calcium and fat-soluble vitamins, and culminates in intestinal mucosal damage (Holtmeier & Caspary, 2006). Gliadin has been determined to be the pathogenic factor responsible meaning the only effective method of treatment has been strict avoidance of gluten, which, in time, allows mucosal recovery (Fasano & Catassi, 2001; Holtmeier & Caspary, 2006).

Gluten is the main structure-forming protein in flour, and is responsible for the elastic characteristics of dough contributing to the appearance and crumb structure of many baked products. Thus, its removal causes problems for bakers and, currently, many gluten-free products available in the market are of low quality, exhibiting poor mouth-feel and flavor (Gallagher, Gormley, & Arendt, 2004; Torbica, Hadnadev, & Dapcevic, 2010). Rice flour is one of the most suitable cereal flours for preparing gluten-free products because of its bland taste, white colour, ease of digestion and hypoallergenic. It also has very low levels of protein, sodium, fat, fibre and high amount of easily digested carbohydrates (Demirkesen et al., 2010). However, the relatively small amounts of protein mean it is difficult to obtain an acceptable yeast-leavened product, such as bread, because of the absence of the network necessary to hold carbon dioxide produced during proofing (Blanco, Ronda, Pérez, & Pando, 2011). Bread has a short shelf-life mostly due to the loss of softness, moisture and flavor. The absence of gluten often results in a liquid batter rather than dough, producing bread with a crumbly texture, poor colour and other post-baking quality defects. Bread dough without gluten can only retain gas if another hydrocolloid replaces the gluten (Torbica et al., 2010) and it is necessary to use emulsifiers, enzymes or dairy products, together with rice flour, to achieve the desired viscoelastic mixture (Demirkesen et al., 2010).

There is, therefore, an urgent need to investigate potential bread-making ingredients, additives and technological aids to develop high-quality gluten-free products at a reasonable price (Blanco et al., 2011). Thus, in recent years, the incorporation of

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starches, dairy proteins and hydrocolloids in gluten-free flour (rice, and corn flour) have been investigated in order to mimic the properties of gluten and improve structure, mouth-feel, acceptability and shelf-life (Lazaridou, Duta, Papageorgiou, Belc, & Biliaderis, 2007; Blanco et al., 2011). But, the supplementation of gluten-free bread dough with additives is difficult because its structure is weaker than wheat bread dough, which contains gluten. The hydrocolloids used as a substitute for gluten seems to be the best alternative for gas retention and provide similar rheological properties to wheat dough (Blanco et al., 2011; Demirkesen et al., 2010). Hydrocolloids are also able to modify starch gelatinization, and to extend the overall quality of the product over time (Rosell, Rojas, & de Barber, 2001). It is known that proteins are good hydrocolloids and they have been used in different formulations, such as whey protein concentrate in unleavened flat bread (parotta) (Indrani, Prabhasankar, Rajiv, & Venkateswara Rao, 2007); soybean flour in gluten-free bread (Ribotta et al., 2004); soy protein isolate, pea protein isolate, egg white protein and casein in rice based gluten free muffins (Matos, Sanz, & Rosell, 2014). Traditionally, bovine plasma protein has not been used in bakery products because plasma proteins have poor sensory qualities. In previous studies, a plasma protein concentrate was obtained by ultrafiltration and freeze-drying, using polysaccharides as a protective agent. The powdered product was easy to use and improved functional and sensory properties (Rodriguez Furlán, Pérez Padilla, & Campderrós, 2010b).

Enrichment of gluten-free bread with dietary fibres has also proved to be necessary since it has been reported that celiac patients have, generally, a low intake of fibres attributed to their gluten-free diet (Lazaridou et al., 2007). It is known that fibres increase calcium absorption, and promote the growth of intestinal bacteria (Griffin, Hicks, Heaney, & Abrams, 2003; Johnson, 2013). In this sense, the oligosaccharide inulin, which behaves as dietary fibre is considered a prebiotic (Rubel, Pérez, Genovese, & Manrique, 2014). This compound was employed in bread with gluten formulations by Poinot et al. (2010).

Therefore, formulations enriched in fibre such as inulin could be developed to improve the nutritional quality of gluten-free bread. Also, the incorporation of inulin may improve the final properties of the gluten-free bread (texture, volume, etc.) as a result of increased water holding capacity, emulsification, etc. (Rodriguez Furlán, Pérez Padilla, & Campderrós, 2010a). Previous studies (Skendi, Biliaderis, Papageorgiou, & Izydorczyk, 2010) demonstrated the addition of fibre to wheat flour had negative effects, specifically weakening the crumb cell structure by the dilution/weakening of the wheat gluten network and impairing gas retention, reducing the volume and changing the texture and appearance of the final product. During storage, bread becomes stale because structural deterioration takes place due to starch recrystallization and loss of moisture (Mandala, Karabela, & Kostaropoulos, 2007).

The resistance of the bread crumb to deformation is referred to as hardness, and is considered an important indication of staling. Because of the role of gluten in the prevention of staling, these problems are more prevalent in gluten-free breads. Mechanical compression tests showing the stress–strain relationship between cell wall elasticity, rigidity and susceptibility to fracture have been used to measure staleness in spongy bakery products (Ahlborn, Pike, Hendrix, Hess, & Huber, 2005). Evaluating the mechanical properties of bread crumb is important not only for staling/shelf-life, but also for assessing the effects of changes in dough ingredients and processing conditions.

In the literature, there are no studies investigating the effect of plasma bovine proteins in combination with saccharides on the properties of bread gluten-free breads. Therefore, the aim of the present study was to use bovine plasma proteins and saccharides (sucrose and inulin) in gluten-free formulations and examine their effects on dough texture properties, as well as on quality parameters (volume, hardness and sensory analysis) on the end-product. The effect of staling during storage on quality attributes was also assessed. Furthermore, the influence of these hydrocolloids on the quality properties of gluten-free breads was evaluated using sensory, mechanical, and microscopic techniques.

2. Materials and methods

2.1. Raw materials

Enhancing agents used in the formulation of gluten-free bread were: bovine plasma protein unprocessed (P) and processed by ultrafiltration and freeze-drying operations (PUF) with the addition of sucrose (PUFS) or inulin (PUFI) as lyo-protective agents (Rodriguez Furlán et al., 2010a, 2010b). The compositions of these concentrates are described in Supplementary Table 1.

2.2. Bread formulations

The basic bread formula per 100 g of gluten-free flour (rice) was: 60.0 g water, 8 g sunflower oil, 1.5 g sugar, 1.3 g salt and 2.0 g powdered yeast species *Saccharomyces cerevisiae*. The enhancing agents (P; PUF; PUFS or PUFI) were added as a function of their protein content to reach concentrations of 0.5% (w/w), 1.5% (w/w), 2.5% (w/w) and 3.5% (w/w) in each sample. A control sample without the addition of enhancers was also baked.

2.3. Breadmaking process

The experiment was carried out following the method described by Lazaridou et al. (2007), Torbica et al. (2010) and Mandala et al. (2007) with several modifications.

Firstly, yeast was dissolved in water at 35 ± 1 °C. This dispersion was added to dry ingredients and sunflower oil and then was mixed with a 5-speed mixer (average mixing speed was 100 rpm) (Santini, Argentina) for 5 min. Approximately 150 g of dough was poured into aluminium rectangular moulds (90 cm^2). Samples were allowed to ferment for 60 min at 25 °C. Baking was carried out in an air electric oven at 200 °C for 20 min (convection/fan). After baking, the breads were removed from the moulds and cooled at room temperature for 30–40 min. Samples were packed in hermetically-sealed bags (Ziploc Brand) and stored at ambient temperature for 3 days.

Each formulation was replicated at least three times, and all the analyses were carried out independently in triplicate.

2.4. Bread quality evaluation

For the baking industry, the benefits expected of enhancers are improved dough handling including greater dough strength, water absorption, crumb structure, brightness of crumb, uniformity in cell size (increased), slicing characteristics of bread, symmetry, gas retention, ovenspring, loaf volume (increased), shelf-life of bread (longer) (Stampfli & Nerden, 1995).

To evaluate the effects of bovine plasma proteins and polysaccharides on gluten free formulations, the following studies were performed:

2.4.1. Moisture content

Moisture content was measured by weighing samples before and after drying for 5 h at 103 °C in a lab dryer. The results are expressed as percent of water on a wet basis (w/w) (Fontanet, Davidou, Dacremont, & Le Meste, 1997).

2.4.2. Measuring crumb mechanical properties

The most commonly used method to measure crumb physical texture is the deformation of a crumb sample between parallel plates in a uniaxial compression test, which can also be used to measure the mechanical properties of bread crumb. The compression test had numerous advantages including simplicity since performing it requires only a small sample size that can be easily prepared, and validity since mechanical properties are measured in a coherent system of units for which standardised testing protocols have been rigorously evaluated (Scanlon & Zghal, 2001). The method consists of compressing a test piece of bread (slices of 2 cm × 4.5 cm, with a thickness of 1.5 cm), with a plate at constant speed to a deformation level above the point of fracture. Therefore, the bread crumbs were subjected to a uniaxial compression between two parallel plates at room temperature (Baiano, Romaniello, Lamacchia, & La Notte, 2009) at 1.1 ± 1 mm/min and were compressed to 80% of the maximum stress (Fontanet et al., 1997). Four replicates were undertaken for each sample during storage. Compressive Young's modulus E, the critical stress σ_c and the Resistance's modulus were extracted from engineering stress σ-strain ε curves (Canet, Alvarez, & Gil, 2007). The results are presented as an average for the four slices (sample replicate) of crumb.

2.4.3. Image texture analysis

A digital image analysis (DIA) system was used to analyse the bread crumb at the cut surface. For this, three elements were necessary: a source of illumination, the specimen and an image sensing device (Scanlon & Zghal, 2001). Images for each slice examined were acquired with a digital camera after 3 h of baking. The images were analysed with Image-Pro Plus 6.0 (Media Cybernetics Inc, Bethesda, USA) and the statistical analysis performed with GraphPad InStat. From these analyses, the mean cell area (mm^2), pore diametric and size distribution of gas cells were obtained.

2.4.4. Yield of baked product

The volume increase of the dough undergoing baking was determined. The experiment was carried out following the method previously described by Rosell et al. (2001). The initial volumes before and after the fermentation and before and after the baking of dough were the parameters used to characterise the samples. The volumes were determined by image acquisition through a digital camera and then analysed by Image-Pro Plus 6.0 software (Media Cybernetics Inc, Bethesda, USA).

2.4.5. Crumb and crust colour (CIELab system)

Crumb colour was measured using a digital spectrophotometer (MiniScan EZ). Colour values (L^*, a^* and b^*) for the control and enhanced bread formulations were recorded, each the average of four measurements at different points in the bread crumb and crust to ensure the reproducibility. L^* is the lightness variable from 100 (white) to zero (black), whilst a^* and b^* are chromaticity, +redness/−greenness and +yellowness/−blueness, respectively (Morales & Van Boeckel, 1999; Skendi et al., 2010).

2.4.6. Sensory analysis

Sensory analyses of gluten-free bread were carried out 3 h after baking in a uniformly illuminated room by 20 untrained panelists, who were 18–55 years old and from various socioeconomic backgrounds, consisting of Food Engineering College staff and students, both male and female. Water was provided for rinsing between samples, to cleanse the palate. A five-point hedonic scale was used to evaluate the overall acceptability of the bread formulations; the panelists scored on a scale of 1 (dislike extremely) to 5 (like extremely). Breads were considered acceptable if their mean scores for overall acceptability were above 3 (neither like nor dislike) (Lazaridou et al., 2007; Torbica et al., 2010).

2.4.7. Scanning electron microscopy

The microstructure of breads was analysed by scanning electron microscopy (SEM, LEO1450VP, Zeiss, Germany). The samples were mounted on double-sided adhesive carbon on aluminium sample holders. The micrographics were determined under VP mode (variable pressure), using 500× and 100× magnifications. The low vacuum mode of SEM is a special type, where the chamber (where the samples are placed) can be maintained at low vacuum at 70 Pa, while that the column remains under vacuum. In this way, it is possible to observe biologically sensitive samples without dehydrating or metalizing with gold (Sammons & Marquis, 1997).

2.5. Statistical analysis

Results are expressed as means with standard deviations of analysis performed in triplicate. One-way analysis of variance and Tukey's test were used to establish the significance of differences among mean values at $P \leqslant 0.05$. The statistical analyses were performed using GraphPad InStat Software Inc.

3. Results and discussion

3.1. Moisture content after baking

Water is the most important plasticizer in foods. Plasticizers work by embedding themselves between the chains of polymers, reducing the force of attraction between them and, thus, significantly lowering the glass transition temperature and making the final product softer (Blasia, D'Souza, Selmin, & DeLuca, 2005). This property is of great relevance in the food field as it can influence processing, shelf-life and sensorial acceptability of products (Pittia & Sacchetti, 2008). In order to achieve a suitable consistency, gluten-free dough requires more hydration than wheat flour dough and better moisture retention, after baking, would improve the gluten-free bread by decreasing hardness (Torbica et al., 2010; Miyazaki, Maeda, & Morita, 2005; Stampfli & Nerden, 1995; Pittia & Sacchetti, 2008).

The results obtained for moisture loss from gluten-free bread after baking are shown in Fig. 1. Incorporation of the enhancers (P, PUF, PUFS and PUFI) reduced moisture loss, which was expected considering the water holding capacity of the hydrocolloids (Rodriguez Furlán et al., 2010b; Rosell et al., 2001; Mandala et al., 2007).

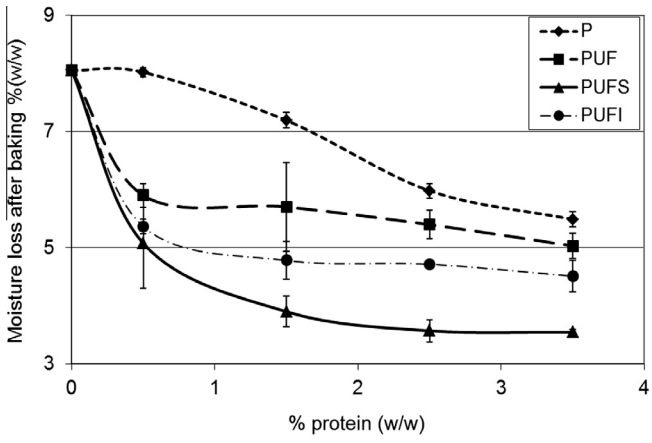

Fig. 1. Moisture loss of gluten-free breads supplemented with different enhancers (P, PUF, PUFS and PUFI) after baking (T = 200 °C; t = 20 min).

Also, due to treatment with ultrafiltration membranes the protein water holding capacity was improved, as found in previous work (Rodriguez Furlán et al., 2010b).

The combination of saccharides and proteins produced better moisture retention after cooking with the highest moisture content in the bread containing inulin and sucrose. In the formulation with incorporation of PUFI, the reduction of moisture loss was caused by the greater number of hydrophilic groups of inulin, which increased water retention through hydrogen bond interactions (Wang, Rosella, & de Barber, 2002; Scanlon & Zghal, 2001), as previously found by Rosell et al. (2001).

3.2. Crumb mechanical properties (compressive testing)

Textural information is important in the design of processes, in determining the functionality of ingredient for the development and improvement of products, quality control of intermediate and final products, in testing shelf-life and assessing properties correlated with sensory analysing (Scanlon & Zghal, 2001; Pittia & Sacchetti, 2008).

Table 1 shows the hardness or yield stress (σ_c), Young's Modulus and Resistance's Modulus obtained for different breads (control, P, PUF, PUFS and PUFI) after 1–3 days of storage.

An optimal value for mechanical properties to protein concentration of 1.5% (w/w) was found ($P < 0.05$) in bread supplemented with P compared with the control. At this concentration, a decrease in Resistance's Modulus was observed. More protein increased the Resistance's Modulus, leading to hardening during storage. There were no statistically significant differences in mechanical properties among samples during the 3 days of storage at the optimal protein concentration (1.5% (w/w)).

For breads supplemented with PUF at 0.5% (w/w), a slight reduction in the mechanical properties compared to the control was observed. However, with increasing protein concentration a significant increase in these values was obtained.

In formulations supplemented with protein and sucrose (PUFS), a statistically significant reduction was obtained in σ_c and Young's Modulus values ($P < 0.001$) for all protein concentrations tested. Furthermore, an optimum concentration in the range 0.5–1.5% (w/w), and a small increase in the properties analysed on the third day, were observed. With respect to the Resistance's Modulus, there were no statistically significant differences between control and samples containing different protein concentrations over the three days analysed. Only a significant increase on day 3 was observed for the highest concentration (3.5% (w/w)).

A statistically significant reduction ($P < 0.001$) in mechanical properties of breads supplemented with protein and inulin (PUFI, 2.5% (w/w)) was observed with respect to controls. Furthermore, in PUFI formulations, no statistically significant difference of the textural properties was determined over the period analysed ($P > 0.05$), (Supplementary Fig. 1).

Comparing the results of the different formulations, the loaves supplemented with PUFI corresponded to the minimum values for the textural properties ($P < 0.05$). Previous studies of Rosell et al. (2001) showed that the addition of hydrocolloids such as k-carrageenan or hydroxypropylmethylcellulose reduced the hardness of breadcrumb; however, it could not match the texture of bread with gluten.

For the sake of comparison, compressive testing assessments on fresh breadcrumb with gluten were carried out, and the following parameters were obtained: σ: 1.64 ± 0.55 KN/m^2; Young's Modulus: 0.09 ± 0.03 and Resistance's Modulus: 13.85 ± 3.01. Similar values were found by Keetels, Visser, van Vliet, Jurgens, and Walstra (1996). These results were in contrast with those obtained for gluten-free breads with the addition of enhancers, whereas for

Table 1
Firmness (σ_c), Young's Modulus and Resistance's Modulus values obtained from compression tests applied on gluten-free breads.[a]

Samples		σ_c = KN/m^2			Young's modulus			Resistance's modulus		
		Day 1	Day 2	Day 3	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3
Control		4.36 ± 0.01^{1,2}	4.36 ± 0.01^{1}	4.91 ± 0.31^{1,8}	0.16 ± 0.01^{1}	0.19 ± 0.02^{1,2}	0.22 ± 0.01^{1,5,7}	34.47 ± 0.40^{1,3}	41.09 ± 3.86^{1,6,8}	42.15 ± 1.56^{1,6,7}
P %(w/w)	0.5	3.27 ± 0.01^{1}	2.73 ± 0.31^{1}	4.36 ± 0.01^{1,8}	0.13 ± 0.01^{1}	0.16 ± 0.00^{1}	0.18 ± 0.01^{1}	26.11 ± 2.04^{1,2,7}	22.05 ± 0.32^{2,7}	34.33 ± 0.68^{1,2}
	1.5	3.63 ± 0.36^{1}	3.27 ± 0.63^{1}	3.82 ± 0.31^{1}	0.23 ± 0.02^{1}	0.25 ± 0.04^{1}	0.18 ± 0.03^{1}	20.30 ± 2.45^{2}	19.63 ± 4.86^{2}	24.22 ± 1.68^{2}
	2.5	4.36 ± 0.01^{1,3}	7.63 ± 0.63^{4,6}	7.09 ± 0.31^{5,4,9}	0.29 ± 0.01^{1,2,5}	0.59 ± 0.10^{2,4}	0.41 ± 0.01^{4,5}	29.93 ± 1.60^{1,2,3}	50.61 ± 0.66^{6}	46.53 ± 3.17^{6}
	3.5	5.45 ± 0.01^{2,3}	7.63 ± 0.63^{6,7}	6.00 ± 0.31^{7,8,9}	0.28 ± 0.03^{1}	0.5 ± 0.09^{3}	0.37 ± 0.02^{1,3,5}	37.42 ± 0.83^{5}	48.79 ± 0.18^{6}	48.04 ± 1.67^{5,6}
PUF %(w/w)	0.5	2.73 ± 0.32^{1}	4.91 ± 0.31^{1,3}	4.91 ± 0.31^{3,8}	0.15 ± 0.01^{1}	0.27 ± 0.01^{2,4}	0.25 ± 0.01^{1,4}	19.09 ± 2.43^{1}	32.94 ± 0.07^{1,6}	39.63 ± 0.40^{6,7,8}
	1.5	3.27 ± 0.01^{1,2}	3.27 ± 0.01^{1,4}	4.86 ± 0.92^{4,8}	0.1 ± 0.01^{1}	0.12 ± 0.01^{1}	0.23 ± 0.06^{1}	31.08 ± 0.96^{1,3}	42.48 ± 1.38^{1,6}	36.64 ± 0.14^{1,8}
	2.5	4.36 ± 0.63^{1,2}	4.13 ± 0.14^{1}	6.83 ± 0.79^{5,7,8}	0.15 ± 0.04^{1}	0.16 ± 0.00^{1,4}	0.43 ± 0.04^{3}	30.65 ± 1.72^{1,3}	47.54 ± 1.70^{1,4,6}	53.36 ± 5.86^{4,7,9}
	3.5	4.91 ± 0.32^{2,6}	4.31 ± 0.03^{1,6}	8.18 ± 0.32^{7}	0.23 ± 0.04^{1}	0.26 ± 0.02^{1}	0.39 ± 0.00^{3}	42.36 ± 1.11^{3}	44.00 ± 3.6^{3,6}	71.82 ± 1.74^{5,9}
PUFS %(w/w)	0.5	3.27 ± 0.01^{3}	4.91 ± 0.31^{1}	4.91 ± 0.31^{1,6}	0.09 ± 0.00^{3}	0.21 ± 0.01^{2,5}	0.25 ± 0.04^{5,7}	38.25 ± 2.18^{1}	45.77 ± 2.45^{1}	42.67 ± 2.23^{1,2}
	1.5	2.18 ± 0.01^{4}	3.27 ± 0.01^{5}	5.45 ± 0.01^{1,7}	0.08 ± 0.00^{3,4}	0.15 ± 0.00^{1,4,5}	0.18 ± 0.00^{5}	25.78 ± 0.20^{1}	36.53 ± 1.97^{1}	55.04 ± 4.32^{2,3}
	2.5	2.73 ± 0.32^{3,4,5}	3.08 ± 0.11^{5}	6.00 ± 0.31^{7,9}	0.10 ± 0.01^{1,3,6}	0.14 ± 0.00^{1,6}	0.29 ± 0.00^{7,8}	29.81 ± 1.84^{1}	35.44 ± 2.47^{1}	53.89 ± 0.53^{2,6}
	3.5	2.73 ± 0.31^{3,4}	3.27 ± 0.01^{5}	6.54 ± 0.01^{9}	0.11 ± 0.02^{1,3}	0.12 ± 0.01^{1}	0.36 ± 0.01^{8}	30.17 ± 3.51^{1}	41.44 ± 1.04^{1}	66.65 ± 3.29^{3}
PUFI %(w/w)	0.5	3.27 ± 0.02^{3}	3.82 ± 0.31^{1,3}	4.91 ± 0.31^{8}	0.11 ± 0.02^{1,3}	0.17 ± 0.03^{1,5}	0.23 ± 0.01^{5}	33.75 ± 1.03^{1}	33.61 ± 2.43^{1}	36.84 ± 1.12^{1}
	1.5	2.18 ± 0.01^{4}	2.73 ± 0.31^{4}	2.80 ± 0.31^{4}	0.07 ± 0.01^{2,3}	0.15 ± 0.01^{1,4}	0.23 ± 0.01^{4,5}	20.15 ± 2.71^{2}	18.87 ± 1.33^{2}	22.14 ± 2.19^{2,4}
	2.5	2.18 ± 0.01^{4}	2.33 ± 0.09^{4}	2.29 ± 0.11^{4}	0.07 ± 0.01^{2,3}	0.09 ± 0.01^{3,4}	0.11 ± 0.01^{3}	16.40 ± 1.62^{2}	15.98 ± 2.50^{2}	16.48 ± 0.30^{2,4}
	3.5	2.18 ± 0.01^{4}	3.27 ± 0.01^{3,4}	3.82 ± 0.31^{3}	0.12 ± 0.01^{1,3}	0.16 ± 0.01^{1,4}	0.23 ± 0.01^{4,5}	12.20 ± 1.27^{2}	20.18 ± 0.88^{2,3}	24.78 ± 2.55^{3,4}

[a] Means with equal superscripts in each group for a same file or column within the different concentrations and the control are not significantly different ($P = 0.05$) by the Tukey's test.

breads supplemented with PUFI, similar values were obtained ($P > 0.05$).

During breadcrumb storage, staling was observed. The determination of hardness with time is a tool to measure bread staling. Crumb hardness is represented by magnitude σ_c. The increase of σ_c ($P < 0.05$) with increasing storage time leads to hardening, which is expected as a result of starch retrogradation phenomena (Lazaridou et al., 2007). In breads supplemented with P, PUF and PUFS, a slight staling was produced during the studied period. However, for the loaves with PUFI, less staling was observed ($P < 0.05$) at the same optimum protein and saccharide concentration (PUFI, 2.5 (w/w)), and also a lower σ_c. Similar results were reported by Korus, Grzelak, Achremowicz, and Sabat (2006), who investigated the influence of inulin on gluten-free breads. These authors analysed the texture profile during 48 h storage, and demonstrated that the addition of 5% (w/w) and 8% (w/w) of inulin reduced the crumb hardening rate during the storage period. Salehifar, Seyedein Ardebili, and Hosein Azizi (2010) found similar results on breads from flour with a higher protein content.

It is known that mechanical properties of foods depend on compositional parameters and arise from the arrangement, by physical forces, of various chemical molecules into distinct micro- and macrostructures. The mechanical behaviours previously observed with the incorporation of the hydrocolloids (proteins and saccharides) within the food matrix depend on the following conditions: their relative concentrations, the physical forces involved in their interactions, and the manner in which these elements are spatially arranged, determining the different physical state and structural characteristics of the food matrix (Pittia & Sacchetti, 2008).

3.3. Bread structure

At macroscopic level, two phases can be identified in bread structure: a solid (wall material) and a gaseous (air cell) one, which are partially connected. So, the nature of their connectivity, their size, uniformity and fraction area determines the structure, and consequently, the mechanical properties of the bread. Therefore, raw materials determine the structure of the bread (Scanlon & Zghal, 2001).

When the dough is optimally developed by the mixer, the proteins appear to form complexes with flour lipids and some carbohydrate components, composing a coherent viscoelastic mass that encapsulates the air (Scanlon & Zghal, 2001). The incorporation of hydrocolloids, such as inulin, sucrose and proteins improves the crumb by stabilizing air cells in the bread dough, and preventing cell coalescence. Nevertheless, the uniform size distribution of gas cells is also important for bread quality.

Fig. 2a shows the area of air cells. The greater gas cell area for the different formulations corresponded with the optimal concentrations previously determined, which was: P: 0.5% (w/w); PUF: 2.5% (w/w); PUFS: 0.5–2.5%; PUFI: 1.5–3.5% (w/w). The maximum area of the air cells for all formulations was 3.5% (w/w) for PUFI.

Fig. 2b presents the distribution profile of the air cell diameters for the control sample, and formulations with PUFI at different concentrations. It shows that the increment in PUFI concentration increased the percentage of air cell of smaller diameter, generating an increase of air cell uniformity, which affects positively the crumb structure and the bread quality. With respect to the other formulations evaluated, no significant differences in distribution were observed. In addition, the Supplementary Table 2 shows that a statistically significant difference was only observed in the average diameter of the air cells between the control sample and the formulation with PUFI (3.5% (w /w)).

Regarding the bread structure, the incorporation of protein and saccharide in the tested formulations improved the matrix structure. In effect, the cracked surfaces in the upper crust of gluten-free

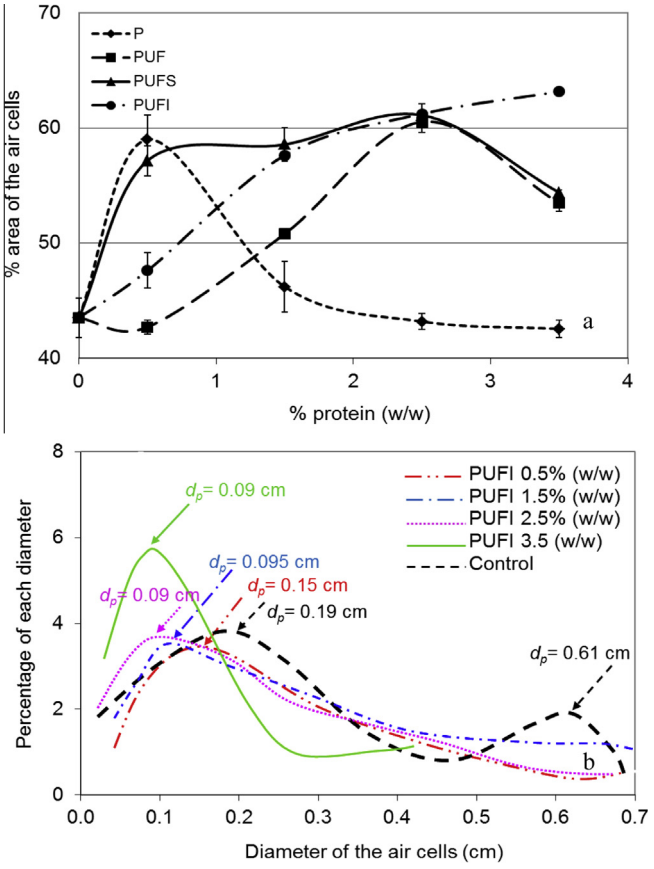

Fig. 2. (a) Area percentage of the air cells of gluten-free breads for the formulations tested for control and breads supplemented with enhancers at different concentrations; (b) percentage distribution profile of the air cells diameter, for control sample and formulations supplemented with PUFI at different concentrations.

control bread disappeared in breads supplemented with PUFI 2.5% (w/w), (Supplementary Fig. 1). Similar results were found by Torbica et al. (2010) when studying gluten-free bread formulations. Furthermore, the cross section and the form of a piece of the sample with PUFI showed a uniformity in the crumb structure and symmetry achieved after cooking (Supplementary Fig. 2).

3.4. Microstructure study of the thickening of walls surrounding the gas cells

In general, the incorporation of saccharides and proteins in bread formulations increased the hardness of the samples. Skendi et al. (2010) relates this behaviour to the thickening of the walls surrounding the gas cells (lamella) for breads fortified with β-glucan.

Fig. 3 shows the thickening of the walls surrounding the gas cells for the control sample and formulations with enhancers in optimal concentrations (P: 0.5% (w/w); PUF: 2.5% (w/w); PUFS: 1.5% (w/w); PUFI: 2.5% (w/w)). In effect, for samples with the addition of P, a thickening of lamella walls was observed ($P < 0.05$), compared with the control. For formulation with PUF, there was no statistically significant difference when compared with the control. For samples with the incorporation of PUFS and PUFI, the thickness of the lamellae decreased ($P < 0.01$), compared with the control. These results corroborated those obtained in hardness testing, in which there was a significant decrease for samples with added protein and saccharide.

As there was a linear relationship between the thickening of the walls surrounding the gas cells and σ_c (related to the hardness of

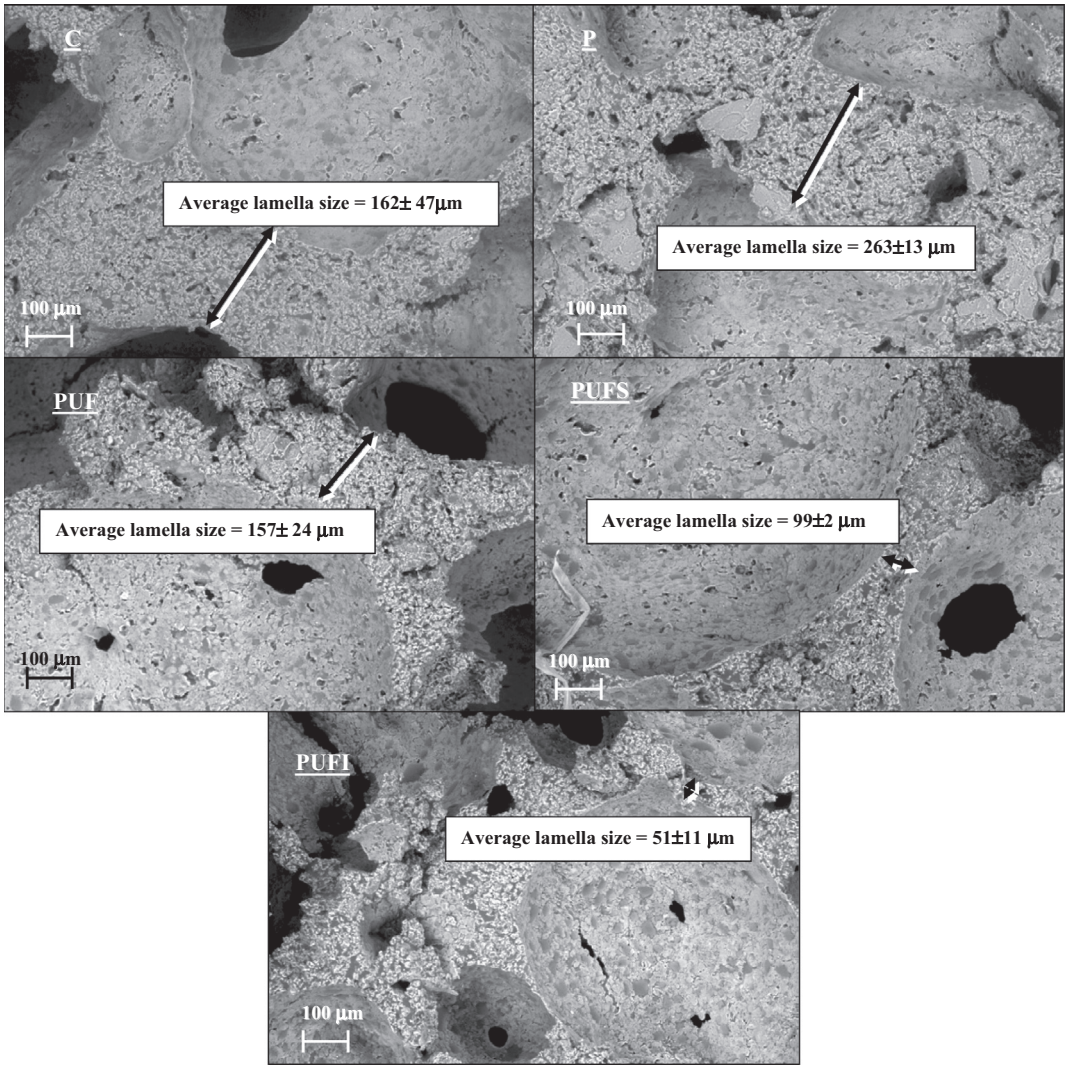

Fig. 3. Scanning electron microscopy (SEM) of gluten-free bread matrix. Average measurements of thickening of the walls surrounding gas cells for gluten-free breads with supplemented to an optimal concentration. (P: 0.5% (w/w); PUF: 2.5% (w/w); PUFS: 1.5% (w/w); PUFI: 2.5% (w/w)). Magnifications 100×.

bread) with a linear regression (R^2) ≈ 0.9, it was assumed that the texture of bread was directly related to the thickening of the walls surrounding the gas cells. Thus, it could be affirmed that the addition of PUFS (1.5% (w/w)) and PUFI (2.5% (w/w)) generates a reduction in the hardness of the bread due to the decrease in the thickening of the walls surrounding the gas cells; PUFI being more effective than PUFS.

3.5. Microstructure study of the starch granule

Starch is the main component of gluten-free dough and, therefore, the characteristics of starch significantly influence the quality of bread (Miyazaki et al., 2005; Korus, Witczak, Ziobro, & Juszczak, 2009).

Staling is a phenomenon that describes the deterioration of bread quality during storage, and is associated with some typical sensorial changes such as loss of flavor and crumb hardness (Stampfli & Nerden, 1995).The mechanism of bread staling, including gelation and recrystallization of starch, is called retrogradation. This phenomenon affects the texture, acceptability and digestibility of food. Starch consists of two polymers: amylase, constituting part of the amorphous zone, and amylopectin, the crystalline zone in the starch granule. The amylopectin fraction in gelatinized starch is transformed and recrystallized during storage, bringing about an increase in hardness and opacity of breads. Only a few compounds can reduce the loaf staling process, restricting starch swelling during baking. When starch granules are less swollen, less solubilisation of starch molecules occurs (Miyazaki et al., 2005).

Using SEM, and based on the calculation of starch granule diameter (take from SEM images), breadcrumb of control and samples containing P; PUF and PUFS was corroborated that no starch granules of diameter ⩾20 μm (≈13.0 ± 1.1 μm). This fact indicates that most starch granules were highly swollen and dispersed during baking. However, breadcrumb containing inulin and protein (PUFI) retained starch granules with diameters greater than 20.2 ± 1.8 μm (Supplementary Fig. 3). These results suggest less swelling of starch and a decrease in the rate of bread staling, due to the large starch granule remnants in the protein network after baking. That could contribute to the decrease in the hardness of breadcrumb, as previously observed.

3.6. Effect of hydrocolloids on baking properties of dough

Fig. 4a shows the increase in volume during the leavening of the control sample (without incorporation of protein and saccharides) and the formulations supplemented with enhancers (P, PUF, PUFS

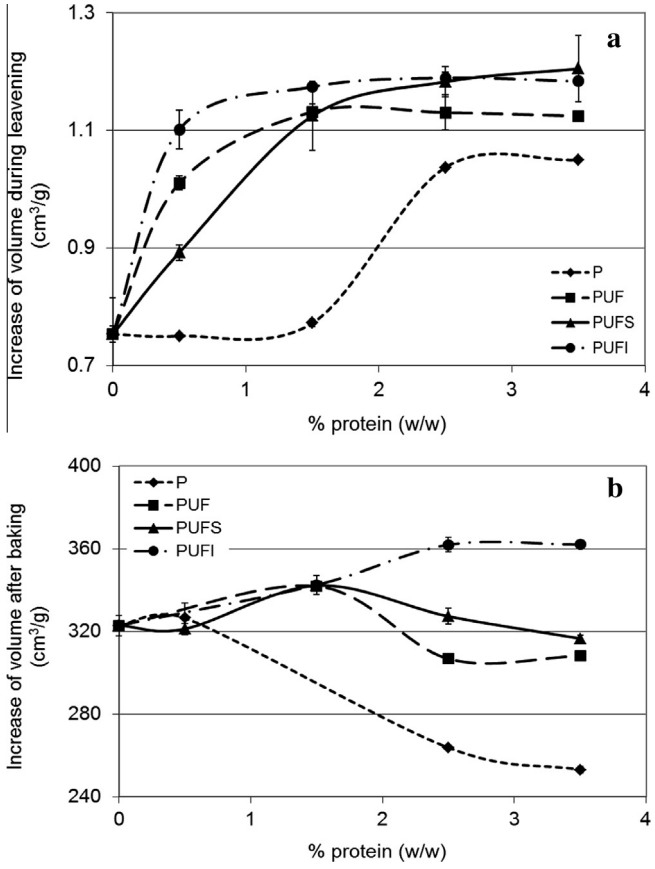

Fig. 4. Increase in volume during: (a) the leavening and (b) baking of the gluten-free bread supplemented with P: 0.5% (w/w); PUF: 2.5% (w/w); PUFS: 1.5% (w/w); PUFI: 2.5% (w/w) compared to the control sample.

and PUFI). In all cases, a significant increase in the volume was observed compared with controls ($P < 0.001$). The smallest growth occurred in those supplemented with P across the range of concentrations studied.

Fig. 4b shows the results obtained with an increase in volume after baking in controls and samples supplemented with different concentrations of P, PUF, PUFS and PUFI. Statistically significant differences were observed when comparing the control with the samples supplemented with concentrations higher than 0.5% (w/w). Again, the worst result was obtained in the sample supplemented with P. This behaviour suggests that the improvement in the performance of the enhancers containing saccharides is due to the upgrading of the functional properties of the plasma processed with UF and freeze-dried with compounds, which act as cryoprotective agents (Rodriguez Furlán et al., 2010a). The greatest increase in volume after baking was for breads supplemented with PUFI at concentrations of 2.5% (w/w) and 3.5% (w/w) ($P < 0.001$).

3.7. Crumb and crust colour

The L^*, a^*, and b^* values for crust and crumb of all prepared gluten-free breads are summarised in Table 2.

The bread formulations supplemented with P, PUF, PUFI resulted in a lighter colour for the crust than the PUFS ($P < 0.01$). However, when comparing L^* values of crust for C and the other samples, no statistically significant differences were found. Similarly, no differences in a^* and b^* for crust among the different gluten-free samples were found.

For breadcrumbs, addition of PUFI produced breads with the highest L^* ($P < 0.001$); this means a whiter crumb when compared with other formulations and controls. a^* for the crumb of control breads and non-supplemented formulations were all close to zero, indicating that the red or green component was negligible. The influence of hydrocolloids on b^* was important compared to the controls, a significant increase was observed for the supplemented formulations, except for PUF 0.5% (w/w). Therefore, these formulations had a greater yellowing, which is desirable for this product. Variations in the colour of gluten-free breads supplemented with hydrocolloids were also observed by Lazaridou et al. (2007) and Mandala et al. (2007).

3.8. Sensory analysis

The sensory evaluation of the fresh gluten-free breads was performed using formulations with the maximum protein concentration (3.5% (w/w)), and carried out by untrained panelists using a hedonic scale of five points for overall acceptability. The parameters evaluated were: colour, aroma, flavor and texture. All gluten-free formulations were acceptable, since they received scores much higher than 2.5, ranging from 3.4 to 4.3. Furthermore, there

Table 2
Effect of hydrocolloid addition on crust and crumb colour of gluten-free bread.[a]

Samples		Crust			Crumb		
		L^*	a^*	b^*	L^*	a^*	b^*
Control		$53.13 \pm 3.05^{1,2}$	$14.84 \pm 2.43^{1,2}$	33.61 ± 0.77^{1}	73.42 ± 0.57^{1}	-1.65 ± 0.02^{1}	12.63 ± 0.23^{1}
P %(w/w)	0.5	65.49 ± 4.39^{1}	10.83 ± 3.16^{1}	35.13 ± 2.41^{1}	76.70 ± 0.60^{2}	-0.78 ± 0.01^{2}	20.29 ± 0.12^{3}
	1.5	$64.00 \pm 1.03^{1,2}$	9.70 ± 0.87^{1}	34.69 ± 0.78^{1}	72.12 ± 0.88^{1}	-1.58 ± 0.02^{1}	12.76 ± 0.10^{1}
	2.5	50.30 ± 2.96^{2}	17.76 ± 1.81^{2}	34.79 ± 1.14^{1}	71.57 ± 0.33^{1}	-1.33 ± 0.02^{1}	14.48 ± 0.14^{2}
	3.5	$63.24 \pm 0.21^{1,2}$	9.99 ± 0.27^{1}	36.64 ± 0.28^{1}	70.93 ± 1.11^{1}	$-1.14 \pm 0.06^{1,2}$	15.65 ± 0.06^{2}
PUF %(w/w)	0.5	66.78 ± 1.93^{1}	5.40 ± 2.03^{3}	28.99 ± 2.79^{1}	$74.63 \pm 0.52^{1,2}$	-1.67 ± 0.08^{1}	12.16 ± 0.23^{1}
	1.5	56.38 ± 1.34^{1}	17.93 ± 0.22^{1}	37.44 ± 0.63^{1}	$75.85 \pm 0.45^{1,2}$	0.09 ± 0.10^{2}	21.41 ± 0.31^{2}
	2.5	59.24 ± 0.68^{1}	16.73 ± 0.21^{1}	38.21 ± 0.6^{1}	$76.21 \pm 0.39^{1,2}$	$0.42 \pm 0.08^{2,3}$	$22.24 \pm 0.21^{2,3}$
	3.5	63.18 ± 1.61^{1}	14.42 ± 0.65^{1}	38.84 ± 0.48^{1}	77.11 ± 0.29^{2}	0.78 ± 0.14^{3}	22.93 ± 0.28^{3}
PUFS %(w/w)	0.5	51.20 ± 2.75^{1}	15.61 ± 1.38^{1}	33.13 ± 0.39^{1}	78.34 ± 0.53^{2}	-0.79 ± 0.06^{2}	19.49 ± 0.19^{2}
	1.5	46.75 ± 4.90^{1}	16.22 ± 0.57^{1}	29.92 ± 3.16^{1}	$75.80 \pm 0.46^{1,2}$	$-0.44 \pm 0.04^{2,3}$	19.20 ± 0.17^{2}
	2.5	38.94 ± 2.20^{1}	18.34 ± 0.24^{1}	25.22 ± 2.22^{1}	$75.66 \pm 0.17^{1,2}$	$-0.39 \pm 0.01^{2,3}$	19.74 ± 0.24^{2}
	3.5	48.86 ± 4.69^{1}	17.00 ± 1.81^{1}	31.51 ± 1.96^{1}	$74.64 \pm 0.44^{1,2}$	-0.14 ± 0.05^{3}	19.91 ± 0.13^{2}
PUFI %(w/w)	0.5	59.82 ± 2.45^{1}	14.82 ± 1.54^{1}	37.89 ± 0.58^{1}	77.73 ± 0.68^{2}	$-1.11 \pm 0.10^{1,3}$	19.57 ± 0.14^{2}
	1.5	62.92 ± 2.08^{1}	14.55 ± 1.12^{1}	40.14 ± 0.31^{1}	78.05 ± 0.61^{2}	-0.19 ± 0.25^{2}	19.73 ± 0.41^{2}
	2.5	62.53 ± 2.75^{1}	12.99 ± 1.86^{1}	35.48 ± 1.05^{1}	78.32 ± 0.04^{2}	$-0.70 \pm 0.03^{3,4}$	19.26 ± 0.16^{2}
	3.5	50.25 ± 2.37^{1}	19.47 ± 0.43^{1}	34.70 ± 1.85^{1}	77.97 ± 0.41^{2}	$-0.38 \pm 0.04^{2,4}$	19.98 ± 0.35^{2}

[a] Means with equal superscripts in each group for the same column within the different concentrations and the control are not significantly different ($P \geqslant 0.05$) by the Tukey's test.

was no statistically significant difference in the attributes evaluated between control (sample without gluten and without additives) and breads with the addition of different supplements. This result is in contrast with those obtained by other authors who studied gluten bread supplemented with inulin. In their reports, a low score for all sensory attributes was observed (Collar, Santos, & Rosell, 2007; Mandala, Polaki, & Yanniotis, 2009; ÓBrien, Mueller, Scannell, & Arendt, 2003; Peressini & Sensidoni, 2009; Wang et al., 2002; Poinot et al., 2010).

4. Conclusions

With the incorporation of hydrocolloids such as bovine proteins, alone or in combination, in particular in the formulation with PUFI in gluten-free breads, several improvements were achieved, including: reduction in the diameter and greater uniformity of the air cells, reduction of the thickness of the lamina surrounding the air cells, increased bread volume with a good symmetry, crumb brightness and yellowing, and water absorption, and a reduction in crumb hardness. The values for textural properties of gluten-free breads (Young's Modulus, the critical stress σ_c and the Resistance's Modulus) were all reduced, approaching those obtained for gluten breads with similar moisture ($P < 0.05$), mainly for formulations with PUFI. In addition, a reduction in hardening of bread over time was achieved. Therefore, based on these results, we suggest it is possible to obtain an improved protein network by incorporating PUFI at (2.5% (w/w)), leading to a gluten-free bread similar to breads with gluten in all quality and sensorial characteristics. Furthermore, the bread formulation supplemented with PUFI is a protein-enriched formulation with a prebiotic inulin.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2014.08.033.

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