



Effect of thermal processing on the antigenicity of allergenic milk, egg and soy proteins

Ana Luiza Soares dos Santos¹ · Pedro Paulo Borges dos Santos¹ · Gustavo de Almeida Amaral¹ · Eduardo Costa Soares¹ · Cláudia Aparecida de Oliveira e Silva² · Scheilla Vitorino Carvalho de Souza¹

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Abstract The detection of allergenic proteins and the influence of processing on the structure and antigenicity of these proteins are relevant topics. Using commercial enzyme-linked immunosorbent assay kits, this study aimed to evaluate the degradation profiles of milk, egg and soy proteins during the processing of semisweet biscuits. The formulations were baked under different conditions according to a complete factorial experiment that included a three-level temperature factor and a six-level time factor. β -lactoglobulin and egg white proteins were severely degraded, the degradation of casein was intermediate, and soy proteins were the most stable. Complete allergen protein degradation was found under only the extreme baking conditions, which resulted in products that were not sensorily acceptable. Residual levels of the proteins were detected after baking, indicating that this thermal processing reduced but did not eliminate the antigenicity of these proteins; thus, baking cannot be considered a strategy to protect allergic consumers.

Keywords Allergen detection · ELISA methods · Food allergen · Baking

Introduction

Food allergies constitute an important public health problem and have been increasing in prevalence in the last two to three decades, disproportionately affecting persons in both industrialized and westernized regions and being more common in children than in adults (Sicherer and Sampson 2018), with prevalences of 6.53% and 3.51%, respectively (Mc Gowan and Keet 2013).

The main treatment for food allergies is exclusion of the allergen from the diet, aiming to avoid the triggering of symptoms, disease progression and worsening of allergic manifestations (Sicherer and Sampson 2018). Careful reading and adequate interpretation of food labels are important aspects of food allergy management.

The first country in the world to establish a law for compulsory labeling of allergens was Japan, which published these regulations in 2002 (Shoji et al. 2018). In the USA, allergens have been declared on the labels of packaged foods since 1996, initially on a voluntary basis. Despite the publication of the Food Allergen Labeling and Consumer Protection Act (FALCPA) in 2004, allergen labeling became mandatory in only 2006, requiring the declaration of common allergens in food (FDA 2013). In the following year, in Europe, similar regulations were enforced through Directive 2007/68/EC, which addressed the declaration of ingredients considered allergenic in commercial foods (Commission 2007). In Brazil, legislation requiring allergen labeling was approved in 2015 and implemented in 2016 (Brasil 2015). Food allergens such as milk, egg and soy are now common in most international regulations (Brasil 2015; FDA 2013; Shoji et al. 2018; Commission 2007).

To ensure compliance with the regulations for labeling allergens in food and to increase consumer protection,

✉ Scheilla Vitorino Carvalho de Souza
scheilla@bromatologiaufmg.com.br

¹ Postgraduate Program in Food Science (ALM/PPGCA), Faculty of Pharmacy (FAFAR), Federal University of Minas Gerais (UFMG), Av. Antônio Carlos, 6627, Campus da UFMG, Pampulha, Belo Horizonte, Minas Gerais 31270-010, Brazil

² Ezequiel Dias Foundation, Health Public Laboratory of Minas Gerais State, Rua Conde Pereira Carneiro, 80, Gameleira, Belo Horizonte, Minas Gerais 30.5010-010, Brazil

quality control and food safety measures that involve the detection of allergenic proteins at even residual levels are necessary (Monaci et al. 2006). In this context, processed foods are considered critical because the processing conditions influence the structure and antigenicity of proteins, as well as their ability to be extracted and detected (Diaz-amigo and Popping 2010; Diaz-amigo 2010; Muraro et al. 2014).

Among the food preservation techniques, thermal processing may cause the most protein denaturation, leading to conformational and chemical modifications that will influence the antigenic capacity of proteins (Culbertson et al. 2006; Diaz-amigo and Popping 2010; Muraro et al. 2014).

Of the milk proteins, caseins are reportedly the most thermostable, whereas whey proteins such as β -lactoglobulin (β LG) are reportedly heat-labile (Monaci et al. 2006; Nowak-Węgrzyn et al. 2008). According to Nowak-Węgrzyn et al. (2008), after ten minutes of heating at 100 °C, a significant reduction in the allergenicity of milk proteins is observed. The caseins remain stable and can be heated at 140 °C for many hours without apparent changes, but aggregation and coagulation may occur after 15–20 min of heating (Culbertson et al. 2006). However, whey proteins are completely denatured by heating at 90 °C for 10 min (Culbertson et al. 2006).

Information on the denaturation temperatures of egg and soy proteins is still limited (Huntington and Stein 2001). Huntington and Stein (2001) reported that ovalbumin has an average denaturation point between 78 and 86 °C, and Goetz and Koehler (2005) reported ovalbumin, conalbumin and ovomucoid denaturation temperatures of 84.5 °C, 61.0 °C and 90 °C, respectively. Huntington and Stein (2001) observed the denaturation of two soy protein fractions, containing glycine and β -conglycine, at temperatures above 80 °C and 70 °C, respectively.

Information on changes in the allergenic potential of proteins due to protein denaturation is relevant for allergen risk assessment in food production and for improving the methodologies for detecting these substances (Muraro et al. 2014). Several studies have described the effects of thermal processing at specific temperatures on the detection of allergens in cookies and pastry products (Bloom et al. 2015; Bugyi et al. 2010; Downs and Taylor 2010; Gomaa and Boye 2013; Khuda et al. 2012; Monaci et al. 2011). However, more precise information on the influence of heat treatment on the antigenicity and allergenicity of allergenic proteins is still lacking. The goal of the present work was to evaluate the degradation profiles of milk, egg and soy proteins during the baking of semisweet biscuits combining different durations and different temperatures and to verify the ability to detect residual levels of these proteins using an enzyme-linked immunosorbent assay (ELISA).

Materials and methods

Biscuit formulations

Formulations of semisweet biscuits (type Maria) were developed using basic ingredients, and the percentages of different ingredients were estimated as the averages of formulations described in the literature (Cauvain and Young 2006; Davidson 2015; Manley 1996), except for the allergens, which were not incorporated as ingredients but in low concentrations to simulate contamination.

The food-grade ingredients used were obtained from companies that supply raw materials to food industries in Brazil: wheat flour enriched with iron and folic acid (Rosa Branca, Rio de Janeiro, Brazil e Agrária, Paraná, Brazil); refined sugar (Guarani, São Paulo, Brazil); margarine (Becel, São Paulo, Brazil); sodium chloride (Cisne, São Paulo, Brazil); ammonium bicarbonate (Bombay, São Paulo, Brazil); sodium bicarbonate (Bombay, São Paulo, Brazil); liquid soy lecithin (Caramuru Alimentos, Goiás, Brazil); sodium metabissulphite (Pryme Foods, São Paulo, Brazil); pasteurized whole milk (Itambé, Minas Gerais, Brazil); egg (Per'fa, Minas Gerais, Brazil); and roasted soy flour (Viver Bem, Minas Gerais, Brazil).

The allergen concentrations in the biscuit formulations were determined considering (i) the proportion of the allergenic proteins in milk, egg and soy; (ii) the quantification range of β LG, casein, egg white proteins and soy proteins declared by the manufacturer of the ELISA kits; and (iii) previous studies of allergen degradation during the baking of cookies (Gomaa and Boye 2015; Khuda et al. 2012; Monaci et al. 2011). Then, semisweet biscuits were formulated with 0% allergen, 0.25% milk (corresponding to 0.012 mg β LG/100 g), 0.20% milk (corresponding to 0.075 mg casein/100 g), 0.022% egg (corresponding to 0.013 mg egg white proteins/100 g) and 0.20% soy flour (corresponding to 0.023 mg soy proteins/100 g). The formulations and origins of the ingredients used to make the biscuits are described in Table 1.

Biscuit processing

Measures were taken in accordance with the Guide established by the National Health Surveillance Agency of Brazil, which defines seven factors as relevant in identifying the critical steps to control the risk of cross contamination: (i) raw materials and ingredients; (ii) production process; (iii) employees and collaborators; (iv) cleaning; (v) packaging; (vi) storage; and (vii) transportation (Brazil 2018). All ingredients used, except pasteurized whole milk, egg and roasted soy flour, were certified as free from the studied allergens. Even though, the main

Table 1 Formulations of the semi-sweet biscuits

Ingredient	Mass (g)				
	Allergen free	0.25% Milk (0.012 mg β LG/100 g)	0.20% Milk (0.075 mg casein/100 g)	0.022% Egg (0.013 mg egg white proteins/100 g)	0.20% Soy (0.023 mg soy proteins/100 g)
Wheat flour enriched with iron and folic acid	100.0	100.0	100.0	100.0	100.0
Refined sugar	23.7	23.7	23.7	23.7	23.7
Margarine	18.9	18.9	18.9	18.9	18.9
Sodium chloride	1.0	1.0	1.0	1.0	1.0
Ammonium bicarbonate	0.7	0.7	0.7	0.7	0.7
Sodium bicarbonate	0.5	0.5	0.5	0.5	0.5
Soy lecithin	0	0.4	0.4	0.4	0
Sodium metabisulphite	0.02	0.02	0.02	0.02	0.02
Pasteurized whole milk	0	0.4	0.3	0	0
Egg	0	0	0	0.03	0
Roasted soy flour	0	0	0	0	0.3
Water	20.9	20.9	20.9	20.9	20.9

Allergenic ingredients indicated in bold

β LG: β -lactoglobulin; All ingredients were food grade: wheat flour enriched with iron and folic acid (Rosa Branca, Rio de Janeiro, Brazil e Agrária, Paraná, Brazil); refined sugar (Guarani, São Paulo, Brazil); margarine (Becel, São Paulo, Brazil); sodium chloride (Cisne, São Paulo, Brazil); ammonium bicarbonate (Bombay, São Paulo, Brazil); sodium bicarbonate (Bombay, São Paulo, Brazil); liquid soy lecithin (Caramuru Alimentos, Goiás, Brazil); sodium metabisulphite (Pryme Foods, São Paulo, Brazil); pasteurized whole milk (Itambé, Minas Gerais, Brazil); egg (Per'fa, Minas Gerais, Brazil); and roasted soy flour (Viver Bem, Minas Gerais, Brazil)

ingredients (wheat flour enriched with iron and folic acid, refined sugar, margarine, liquid soy lecithin, and sodium metabisulphite) were tested individually for the studied allergens. For liquid soy lecithin only egg and milk proteins were evaluated. All the material used in the production of biscuits (such as bowls, rollers, mechanical openers, molds, trays, spatulas and packages) and the cleaning supplies were for first use. Also, these materials were purchased in sufficient quantity to be divided into four different biscuit production lines: allergen-free, milk, egg and soy. Prior to the formulation of the biscuits, all materials were rinsed with water, soak in neutral detergent solution, washed thoroughly, washed in tap water ten times, rinsed with deionized water and then dried at room temperature. After that, the material was disinfected with 70% ethyl alcohol. Equipment and countertops were also washed and disinfected with 70% ethyl alcohol. All collaborators were previously trained, used the necessary vestments and followed principles of good manufacturing practices to avoid contamination of formulated products.

The food-grade ingredients used in the production of the biscuits were weighed individually on a calibrated analytical balance with a resolution of 0.0001 g and maximum capacity of 220,000 g (Shimadzu AUX 220, Brazil) and on a balance with a resolution of 0.001 g and maximum capacity of 310.0 g (Gehaka BK 300, São Paulo, Brazil).

The ingredients were transferred to a stainless steel bowl, and ammonium bicarbonate was dissolved in water before being incorporated into the dough (Manley 2001). The dough was then mixed using a planetary mixer (Arno, São Paulo, Brazil) for 5 min, followed by manual kneading to obtain a uniform mass that reached approximately 30 °C. Subsequently, the dough was laminated with the help of a stainless steel roller and mechanical openers until it reached a thickness of 5 mm. Then, the dough was cut with round stainless steel molds 4.0 cm in diameter, resulting in raw biscuits of approximately 15 g each.

The biscuits were arranged on stainless steel baking trays and baked in a conventional electric oven (Layr, São Paulo, Brazil) under the time and temperature conditions established in the factorial experiment. The temperature was monitored by a calibrated infrared thermometer (Instruemp ITTI 380, São Paulo, Brazil), and the time was monitored by a calibrated chronometer. The baked biscuits, approximately 11.3 g each, were cooled to room temperature (20 °C–25 °C), removed from the tray using stainless steel spatulas and ground and homogenized in a sample processor (Cutter Sire, Filizola, São Paulo, Brazil) for 1 min, followed by sieving (25 mesh).

Some biscuits were not baked and remained raw. Such biscuits were homogenized manually.

The baked and unbaked samples were packaged in 10-g low-density polyethylene terephthalate *stand-up pouch* sachets, sealed using a vacuum sealer (Ez Home 201, São Paulo, Brazil) and stored in a refrigerator (2 °C–8 °C) until the time of the tests.

Factorial design for the baking conditions

Each formulation was produced in two different batches. In each batch, the formulations containing allergens were divided and baked under different conditions according to the complete factorial experiment that included a three-level temperature factor (150, 180 and 210 °C) and a six-level time factor (5, 10, 15, 20, 25 and 30 min). For casein and soy, the time point of 30 min was not evaluated.

The allergen-free formulation was divided into two portions: one was kept raw, and the other was baked under the milder study conditions (150 °C for 5 min). These samples, in addition to the individual raw materials (wheat flour enriched with iron and folic acid, refined sugar, margarine, liquid soy lecithin, and sodium metabisulphite), served as the negative controls of the experiment (Fig. 1).

Determination of allergens by enzyme-linked immunosorbent assay (ELISA)

The allergen analysis was performed in duplicate for the tested ingredients and each treatment of each batch using: (i) a RIDASCREEN FAST β -Lactoglobulin® ELISA kit (R4902) with a limit of detection of 0.19 mg of β LG/kg, limit of quantification of 0.5 mg of β LG/kg and standard

solutions for calibration curve of 0, 0.5, 1.5, 4.5 and 13.5 mg of β LG/kg; (ii) a RIDASCREEN FAST Casein® ELISA kit (R4612) with a limit of detection of 1.36 mg of casein/kg, limit of quantification of 2.5 mg of casein/kg and standard solutions for calibration curve 0, 0.5, 1.5, 4.5 and 13.5 mg of casein/kg; (iii) a RIDASCREEN FAST Egg® ELISA kit (R6402) with a limit of detection of 0.03 mg of egg white protein/kg, limit of quantification of 0.13 mg of egg white protein/kg and standard solutions for calibration curve of 0, 0.13, 0.40, 1.18 and 3.55 mg of egg white protein/kg; and (iv) a RIDASCREEN FAST Soya® (R7102) ELISA kit with a limit of detection of 0.31 mg of soy protein/kg, limit of quantification of 2.5 mg of soy protein/kg and standard solutions for calibration curve of 0, 2.5, 5, 10 and 20 mg of soy protein/kg.

The sample extraction procedure for ELISA analysis is described below for the different kits.

β -Lactoglobulin and casein—1 g of each sample previously ground, sieved and homogenized was weighed in a 50 mL Falcon tube. 4 mL of the extraction solution 2 were added to the tube, which was vigorously shaken in a Vortex-type shaker for 1 min. The tube was then heated in a thermostatic bath at 100 °C for 10 min, followed by cooling in an ice bath. Then, 16 mL of allergen extraction buffer containing additive 1 (A-AEP solution) previously heated to 60 °C were added to the tube, which was shaken in a Vortex-type shaker for 1 min. The tube was heated in a thermostatic bath at 60 °C for 10 min, followed by cooling in an ice bath and centrifugation for 10 min at a rotation of 2500 xg. The extract was obtained by diluting the

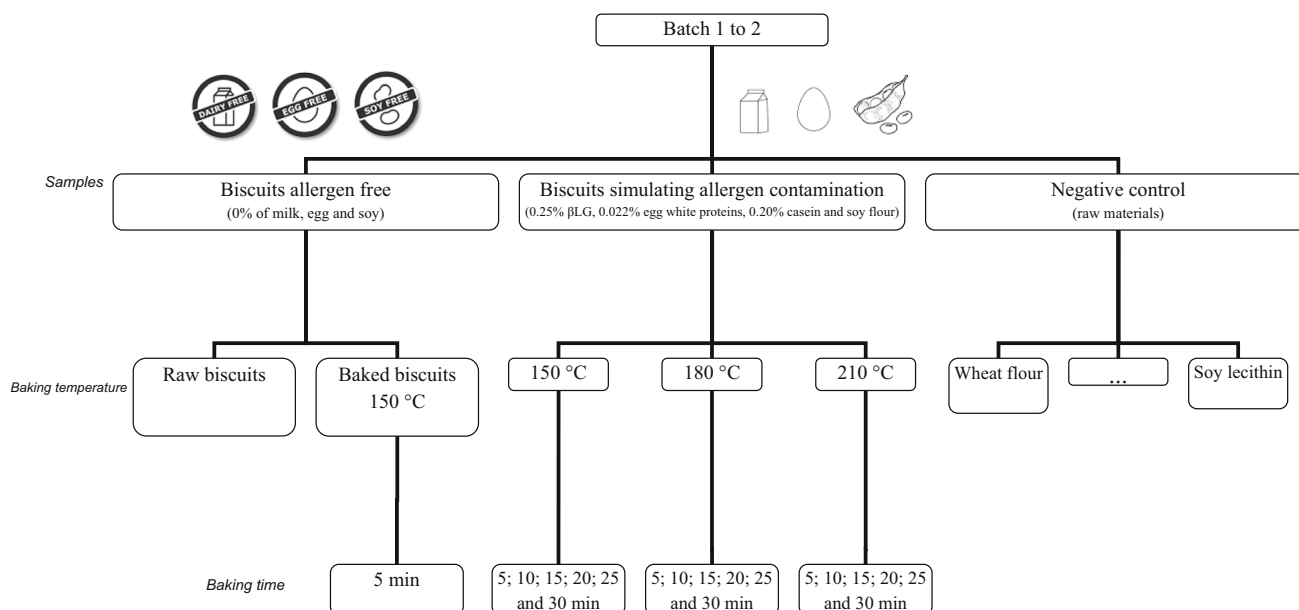


Fig. 1 Experimental design for the study of the degradation of β -lactoglobulin (β LG), casein, egg white proteins and soy proteins in the baking of semi-sweet biscuits

supernatant at a ratio of 1:5 (v/v) with an extraction buffer diluted without additive, in an Eppendorf tube.

Egg white protein—1 g of each sample previously ground, sieved and homogenized was weighed in a 50 mL Falcon tube. 20 mL of diluted extraction buffer (1:10, previously heated to 60 °C) were added and vigorously shaken in a Vortex-type shaker for 1 min. The tube was heated in a thermostatic bath at 60 °C for 10 min, followed by cooling in an ice bath and centrifugation for 10 min at a rotation of 2500 $\times$ g at 4 °C. The supernatant was transferred for an Eppendorf tube.

Soy protein—1 g of each sample previously ground, sieved and homogenized was weighed in a 50 mL Falcon tube. 2.5 mL of extraction solution 3 and 17.5 mL of diluted allergen extraction buffer solution (previously heated to 60 °C) were added and vigorously shaken in a Vortex-type shaker for 1 min. The tube was then heated in a thermostatic bath at 100 °C for 10 min, followed by cooling in an ice bath. Then, the sample was centrifuged for 10 min at a rotation of 2500 $\times$ g at 4 °C. The extract is obtained by diluting the supernatant at a ratio of 1:5 (v/v) with a diluted extraction buffer, in an Eppendorf tube.

For ELISA, 100 μ L aliquots of the sample extracts and standard solutions were transferred to microplate wells using a micropipette. The microplate was incubated for 10 min at room temperature (20 °C–25 °C). Then, the liquid contents of the microplate were washed with wash buffer using an automatic washing machine (Biotek 50TS12, São Paulo, Brazil) with a program consisting of three cycles of 250 μ L. Then, 100 μ L of the enzyme conjugate was added to the wells using a micropipette, followed by manual homogenization and incubation for 10 min at room temperature (20 °C–25 °C). The liquid contents of the microplate were washed with wash buffer, with three cycles of 250 μ L in the automatic washer. Then, 100 μ L of the chromogen solution was added to each well using a micropipette, followed by manual homogenization and incubation for 10 min at room temperature (20 °C–25 °C) in the dark. To conclude the test, 100 μ L of stop solution was added to each well. The plate was homogenized manually, and the absorbance at 450 nm was measured on a microplate reader (Biotek 800TSUVI, São Paulo, Brazil) within 10 min. The absorbances recorded for the samples and standards were typed into a RIDASOFT® Win program spreadsheet, and the results were automatically generated according to the models proposed by the manufacturer.

Statistical analysis

The results obtained from the factorial experiment were evaluated for their distribution by the Ryan-Joiner test and were then submitted to analysis of variance (ANOVA), and

the means estimated for the different treatments were discriminated by Tukey's test (Pimentel-Gomes 2009). The level of significance adopted in the hypothesis tests was $\alpha = 0.05$. The programs used for the statistical analyses were Microsoft Excel 2010® and Minitab®.

Results and discussion

Decrease in the contents of allergenic proteins

Negative assay results were obtained for both the raw allergen-free biscuit samples and allergen-free biscuit samples baked at 150 °C for 5 min, as well as for the raw materials (wheat flour enriched with iron and folic acid, refined sugar, margarine, liquid soy lecithin, and sodium metabisulphite), ensuring the absence of contamination and the adequacy of the good manufacturing practices used to prepare the biscuits. However, for soy proteins, positive results were obtained for the samples of allergen free formulation raw and baked at 150 °C for 5 min and for the individual samples of wheat flour enriched with iron and folic acid, suggesting contamination of the raw material wheat flour by soy proteins. Notably, the flour used was certified to be allergen-free. Soy proteins were not detected in the other raw materials (refined sugar, margarine and sodium metabisulphite).

The decay profiles of β LG, casein, egg white proteins and soy proteins were estimated from the contents quantified after baking for 5 min at 150 °C, which was the mildest condition used in the experiment.

Under all conditions tested, the egg white proteins, followed by β LG, casein, and soy proteins, showed greater decay than the other proteins studied. At 150 °C, a progressive increase in the decay of the studied proteins was observed as a function of time, reaching a maximum decay of 95%, 90% and 51% for the egg white proteins, β LG and casein, respectively. However, for the soy proteins no decay was observed. After 5 min of baking at 180 °C, egg white protein decay began, whereas a reduction in casein, β LG and soy proteins only started after 10, 10 and 25 min of baking, respectively. The maximum decay reached at 180 °C was 100% for egg white proteins, 95% for β LG, 82% for casein and 47% for soy proteins. In the biscuits baked at 210 °C, 100% degradation was observed for egg white proteins, and the antigenicity of β LG was reduced (Fig. 2).

Despite the complete degradation of egg white proteins baked at 180 °C for 30 min and at 210 °C for up to 20 min and of β LG baked at 210 °C for up to 25 min, the products obtained under these conditions were burned and not sensorily acceptable for consumers, as illustrated in Fig. 2. Therefore, such conditions could not be applied to prepare

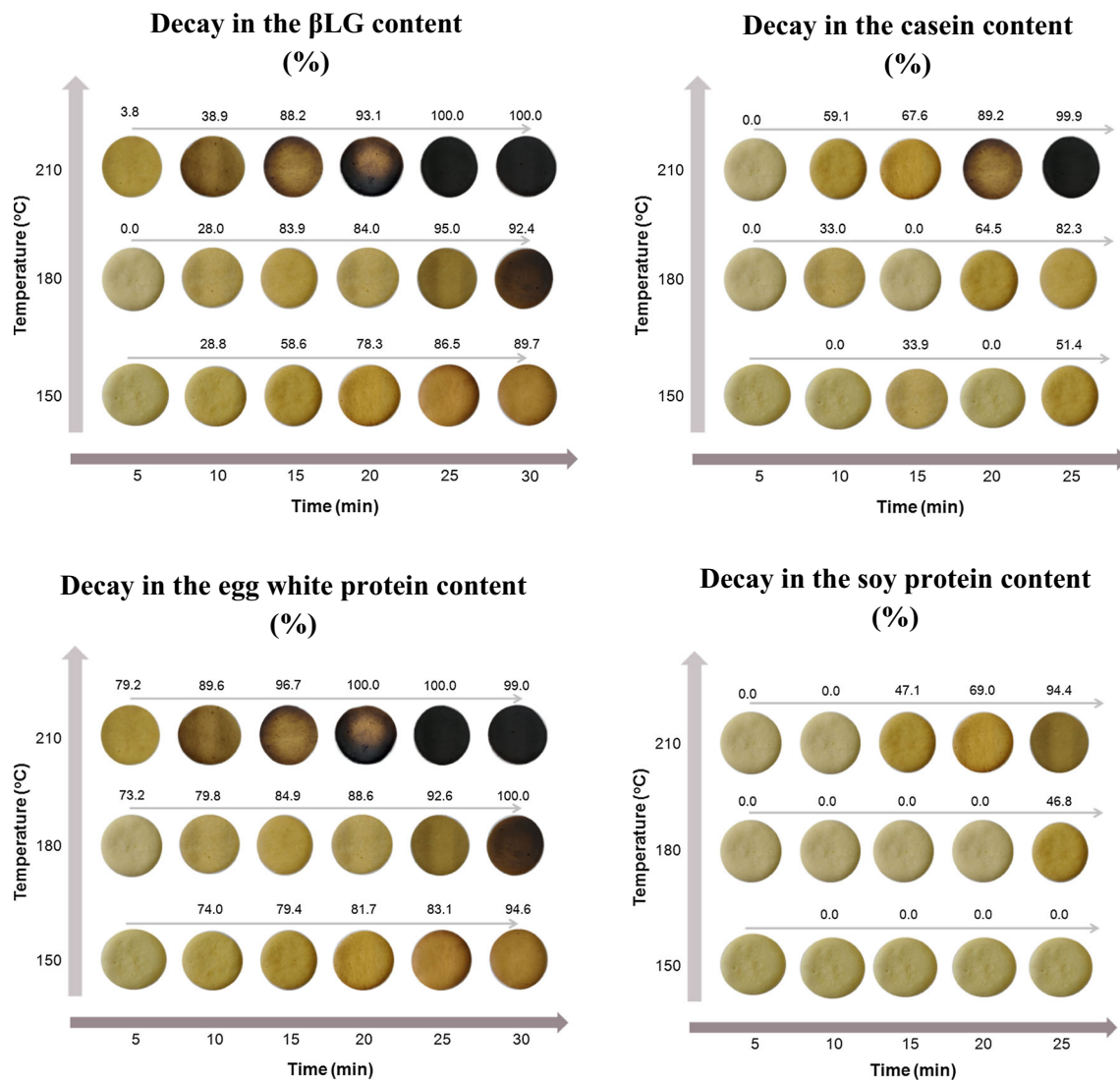


Fig. 2 Profile of the baked products under different baking time and temperature conditions and the percentage of decay in the content of β -lactoglobulin (β LG), casein, egg white proteins and soy proteins

allergen-free biscuits, highlighting the need for the detection of residual allergenic proteins.

Detection of allergens by ELISA

The obtained results followed a normal distribution, with Ryan-Joiner coefficients of 0.959 for β LG, 0.972 for casein, 0.991 for egg and 0.942 for soy proteins ($p > 0.05$), as shown in Fig. 3.

The time and temperature factors were significant for all the studied allergens, and the interactions between them were significant for casein, egg white proteins and soy proteins ($p < 0.05$), as shown in the ANOVA results represented in Table 2.

At 150 °C, there was a significant reduction ($p < 0.05$) in the content of β LG and, consequently, in the antigen–

antibody binding capacity in the biscuits baked for 5, 10 or 15 min. Twenty minutes of baking did not differ significantly ($p > 0.05$) from 15, 25 and 30 min of baking. However, after the latter two durations, the β LG content was lower than that after baking for the other durations ($p < 0.05$), but there was no difference between the baking durations of 25 and 30 min ($p > 0.05$). At 180 °C, although a significant decay occurred after 5, 10 and 15 min of baking ($p < 0.05$), the content of β LG eventually stabilized ($p > 0.05$). At 210 °C, the content of β LG did not differ significantly between 5 and 10 min of baking ($p > 0.05$) but was significantly lower at 15 min ($p < 0.05$), after which it was stabilized ($p > 0.05$). No significant difference ($p > 0.05$) was observed in the casein content after 5, 10, 15 and 20 min of baking at 150 °C. The casein content was lowest after 25 min of baking but did

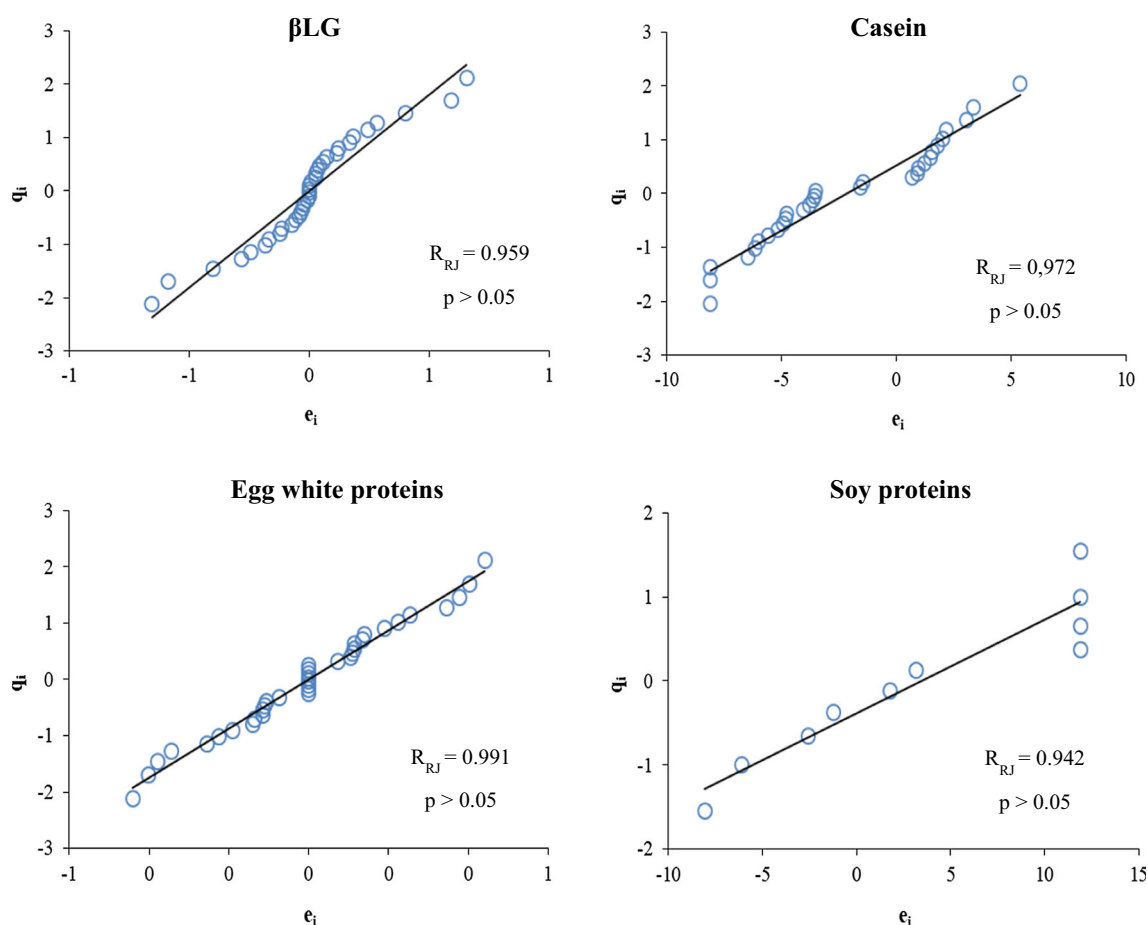


Fig. 3 Probability of a normal (Q-Q Plot) for the results of the degradation of β -lactoglobulin (β LG), casein, egg white proteins and soy proteins. R_{RJ} : Ryan-Joiner correlation coefficient, p : probability

not differ significantly between the 15- and 20-min time points ($p > 0.05$). At 180 °C, the casein contents after baking for 5, 10 and 15 min were not significantly different ($p > 0.05$). Baking for 20 or 25 min resulted in significantly lower ($p < 0.05$) casein contents than baking for 5 or 15 min. At 210 °C, the casein content of biscuits baked for 5 min differed significantly from that of biscuits baked for the other durations ($p < 0.05$), and although the casein content decreased after 15 min of baking, it did not differ significantly ($p > 0.05$) between the 20- and 25-min time points (Fig. 4).

At 150 °C, there was a significant reduction in the egg white protein content after 10 min, which differed significantly ($p < 0.05$) from that after 5 min. Although the egg white protein content decreased with increasing baking time, no significant differences ($p > 0.05$) were observed among the 10-, 15- and 20-min time points or between the 20- and 25-min time points. However, the egg white protein content of the biscuits baked for 30 min was significantly different from that of biscuits baked for the other durations ($p < 0.05$). At 180 °C, there were no significant

differences in the egg white protein content between 5 and 10 min; among 10, 15 and 20 min; between 20 and 25 min; and between 25 and 30 min ($p > 0.05$). However, the egg white protein content at 25 min differed significantly from those at 5 and 10 min ($p < 0.05$), and that at 30 min differed significantly from those at 5, 10, 15 and 20 min ($p < 0.05$). At 210 °C, the egg white protein content of biscuits baked for 5 min differed significantly from those in biscuits baked for the other durations ($p < 0.05$), and that of the biscuits baked for 10 min significantly differed from those of the biscuits baked for 20, 25 or 30 min ($p < 0.05$). No differences were observed between 15 and 10 min or among the baking times of 15, 20, 25 and 30 min ($p > 0.05$) (Fig. 4).

The soy protein content was not altered by baking at 150 °C for the different durations evaluated ($p > 0.05$) but significantly decreased after only 20 min of baking at 180 °C ($p < 0.05$). At 210 °C, there was a gradual but significant decrease in the soy protein content while baking for 10 min ($p < 0.05$) (Fig. 4).

Table 2 Analysis of variance in the results of the factorial experiment

Allergen protein	Source of variation	Degrees of freedom	Sum of squares	Middle square	F	<i>p</i>	Fc	Significance
βLG	Block	1	0.03	0.03	0.2	0.7	4.5	<i>p</i> > 0.05
	Time	5	91.0	18.2	132.2	5.5×10^{-13}	2.8	<i>p</i> < 0.001
	Temperature	2	2.3	1.1	8.3	3.1×10^{-3}	3.6	<i>p</i> < 0.01
	Temperature and time	10	1.2	0.1	0.9	0.6	2.4	<i>p</i> > 0.05
	Treatment	17	94.5					
	Residue	17	2.3	0.1				
	Total	35	96.8					
Casein	Block	1	4.5	4.5	2.0	1.8×10^{-1}	4.6	<i>p</i> > 0.05
	Time	4	174.7	43.7	19.2	1.4×10^{-5}	3.1	<i>p</i> < 0.001
	Temperature	2	102.8	51.4	22.6	4.2×10^{-5}	3.7	<i>p</i> < 0.001
	Temperature and time	8	128.8	16.1	7.1	8.2×10^{-4}	2.7	<i>p</i> < 0.001
	Treatment	14	406.2					
	Residue	14	31.9	2.3				
	Total	29	442.6					
Egg white proteins	Block	1	0.01	0.01	0.1	0.7	4.5	<i>p</i> > 0.05
	Time	5	153.7	30.7	305.5	5.1×10^{-16}	2.8	<i>p</i> < 0.001
	Temperature	2	73.7	36.9	366.1	1.1×10^{-14}	3.6	<i>p</i> < 0.001
	Temperature and time	10	90.5	9.0	89.9	0.0	2.4	<i>p</i> < 0.001
	Treatment	17	317.8					
	Residue	17	1.7	0.1				
	Total	35	319.5					
Soy proteins	Block	1	0.04	0.04	0.1	7.3×10^{-1}	4.6	<i>p</i> > 0.05
	Time	4	367.3	91.8	303.9	1.9×10^{-13}	3.1	<i>p</i> < 0.001
	Temperature	2	393.3	196.7	650.9	1.5×10^{-14}	3.7	<i>p</i> < 0.001
	Temperature and time	8	339.8	42.5	140.6	4.9×10^{-12}	2.7	<i>p</i> < 0.001
	Treatment	14	1100.4					
	Residue	14	4.2	0.3				
	Total	29	1104.7					

βLG: β-lactoglobulin; F: calculated F statistic; *p*: significance; Fc: critical F statistic

For βLG, the estimated mean content was not significantly different in biscuits baked for 5 min at each of the three temperatures, and the same was true for baking times of 10 and 15 min (*p* > 0.05). At 20 min, there was a significant difference in the βLG between 150 and 210 °C, with a lower content observed at the latter temperature (*p* < 0.05); however, the content determined after baking for 20 min at 180 °C did not differ from that determined after baking at the other temperatures (*p* > 0.05). At 25 min, lower βLG contents were significantly associated with higher temperatures (*p* < 0.05). At 30 min, only the βLG content at 210 °C differed significantly from that at the other temperatures (*p* < 0.05). Notably, after 25 and 30 min of baking at 210 °C, no βLG was detected by the ELISA kit, which suggested complete elimination of the antigenic potential of this protein (Fig. 4).

The casein content was not significantly different in biscuits baked for 5 min at each of the three temperatures, and the same was true for a baking time of 10 min (*p* > 0.05). At 15 min, there was a significant difference in the casein content between 180 and 210 °C (*p* < 0.05), but the content at 150 °C did not differ from those at 180 °C or 210 °C (*p* > 0.05). At 20 min, the content at 150 °C differed significantly (*p* < 0.05) from those at 180 °C and 210 °C, but the contents at these latter two temperatures did not differ from each other (*p* > 0.05). At 25 min, the casein content at 210 °C was significantly lower than that at 150 °C (*p* < 0.05) but not significantly different from that at 180 °C (*p* > 0.05) (Fig. 4).

The content of egg white proteins was not significantly different in biscuits baked for 5 min at each of the three temperatures, and the same was true for baking times of 10 and 15 min (*p* > 0.05). At 20 min, the content was

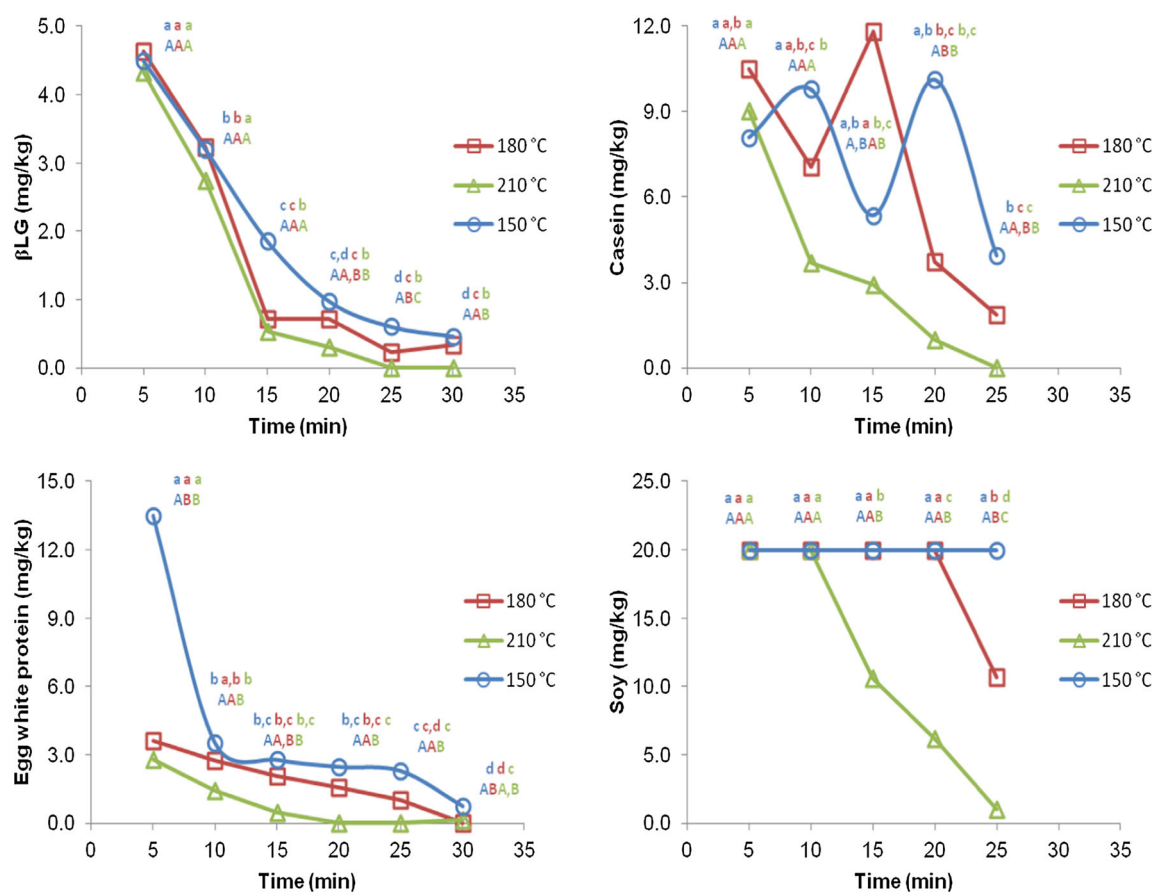


Fig. 4 Degradation profile of allergenic proteins in baked semi-sweet biscuits under different baking time and temperature conditions in a complete factorial experiment. βLG: β-lactoglobulin. Averages indicated by the same letter do not differ by Tukey's test, significance

significantly lower at 210 °C than at 150 °C ($p < 0.05$), but the content at 180 °C was not different from those at the other temperatures ($p > 0.05$). At 25 min, the content of egg white proteins and the antigenicity decreased as the baking temperature increased ($p < 0.05$). At 30 min, only the content at 210 °C differed significantly from that at the other temperatures ($p < 0.05$). Notably, after baking for 25 or 30 min at 210 °C, no egg white protein was detected by the ELISA kit, which suggested complete elimination of the antigenic potential of this protein (Fig. 4).

The soy protein content was not significantly different in biscuits baked for 5 min at each of the three temperatures, and the same was true for a baking time of 10 min ($p > 0.05$). At 15 and 20 min, only the content at 210 °C differed significantly from that at the other temperatures ($p < 0.05$). At 25 min, the soy protein content significantly decreased as the baking temperature increased ($p < 0.05$) (Fig. 4).

By means of three sandwich ELISA kits, Downs and Taylor (2010) evaluated the effect of thermal processing on the detection of total milk proteins, βLG and casein in

level $\alpha = 0.05$. Lowercase letters indicate the comparison of different times at the same temperature, while uppercase letters represent the comparison of different temperatures at the same time

pastry supplemented with powdered milk at concentrations of 0, 10, 25, 50, 100 and 250 mg/kg. Baking under a single time and temperature condition (30 min at 190 °C) was one of the thermal processes studied by the authors. The results were submitted to ANOVA with two factors and a significance level of $\alpha = 0.05$, although a test for discrimination of the treatment results was not reported. For each commercial kit, two independent extracts were prepared and analyzed in triplicate. The βLG and casein contents were reduced by approximately 96% and 79%, respectively, by the thermal treatment.

The behavior of milk proteins observed by Downs and Taylor (2010) was similar to that found in our study. According to these authors, βLG was the milk protein that decreased in abundance the most after heat treatment, as it was detected at only the two highest milk concentrations. Casein was shown to be more resistant to heat treatment and was detected in the pastries with the lower concentrations of added milk (Downs and Taylor 2010).

Monaci et al. (2011) also investigated the influence of baking on the detection of allergenic milk proteins in

cookies by using four commercial ELISA kits for total proteins, casein and β LG. Samples were supplemented with skimmed milk powder at 1000 mg/kg. Cookies were baked at a single temperature of 180 °C for 3, 6, 9, 12, 15, 20, 25, 30, 35 or 40 min. Each cookie was analyzed in triplicate, although the statistical analysis was not presented. The researchers demonstrated that casein was more stable than β LG, as evidenced in the present study. Decreases in the contents of β LG and casein of approximately 80% and 30%, respectively, were observed in the first 9 min of baking, whereas under similar conditions in the present study, reductions of 28% and 33% were observed in β LG and casein, respectively. After 9 min, the β LG and casein contents continued to decline, corroborating the results of the present work.

Bugyi et al. (2010) investigated the effect of processing on the ability to quantify allergenic proteins in cookies containing powdered milk and eggs at concentrations of 100 and 1000 mg/kg, respectively. Cookies were baked at 180 °C for 16 min and analyzed by a commercial ELISA kit. Although these authors used only one baking condition and allergen concentrations higher than those evaluated in the present study, the decrease in the concentration of egg protein was considerable, similar to that reported here, reaching close to zero. Their results for casein did not agree with the present study.

Khuda et al. (2012) also evaluated the effect of thermal processing on the determination of allergenic milk and egg proteins in cookies using ELISA kits. The studied samples were supplemented with skimmed milk powder and egg powder certified reference materials at concentrations of 0, 0.25, 5, 10, 25, 100, and 500 mg/kg. A single temperature and two baking times (190 °C at 25 and 30 min) were evaluated. For each sample, four aliquots were used to quantify the allergen concentrations. Single-factor ANOVA was employed. All the kits studied showed less recovery of the allergens after baking. Using all the kits, the authors found mean reductions in β LG of 35.2% and 93.8% and in casein of 16.8% and 57.4% at 25 and 30 min, respectively. For egg proteins, a reduction of 79.5% was found after 25 min of baking, and none of the kits were able to detect egg proteins in the cookies baked for 30 min. Khuda et al. (2012) also used R-Biopharm® kits and obtained results consistent with those obtained in the present study for β LG, casein and egg proteins.

Gomma and Boye (2013) investigated the effects of baking and matrix size on the detection of casein, egg and soy proteins in cookies baked at 170 °C for 10, 15 or 25 min. The samples were analyzed in duplicate, and the results were submitted to single-factor ANOVA. Duncan's test with a significance level of $\alpha = 0.05$ was used to compare the means. As in the present study, a reduction in the recovery of proteins from all samples was observed

with increased baking time. On the other hand, casein was relatively more resistant to the effects of thermal processing than egg and soy proteins, which were more degraded, and no kits detected soy proteins in biscuits baked for 25 min.

Török et al. (2015) analyzed the effect of heat treatment on cookies made with egg and soy proteins each at 50 mg/kg and baked at 180 °C for 16 min. The results were analyzed by ANOVA and submitted to t tests with a significance level of $\alpha = 0.05$. The authors observed complete degradation of the proteins analyzed after baking. In contrast to our findings, egg white protein degradation was observed only after baking at 180 °C for 30 min or at 210 °C for 20, 25 or 30 min, and 94% of the soy protein was degraded by baking at 210 °C for 25 min.

Although the above studies did not evaluate the degradation of β LG, casein, egg white proteins and soy proteins under the same combined time and temperature conditions as those evaluated herein and tested protein contents higher than those investigated here, these studies corroborate the findings of the present study in relation to the influence of heat treatment on the detection and antigenic potential of these proteins. It is relevant to consider that, as in the present paper, the studies reported above were conducted only based on the results of the ELISA technique. It would be important to develop further studies using a second confirmatory technique.

The protein degradation profiles generated in this study were supported by the conformational changes in the structures of the proteins due to thermal denaturation. Typically, most proteins undergo thermal denaturation at elevated temperatures. The temperature-induced denaturation mechanism mainly involves the effect of temperature on the stability of non-covalent interactions. In this way, hydrogen bonds and electrostatic interactions, which are exothermic in nature, are destabilized, while hydrophobic interactions, which are endothermic in nature, are stabilized with increasing temperature (Culbertson et al. 2006). It has been reported that β LG denatures at temperatures between 70.5 and 81.5 °C (Monaci et al. 2006) and that ovalbumin denatures between 78 and 86 °C (Huntington and Stein 2001). Casein and soy proteins are denatured at temperatures above 140 °C (Culbertson et al. 2006) and 80 °C (Huntington and Stein 2001), respectively. However, these reported temperatures for the degradation of these proteins are lower than those studied here and those commonly used in biscuit processing.

It is also important to consider that some authors have reported increased allergenic protein antigenicity after heating at temperatures of 50 °C–120 °C for 20 min and at 90 °C for 90 min (Bu et al. 2009; Kleber et al. 2004). In a study by Fiocchi et al. (1998) involving thirteen children with meat hypersensitivity, heat treatments (103 °C and

98 °C for 20 min for beef and 100 °C for 5 min for bovine serum albumin) decreased allergic sensitization in skin tests, but the difference was not significant. Souza and Rosario (2012) argued that industrial processing, such as pasteurization and high-temperature processing, could minimally reduce the allergenic potential of these proteins.

Even though the protein degradation profiles due to conformational changes could explain the antigenicity of the studied proteins, it would be necessary to establish allergenic safety thresholds, i.e., the lowest dose of a given allergen capable of producing objective effects in highly sensitive individuals (Monaci et al. 2006). As there are still insufficient data to define such thresholds (Brasil 2014; Monaci et al. 2006), assessing residual allergen levels remains important for allergen management and control.

Conclusion

The present study evaluated the antigenicity of different proteins in biscuits containing allergenic ingredients at concentrations simulating cross contamination in a complete factorial design with different baking durations and temperatures. A significant reduction in the antigenicity of these proteins was observed after thermal treatment, and the structural changes resulted in lower recoveries of the proteins detectable by ELISA.

The decay profiles of the evaluated proteins differed: egg proteins were more unstable than the other proteins during heat treatment. Soy proteins had the highest thermal stability, with a significant reduction in their antigenicity after heat treatment under only the most extreme conditions evaluated. Of the milk proteins, casein was more resistant to heat treatment than β LG.

After heat treatment corresponding to typical baking conditions, the levels of the allergenic proteins were residual, i.e., their antigenic activity was reduced but not eliminated. Therefore, baking is not a possible strategy for obtaining allergen-free biscuits. Methods with high sensitivity and capable of detecting allergens at low concentrations should be established and validated for the protection of consumers with food allergies.

Author contributions ALSS: validation, formal analysis, investigation, data curation, writing original draft. PPBS: formal analysis, investigation. GA: investigation. ES: investigation. COS: supervision, resources. SVCS: conceptualization, resources, project administration, review and editing the work.

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Declarations

Conflict of interest The authors declare that they have no conflicts of interest.

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