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# Impact of thermal processing time and cookie size on the detection of casein, egg, gluten and soy allergens in food

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## ARTICLE INFO

### Article history:

Received 23 August 2012

Accepted 8 January 2013

Available online xxxx

### Keywords:

Allergen detection

ELISA

Flow cytometry

Thermal effect

Matrix effect

## ABSTRACT

Thermal processing can affect allergen detection by inducing chemical modifications as well as changes to protein structure and solubility. The objectives of this research were to investigate the effects of baking time, temperature profile and cookie dimensions and weight on the detection of four allergens (casein, egg, gluten and soy) simultaneously incurred in a non-wheat flour cookie using enzyme linked immunosorbent assay (ELISA) and flow cytometry. In general, allergen recovery decreased as baking time increased and cookie size was decreased. Temperatures at the center of the cookies also increased with decreasing cookie size and increasing baking time. Casein recoveries were: 88 to 35%, 77 to 21%, and 75 to 19% with the Ridascreen ELISA kit, Veratox ELISA kit and flow cytometry, respectively. Egg recoveries were: 48 to 8%, 4 to 0% and 5 to 0%, with the Morinaga ELISA kit, Veratox ELISA kit, and flow cytometry, respectively. Gluten recoveries were: 93 to 31%, 72 to 27% and 75 to 21%, with the Ridascreen ELISA kit, Veratox ELISA kit, and flow cytometry, respectively. Finally, soy recoveries were: 33 to 1%, 1 to 0% and 21 to 0%, with the Veratox ELISA kit, ELISA systems kit, and flow cytometry, respectively. No recoveries were obtained for soy and egg in some of the thermally processed samples.

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## 1. Introduction

Food allergies are increasing in prevalence (Sicherer, 2011). It is estimated that about 5% of young children and 3% to 4% of adults have food allergies (Sicherer & Sampson, 2010). Among the major allergens believed to be responsible for up to 90% of food allergies, milk and egg allergens are the most prominent. Indeed, about 2.5% of young children and 0.3% of adults are allergic to milk, 1.5% of young children and 0.2% of adults are allergic to eggs, while wheat and soy allergies in totality affect an estimated 0.4% of young children and 0.2% of adults (Sicherer & Sampson, 2010).

Food allergy is an IgE-mediated immunological response that is initiated and provoked by allergenic foods (Taylor, 1992). In some instances, the immunological response may involve cell-mediated mechanisms (Sampson, 1999). Allergic reactions are usually triggered by antigenic proteins with molecular masses of 10–70 kDa in offending foods (Soler-Rivas & Wichers, 2001). Protein sites recognized by antibodies are known as epitopes and can be divided into conformational epitopes, which interact with the allergen receptors based on their three dimensional conformation (Mills, Madsen, Shewry, & Wichers, 2003) and, continuous epitopes, which are short peptides of 12–18 amino acid residues of which only the primary sequence of the polypeptide is involved in antibody binding (Soler-Rivas & Wichers, 2001). Due to their molecular structure, processes that induce changes to the conformation or

accessibility of epitopes (e.g., thermal processing) can influence antibody binding and allergen detection.

Despite the growing interest in the effect of thermal processing on allergen quantitation with respect to the detection of allergenic food residues at low levels (Albillos, Al-Taher, & Maks, 2011; Tong et al., 2012), only few studies have investigated the processing effects on allergen detection and quantification in food matrices (Downs & Taylor, 2010) and, most of these studies have dealt with single allergen detection (Khuda et al., 2012b).

Due to its simplicity, sensitivity, and accuracy for native proteins, the most common method used for allergen detection is the enzyme-linked immunosorbent assay (ELISA). A major drawback of ELISA, however, is that it detects only one allergen at a time. Additionally, the performance of the kits is variable and depends on the antibody specificity, composition of the extraction buffer and kit construction. For processed foods, extraction of denatured or altered proteins tends to be difficult due to their reduced solubility as compared to native proteins. Flow cytometry has been proposed as another immunoassay that could be employed in allergen detection which provides results comparable to ELISA with the additional advantage of simultaneous multiple allergen detection (Gomaa, Ribereau, & Boye, 2012).

The objectives of this research were to investigate the effects of baking time, temperature profile and food matrix size (cookie dimensions and weight) on the detection in baked cookies, of each of the four allergens (casein, egg, gluten and soy), simultaneously incurred in a non-wheat flour matrix using ELISA and flow cytometry as detection methods.

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## 2. Materials and methods

### 2.1. Materials

Casein (90% protein), gluten (80% protein) and whole egg powder (50% protein) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Soy protein concentrate (81.5% protein) was prepared in-house from press-cake provided from La Ferme Jirah (Ormstown Québec, Canada). PBS (phosphate buffered saline) was provided with the Veratox ELISA kits. Bio-Plex promagnetic COOH beads and amine coupling kit were obtained from Bio-Rad (USA). Captured antibodies used were as follows: rabbit polyclonal anti-casein (immunogen – synthetic peptide; specificity – casein alpha S1) was obtained from GeneTex Inc. (Irvine, CA, USA); rabbit polyclonal anti-ovalbumin (immunogen – ovalbumin [hen egg white]; specificity – purified and partially purified ovalbumin [hen egg white]), rabbit polyclonal anti-gluten (immunogen – gliadin-related peptide Lys57–Glu65-[alpha-gliadin (58–73)] KLQPFQPELPYPQPQ coupled to deamidogenic carrier protein; specificity – reacts specifically with the deamidated peptide KLQPFQPELPYPQPQ when coated directly in an immunoplate), and rabbit polyclonal anti-soy protein (immunogen – full length protein [soybean]; specificity – purified and partially purified trypsin inhibitor [soybean]), were obtained from Abcam (Cambridge, MA, USA). Detection (reporter) antibodies were obtained by conjugating capture antibodies to phycoerythrin (PE) using a Phycolink R-Phycoerythrin (RPE) conjugation kit and following the manufacturer's recommendations (Promega, USA). N-hydroxysulfosuccinimide (Sulfo-NHS) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) were obtained from Fisher Scientific (Ottawa, Ontario, CA). ELISA kits were purchased from commercial companies as described in Table 1. All other chemicals used were of analytical grade.

### 2.2. Preparation of incurred flour and cookie matrix (baked cookie)

Allergen-free dry mix samples comprising a non-wheat gluten flour mix of buckwheat flour (180 g), rice flour (90 g), sorghum flour (90 g), and tapioca starch (90 g), were mixed with sugar (180 g), salt (1.3 g), sodium bicarbonate (2.23 g) and baking soda (1.8 g) using an electric mixer (Mix Master, Sunbeam Products Inc, Boca Raton FL USA). The dry mix sample was incurred with casein, gluten, whole egg powder and soy protein concentrates which are listed in the materials section (Section 2.1) to obtain an incurred flour mix containing 1000 µg of each allergenic protein/g (i.e., 1000 ppm incurred samples). The incurred samples were mixed in a 'V' shaped blender (Arnold Equipment Company, Twinsburg, OH, USA) as described by Gomaa et al. (2012). Briefly, the mixing time required to achieve a homogenous distribution, was visually estimated using casein at a concentration of 1000 ppm that had been dyed with a coloring agent prior to mixing. The mixing time for homogenous distribution was determined to be

30 min. For further assurance, each sample was mixed for a total of 2 h to ensure homogenous distribution of the allergens. Homogeneity testing yielded coefficient of variation values (CV) below 17%. The samples were packaged in sterile sample bags (VMR, Mississauga, ON, CA) and stored at 4 °C until use.

Allergen-free and incurred cookies (cooked samples) were prepared from the allergen-free or incurred dry mix sample by adding 50 g of the flour mix to 90 g of sunflower oil, to which 150 g of water was then added. Cookies were shaped with different dimensions and weights as shown in Table 2, and baked in the center of an oven pre-heated at 177 °C for 10, 15 and 25 min. The baked cookies were cooled to room temperature and subsequently ground into fine powders. Taking into consideration moisture loss during baking, the final concentration of each allergen in the baked cookies were calculated. The employed samples and their respective dimensions and final allergen concentrations are summarized in Table 2.

### 2.3. ELISA method

Allergens were detected with ELISA kits according to the manufacturer instructions. A description of the test kits used is provided in Table 1. Quantitative data were obtained by measuring the optical density at the wavelength specified by the manufacturer using a microplate spectrophotometer Epoch (Biotek, Winooski, Vermont, USA). All standard curves were generated with the standard solutions provided with the test kits. Additionally, data analysis was undertaken using the microplate and data collection analysis software Gen5 (Biotek, Winooski, Vermont, USA). Calibration curves were constructed from three replicates at each point of the standard curve.

ELISA measurements were obtained in duplicate for each sample and all experiments were performed twice. In order to determine the percentage recovery, the following conversion factors were used for each allergen: Neogen (Veratox) ELISA kit for casein expresses the results as non-fat dried milk which was estimated to contain 33.3% casein, Morinaga ELISA kit for egg expresses the results as total egg protein which was estimated to be 50% of the total egg powder and Neogen (Veratox) ELISA kit for soy expresses the results as soy flour which was estimated to contain 37.3% soy protein.

### 2.4. Flow cytometry

A competition immunoassay flow cytometry was performed as described previously (Gomaa et al., 2012). Briefly, standard allergens were prepared in phosphate buffered saline (PBS) solution and coupled to the pre-activated magnetic COOH beads. Protein extracts were prepared by mixing 1 g of sample with 9 mL of phosphate buffered saline (PBS) pH 7.4. The mixture was shaken for 15 min in a stirring water bath at 60 °C. Subsequently, the mixture was centrifuged at 1500 g for 15 min. The protein extracts were diluted using PBS

**Table 1**  
Description of ELISA test kits employed for allergen detection.

| Target allergen | Limit of detection (ppm) | Quantification range (ppm) | Results expressed as ppm of  | Extraction buffer, pH and temperature                                                      | Supplier and name of kit                     |
|-----------------|--------------------------|----------------------------|------------------------------|--------------------------------------------------------------------------------------------|----------------------------------------------|
| Casein          | 2.5<br>0.5               | 2.5–15<br>0.5–13.5         | Non-fat dried milk<br>Casein | PBS, pH 7.4 and specific allergen extraction additive<br>Provided with kit (contains 2-ME) | Neogen (Veratox)<br>R-biopharm<br>Ridascreen |
| Soy protein     | 2.5<br>2.5               | 2.5–25<br>2.5–25           | Soy flour<br>Soy protein     | PBS, pH 7.4 and allergen extraction additive<br>Provided with kit                          | Neogen (Veratox)<br>ELISA Systems            |
| Egg             | 0.5                      | 0.5–13.5                   | Total egg powder             | Provided with kit                                                                          | R-biopharm<br>Ridascreen                     |
|                 | 0.3                      | 0.3–20                     | Total egg protein            | Provided with kit (contains 2-ME)                                                          | Morinaga<br>(Morinaga Institute)             |
| Gluten          | 3                        | 5–160                      | Gluten                       | Provided with kit (contains 2-ME)                                                          | R-biopharm<br>Ridascreen                     |
|                 | 5                        | 5–50                       | Gluten                       | 40% ethanol and an extraction cocktail solution contains 2-ME                              | Neogen (Veratox)                             |

**Table 2**

Description of samples employed for allergen detection including dimensions and final allergen concentrations.

| Sample                 | Cooking time (min) | Dimensions (mm) |          | Weight (g) | Allergen concentration (ppm) |
|------------------------|--------------------|-----------------|----------|------------|------------------------------|
|                        |                    | Thickness       | Diameter |            |                              |
| Blank flour            | –                  |                 |          |            | 0                            |
| Incurred flour         | –                  |                 |          |            | 1000                         |
| Blank cookie (large)   | 10                 | 10              | 76       | 43.5       | 0                            |
| Small size (small)     | 10                 | 10              | 38       | 9.5        | 625                          |
|                        | 15                 | 10              | 38       | 9.5        | 667                          |
|                        | 25                 | 10              | 38       | 9.5        | 774                          |
| Medium size (mid-size) | 10                 | 10              | 58       | 23.5       | 668                          |
|                        | 15                 | 10              | 58       | 23.5       | 681                          |
|                        | 25                 | 10              | 58       | 23.5       | 786                          |
| Large size (large)     | 10                 | 10              | 76       | 43.5       | 739                          |
|                        | 15                 | 10              | 76       | 43.5       | 773                          |
|                        | 25                 | 10              | 76       | 43.5       | 790                          |

pH 7.4 and used immediately for analysis. Standard solutions were prepared using the allergens listed in the materials section and extracted according to the same protocol as the samples.

The validation method for coupling was based on the detection of the coupled protein with phycoerythrin (PE)-conjugated secondary antibody. All detection antibodies were conjugated to phycoerythrin (PE) using a kit and following the manufacturer's recommendations (Prozyme, USA). The produced PE conjugated antibody (reporter antibody) was used to estimate the density of the antibody coupled to the beads. Each coupling reaction was repeated in triplicate in order to confirm the reproducibility of the data. The coupled beads were analyzed using a Bio-Plex 200 Luminex analyzer (Bio-Rad) according to the system manual.

Flow cytometry data analysis and interpretation were performed by generating a standard curve for each allergen using extracts of the standard allergens in the range of 2.5 to 0.04 ppm protein based on the initial content. The standard curve was prepared using the standard allergens listed in the materials section (Section 2.1) and consisted of a plot of the allergen standard concentration on the X-axis versus the mean fluorescence value on the Y-axis and a logarithmic curve fit of the data. Sample extracts with a fluorescence response lower than the response of the 2.5 ppm standard were further diluted as appropriate.

The calculation of the concentration of each allergen in ppm based on the soluble protein was estimated directly from the standard curve and determined using the formula:

$$\text{Allergen protein content (ppm)} = \text{EC} \times \text{Dilution A} \times \text{Dilution B}$$

where:

EC estimated concentration from standard curve (protein content in ppm).

Dilution A dilution fold during extraction (nominal 10-fold used for all samples).

Dilution B further dilutions as required to bring concentration within the standard curve.

All results were expressed based on the total soluble protein content.

## 2.5. Temperature profile during baking

A digital thermocouple thermometer (Cole-Parmer, NY, USA) was inserted in the center of each cookie during baking in order to monitor the temperature profile for each cookie size as a function of baking time.

## 2.6. Statistical analyses

Experimental results were recorded as mean  $\pm$  coefficient of variation. Data were analyzed using SAS for Windows (version 9.1) following an analysis of variance (ANOVA) one-way linear model. Mean comparisons were performed using the Duncan test, and a level of  $p \leq 0.05$  was considered to indicate significance.

## 3. Results and discussion

### 3.1. Detection of allergens by ELISA and flow cytometry

#### 3.1.1. Casein allergens

Fig. 1 shows the percentage recoveries of casein as detected by ELISA and flow cytometry. The results show that allergen recoveries were high, at 90% or higher, for the non-baked samples when detected using ELISA (Veratox kit and Ridascreen kit) and flow cytometry. Casein allergen recoveries in non-baked samples were: 93% using the Veratox kit, 95% using the Ridascreen kit, and 90% using flow cytometry.

For the baked samples, both ELISA kits and flow cytometry were able to detect casein allergens under all processing conditions. However, the recovery was highly variable and depended on the baking time and the cookie size whereby it decreased with either increasing baking time or decreasing cookie size. The recovery range was 89–35% for the Ridascreen kit, 77–21% for the Veratox kit and 75–19% for flow cytometry. For the Ridascreen kit, casein allergen recoveries as a function of cookie size and baking time decreased as follows: large cookies: 89%, 78%, and 49%; mid-size cookies: 87%, 75%, and 44%; and small cookies: 63%, 56%, and 35%; for baking times of 10, 15 and 25 min, respectively. Casein allergen recoveries using the Veratox kit were as follows: large cookies: 77%, 68%, and 34%; mid-size cookies: 67%, 61%, and 31%; and small cookies: 65%, 46%, and 21% for baking times of 10, 15 and 25 min, respectively, whereas flow cytometry provided the following recoveries: large cookies: 75%, 57%, and 43%; mid-size cookies: 58%, 63%, and 31%; and small cookies: 67%, 54%, and 19%, for baking times of 10, 15 and 25 min, respectively. Although the Veratox kit and flow cytometry provided lower recoveries than the Ridascreen kit, the differences were not statistically significant except for the mid-size and small samples baked for 15 min; for these samples, despite the low recoveries, the Ridascreen kit gave the highest results.

#### 3.1.2. Egg allergens

Fig. 2 shows the percentage recoveries of egg allergens as detected by ELISA and flow cytometry. For the non-baked samples, the Veratox kit and flow cytometry provided better recoveries than the Morinaga kit. The recoveries were as follows: 81% using the Veratox kit, 42% using the Morinaga kit, and 92% using flow cytometry.

The recoveries of egg allergens in the baked cookies were less than 50% for the ELISA kits (Morinaga and Veratox kits) and flow cytometry. Nevertheless, the Morinaga kit had significantly higher recoveries of egg allergens in the baked cookies than either the Veratox kit or flow cytometry. Whereas the Morinaga kit had the maximum allergen recovery at 48% for the large cookies baked for 10 min, recoveries with the Veratox kit and flow cytometry did not exceed 5%. Furthermore, no recoveries were obtained with the Veratox kit and flow cytometry for the small cookies baked for 10 min, and for all sizes at baking times of 15 min and 25 min. The recovery range was 48–8% for the Morinaga kit, 5–0% for the Veratox kit and 5–0% for flow cytometry. For the Morinaga kit, egg allergen recoveries as a function of cookie size and baking time decreased as follows: large cookies: 48%, 40%, 11%; mid-size cookies: 42%, 41%, 10%; and small cookies: 43%, 36%, 8%; for baking times of 10, 15 and 25 min, respectively. Allergen recoveries using the Veratox kit were as follows: large cookies: 4%, 2%, 0%; mid-size cookies: 5%, 2%, 1%; and small cookies: 2%, 1%, 0% for baking times of 10, 15 and 25 min, respectively. Flow cytometry provided the following recoveries: large cookies: 5%, 0%, and 0%; mid-size

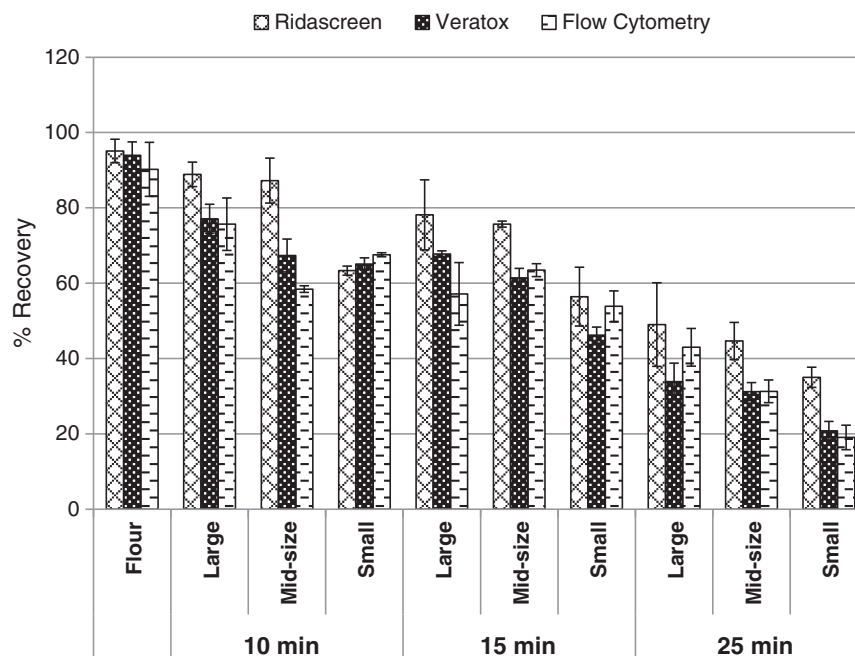


**Fig. 1.** Percentage recoveries of casein in incurred cookie as detected by ELISA and flow cytometry. (Large, mid-size, and small refers to cookie sizes of 10 mm thickness and 38, 58 and 76 mm diameters, respectively; 10, 15, 25 min refers to cookie baked for these different times in an oven at 177 °C).

cookies: 3%, 0%, and 0%; and small cookies: 0%; 0%; and 0%, for baking times of 10, 15 and 25 min, respectively. Thus, for the baked cookies the values for the recoveries detected with the Morinaga kit were significantly higher than those detected with the Veratox kit and flow cytometry.

Compared to the Veratox kit and flow cytometry, the high recovery values obtained with the Morinaga kit could be due to the higher quantity of protein extracted with the kit buffers, which contain denaturant and surfactant, as well as the protocol employed (Diaz-Amigo, 2010a, 2010b; Fæste, Løvberg, Lindvik, & Egaas, 2007; Khuda et al., 2012a; Khuda et al., 2012b). Indeed, Diaz-Amigo (2010b) also reported better recoveries for egg allergens in cookies baked between 5 and 15 min

with the Morinaga kit (144%–58%) as compared with three other ELISA kits (r-Biopharm, Tepnel, Neogen), which collectively had recoveries of 72%–1%.

### 3.1.3. Gluten allergens

Fig. 3 shows the percentage recoveries of gluten as detected by ELISA and flow cytometry. The data show high gluten allergen recoveries in the non-baked samples of: 96% for the Ridascreen kit, 86% for the Veratox kit, and 95% for flow cytometry. For the baked samples, the ELISA kits and flow cytometry were able to detect gluten allergens under all processing conditions with recoveries of: 93–31% for the Ridascreen kit, 72–27% for the Veratox kit, and 75–21% for flow

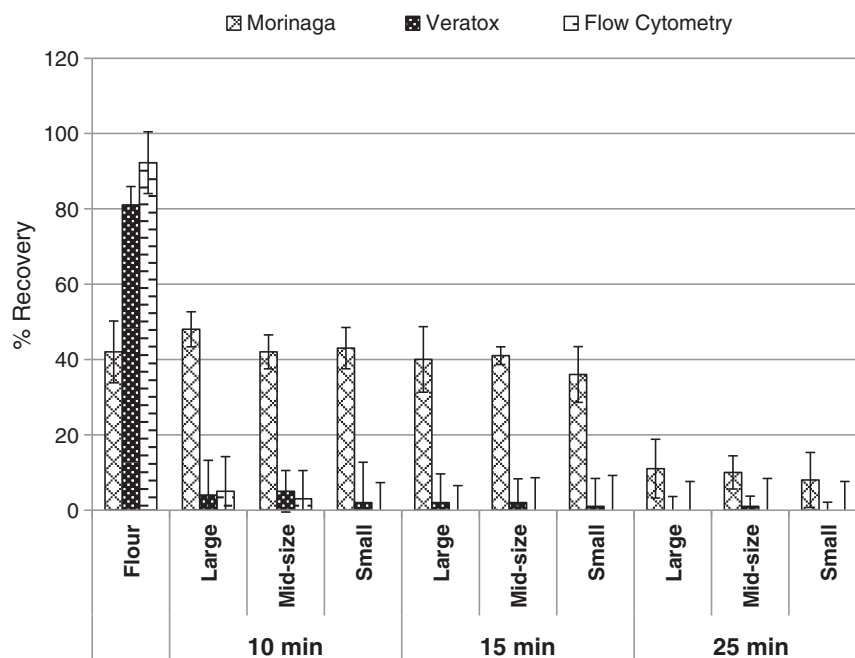


**Fig. 2.** Percentage recoveries of egg in incurred cookie as detected by ELISA and flow cytometry. (Large, mid-size, and small refers to cookie sizes of 10 mm thickness and 38, 58 and 76 mm diameters, respectively; 10, 15, 25 min refers to cookie baked for these different times in an oven at 177 °C).

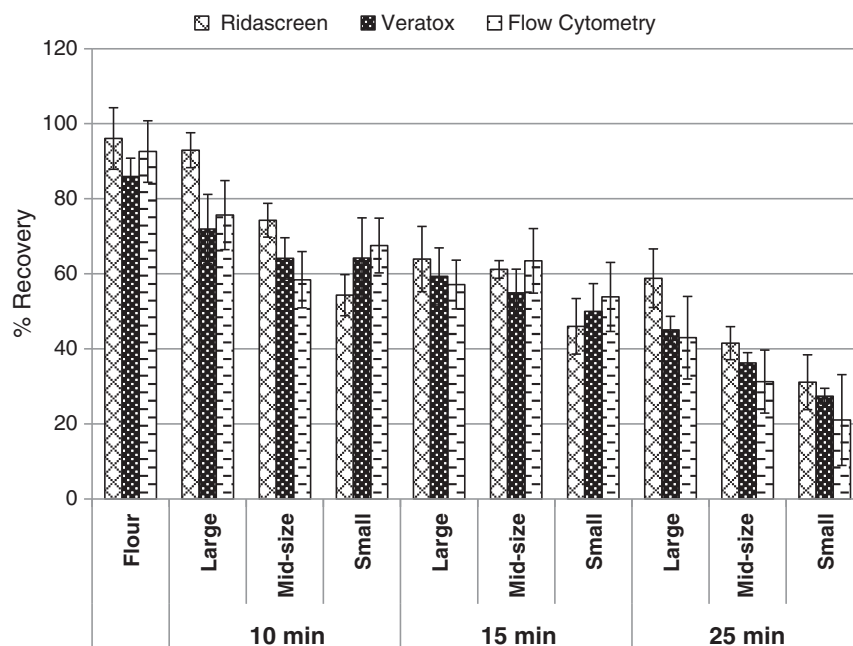


**Fig. 3.** Percentage recoveries of gluten in incurred cookie as detected by ELISA and flow cytometry. (Large, mid-size, and small refers to cookie sizes of 10 mm thickness and 38, 58 and 76 mm diameters, respectively; 10, 15, 25 min refers to cookie baked for these different times in an oven at 177 °C).

cytometry. Similar to casein, recoveries for baked samples decreased with increasing baking time for each cookie size, and also decreased with decreasing cookie size at each baking time. For the Ridascreen kit, gluten allergen recoveries as a function of cookie size and baking time decreased as follows: large cookies: 93%, 64%, and 59%; mid-size cookies: 74%, 61%, and 41%; and small cookies: 54%, 46%, and 31%, for baking times of 10, 15 and 25 min, respectively. Allergen recoveries using the Veratox kit were as follows: large cookies: 72%, 59%, and 45%; mid-size cookies: 64%, 55%, and 36%; and small cookies: 64%, 50%, and 27%, for baking times of 10, 15 and 25 min, respectively, whereas flow cytometry provided the following recoveries: large cookies: 75%, 57%, and 43%; mid-size cookies: 58%, 57%, and 31%; and small cookies: 67%, 54%, and 21%, for baking times of 10, 15 and 25 min, respectively. All three detection methods gave positive recoveries (i.e., no 0% recoveries) for gluten allergen detection under the conditions studied.

### 3.1.4. Soy allergens

Fig. 4 shows the percentage recoveries of soy as detected by ELISA and flow cytometry. The recoveries for the non-baked samples were as follows: Veratox kit: 86%, ELISA systems kit: 74%, and flow cytometry: 89%.

For the baked samples, the Veratox kit and flow cytometry were able to detect soy allergens in the small, medium and large cookies baked for 10 min and 15 min. The recovery range was 33–1% for the Veratox kit and 21–0% for flow cytometry. The maximum recovery detected using the ELISA System kit was 1% for the cookies of different sizes baked for 10 min and 15 min. Similar to casein and gluten, recoveries for baked samples decreased with increasing baking time for each cookie size, and also decreased with decreasing cookie size at each baking time under the conditions for which values were obtained. The detection methods employed failed to detect soy allergens in cookies baked for 25 min and provided 0% recoveries. For the Veratox kit, soy allergen recoveries as a function of cookie size and baking time decreased as follows: large cookies: 33%, 22%, and 1%; mid-size cookies: 26%, 19%, and 1%; and small cookies: 22%, 13%, and 1%, for baking times of 10, 15 and 25 min, respectively. Allergen recoveries using the ELISA system were as follows: large cookies: 1%, 1%, and 0%; mid-size cookies: 1%, 1%, and 0%; and small

cookies: 1%, 0%, and 0%, for baking times of 10, 15 and 25 min, respectively, whereas flow cytometry provided the following recoveries: large cookies: 21%, 15%, and 0%; mid-size cookies: 16%, 16%, and 0%; and small cookies: 0%; 0%; and 0%, for baking times of 10, 15 and 25 min, respectively. Cucu et al. (2011) also reported low recovery values of 1.5%–7% for soy in cookies baked at 205 °C for 10 min with diameter of 8 cm and a thickness of 1 cm.

### 3.2. Temperature profile during baking

The temperature employed during baking can affect protein structure and functionality, which in turn can affect protein extraction and allergen detection. Fig. 5 presents the temperature profile during baking of cookies of different sizes (i.e., same thickness but varying diameter and weight). Although baking was done at 177 °C and the thickness was the same for all cookies, cookie size and baking time affected the temperature profile as well as the final temperature. Temperatures of 111 °C, 98 °C, and 90 °C at 10 min were attained for small, mid-size and large cookies, respectively. After 15 min, the baking and final temperatures were 118 °C, 104 °C, 97 °C for the small, mid-size and large cookies, respectively. After 25 min, the temperatures were 155 °C, 130 °C and 109 °C for the small, mid-size and large cookies, respectively. As the thickness was the same for all samples, differences in the temperature profile and final temperature could be attributed to variations in lateral thermal transfer and temperature distribution due to the differences in the diameters of the cookies. For all the baking times studied, the interior temperature of the cookies increased with decreasing cookie size and increasing baking time. The temperature, however, never reached 177 °C.

The decrease in allergen recovery as cookies were baked for longer periods could, in part, be attributed to structural changes that occur in proteins with heating. They include loss of secondary and tertiary structures, cleavage and rearrangements of disulphide bonds, and formation of intra-molecular disulphide bonds. These structural changes can reduce allergen recoveries with ELISA and flow cytometry because they can affect allergenic epitopes and, in turn, antigen-antibody binding (Downs & Taylor, 2010). Indeed, Tong et al. (2012) noted that changes in the protein secondary structure as well as sulfhydryl-disulfide

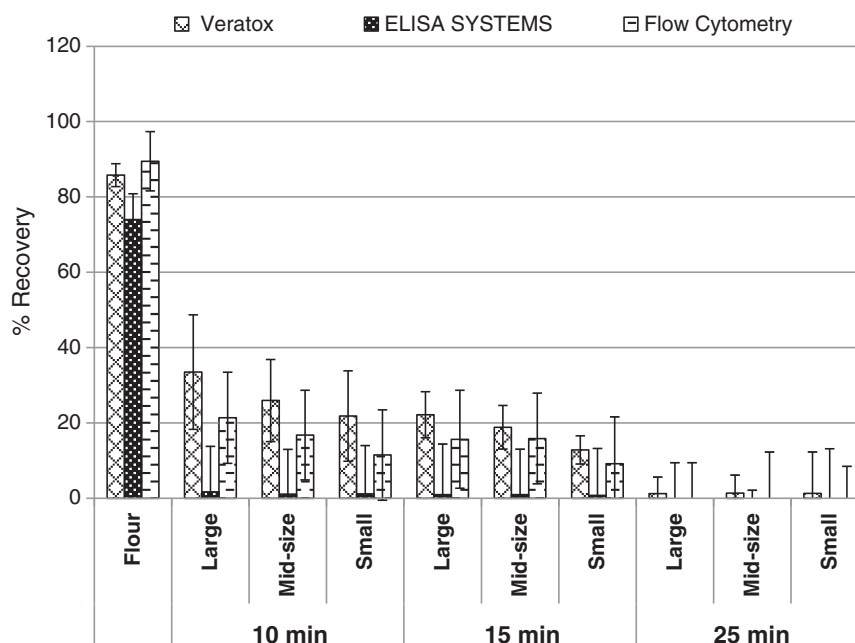


**Fig. 4.** Percentage recoveries of soy in incurred cookie as detected by ELISA and flow cytometry. (Large, mid-size, and small refers to cookie sizes of 10 mm thickness and 38, 58 and 76 mm diameters, respectively; 10, 15, 25 min refers to cookie baked for these different times in an oven at 177 °C).

interchange reactions that occur during thermal treatment of egg proteins affect protein antigenicity.

Between 90 and 100 °C, which was the temperature range achieved for the medium-sized cookies baked for 10 min and large size cookies cooked for 15 min, additional protein structural changes such as formation of insoluble aggregates are expected to occur (Poms & Anklam, 2004). Therefore, under these temperature conditions, the allergens can become insoluble and less-detectable by immunoassays such as ELISA and flow cytometry (Albillos et al., 2011).

Above 100 °C, as was recorded for the small cookies baked at 10, 15 and 25 min, the mid-size cookies baked for 15 and 25 min, and the large cookies baked for 25 min, increased chemical modifications

(e.g., enhanced Maillard reaction) occur (Poms, Klein, & Anklam, 2004). Protein-carbohydrate interactions resulting from Maillard reactions can modify allergenic epitopes and can, thereby, further reduce allergen detection (Oste, Brandon, Bates, & Friedman, 1990). Downs and Taylor (2010) have reported that Maillard reactions affected the solubility and immunoreactivity of antigens detected using ELISA kits.

In summary, two different immunoassays (ELISA and flow cytometry) were used in this research. Flow cytometer is a multiplex immunoassay that enables multiple allergen detection which enhances ease-of-use and automation and can markedly reduce the time and labor compared with ELISA. Differences in specificity of antibodies and employed extraction protocols may account for some of the variances between flow cytometry and ELISA results. For the four tested allergens, flow cytometry results were not significantly different from the ELISA results except for processed egg samples when measured using Morinaga ELISA kit where recoveries were significantly higher than the other ELISA kit and flow cytometry likely due to the improved extraction buffer employed.

Among the ELISA kits, the lowest detection obtained was for soy using the ELISA Systems kit where the recovery did not exceed 1% for all thermally processed samples. These results are of interest as the extensive use of soybean proteins in processed foods can pose a problem of hidden allergens for allergic consumers (L'Hocine & Boye, 2007).

Overall, the results presented in this study which show reduced recoveries of each allergen following thermal processing are similar to some recently published data. Specific examples include low recoveries for: egg allergens in six different heat processed food matrices (Fæste et al., 2007); milk allergens in a pastry dough food matrix processed by boiling, baking, frying and retorting (Downs & Taylor, 2010); peanut, egg and milk allergens in a cookie and dark chocolate matrix (Khuda et al., 2012a, 2012b); soy in cookies (Cucu et al., 2011) and hazelnut in cookie (Cucu et al., 2012). The low allergen recoveries for thermally processed food were attributed to: (i) conformational modifications of the epitopes that reduced antibody binding during ELISA analysis inducing changes to the conformation of the conformational epitopes thus altering the antigen-antibody interaction and reducing antigenicity (Fæste et al., 2007; Platteau et al., 2011);

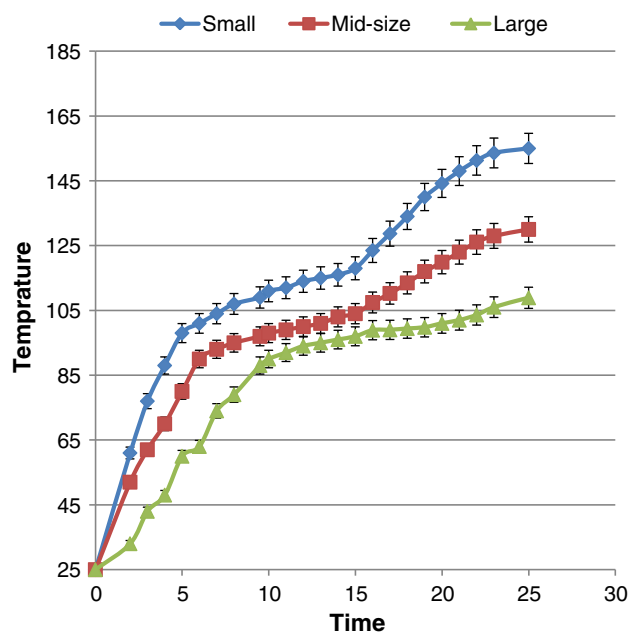


**Fig. 5.** Interior temperature profile during baking of different sizes of cookies. (Large, mid-size, and small refers to cookie sizes of 10 mm thickness and 38, 58 and 76 mm diameters, respectively).

(ii) decreasing the solubility of and in turn decreasing protein extractability (Schmitt, Nesbit, Hurlburt, Cheng, & Maleki, 2010) and the formation of insoluble protein aggregates (Kato, Oozawa, & Matsuda, 2001); and (iii) enhancing and activating protein modification including through the Maillard reaction (Downs & Taylor, 2010; Platteau et al., 2011; Poms & Anklam, 2004). Additionally, physiochemical changes due to interactions occurring between allergens and other food components in food matrices during thermal treatment can affect allergen detection by causing changes to pH and the solubility and/or structure of allergenic proteins; furthermore interferences or cross-reactivities with kit reagents can also occur (Cucu et al., 2011; Khuda et al., 2012b; Monaci & Visconti, 2010). As an example, interactions between soy and flour proteins during dough mixing and subsequent formation of a high molecular weight complex between soy 11S globulin and prolamins of wheat, in a dough containing both soy and wheat contributed to underestimation of soy allergen recovery (Ribotta, León, Pérez, & Añón, 2005).

#### 4. Conclusion

Low estimation of thermally processed allergens does not necessarily reflect decreased allergenicity (Taylor et al., 2002) which poses a risk for allergenic consumers of falsely declared allergen-free foods. Processing time during thermal treatment and product dimensions are two important factors that can affect allergen recovery and detection as demonstrated by this study. Allergen recovery decreased with increasing processing time and decreasing product size. These effects may be higher for specific allergens, e.g. soy and egg, resulting in lower levels of detection using methods that usually provide high recoveries for native proteins. This, evidently, can put sensitive consumers at risk. Furthermore, this research shows that the sensitivity of a detection method to a specific allergen is influenced by the extraction buffer and protocol employed. ELISA kits therefore need to be optimized to improve their suitability for the detection of specific allergens in thermally processed foods.

#### References

- Albillos, S. M., Al-Taher, F., & Maks, N. (2011). Increasing extractability of protein for allergen detection after food processing. *Food Chemistry*, 127(4), 1831–1834.
- Cucu, T., Devreese, B., Kerkaert, B., Rogge, M., Vercruyssen, L., & De Meulenaer, B. (2011). ELISA-based detection of soybean proteins: A comparative study using antibodies against modified and native proteins. *Food Analytical Methods*, 1–10.
- Cucu, T., Devreese, B., Trashin, S., Kerkaert, B., Rogge, M., & De Meulenaer, B. (2012). Detection of hazelnut in foods using ELISA: Challenges related to the detectability in processed foodstuffs. *Journal of AOAC International*, 95(1), 149–156.
- Diaz-Amigo, C. (2010a). Towards a comprehensive validation of elisa kits for food allergens. Case 2 milk. *Food Analytical Methods*, 3(4), 351–356.
- Diaz-Amigo, C. (2010b). Towards a comprehensive validation of ELISA kits for food allergens: Case 1—Egg. *Food Analytical Methods*, 3(4), 344–350.
- Downs, M. L., & Taylor, S. L. (2010). Effects of thermal processing on the enzyme-linked immunosorbent assay (ELISA) detection of milk residues in a model food matrix. *Journal of Agricultural and Food Chemistry*, 58(18), 10085–10091.
- Fæste, C. K., Lovberg, K. E., Lindvik, H., & Egaas, E. (2007). Extractability, stability, and allergenicity of egg white proteins in differently heat-processed foods. *Journal of AOAC International*, 90(2), 427–436.
- Goma, A., Ribereau, S., & Boye, J. (2012). Detection of allergens in a multiple allergen matrix and study of the impact of thermal processing. *J Nutr Food Sci* (S9:001).
- Kato, Y., Oozawa, E., & Matsuda, T. (2001). Decrease in antigenic and allergenic potentials of ovomucoid by heating in the presence of wheat flour: Dependence on wheat variety and intermolecular disulfide bridges. *Journal of Agricultural and Food Chemistry*, 49(8), 3661–3665.
- Khuda, S., Slate, A., Pereira, M., Al-Taher, F., Jackson, L., Diaz-Amigo, C., et al. (2012a). Effect of processing on recovery and variability associated with immunochemical analytical methods for multiple allergens in a single matrix: Dark chocolate. *Journal of Agricultural and Food Chemistry*, 60(17), 4204–4211.
- Khuda, S., Slate, A., Pereira, M., Al-Taher, F., Jackson, L., Diaz-Amigo, C., et al. (2012b). Effect of processing on recovery and variability associated with immunochemical analytical methods for multiple allergens in a single matrix: sugar cookies. *Journal of Agricultural and Food Chemistry*, 60(17), 4195–4203.
- L'Hocine, L., & Boye, J. I. (2007). Allergenicity of soybean: New developments in identification of allergenic proteins, cross-reactivities and hypoallergenization technologies. *Critical Reviews in Food Science and Nutrition*, 47(2), 127–143.
- Mills, E. N. C., Madsen, C., Shewry, P. R., & Wichers, H. J. (2003). Food allergens of plant origin – Their molecular and evolutionary relationships. *Trends in Food Science and Technology*, 14(4), 145–156.
- Monaci, L., & Visconti, A. (2010). Immunochemical and DNA-based methods in food allergen analysis and quality assurance perspectives. *Trends in Food Science and Technology*, 21(6), 272–283.
- Oste, R. E., Brandon, D. L., Bates, A. H., & Friedman, M. (1990). Effect of Maillard browning reactions of the Kunitz soybean trypsin inhibitor on its interaction with monoclonal antibodies. *Journal of Agricultural and Food Chemistry*, 38(1), 258–261.
- Platteau, C., Cucu, T., De Meulenaer, B., Devreese, B., De Loose, M., & Taverniers, I. (2011). Effect of protein glycation in the presence or absence of wheat proteins on detection of soybean proteins by commercial ELISA. *Food Additives & Contaminants: Part A*, 28(2), 127–135.
- Poms, R. E., & Anklam, E. (2004). Effects of chemical, physical, and technological processes on the nature of food allergens. *Journal of AOAC International*, 87(6), 1466–1474.
- Poms, R. E., Klein, C. L., & Anklam, E. (2004). Methods for allergen analysis in food: A review. *Food Additives and Contaminants*, 21(1), 1–31.
- Ribotta, P. D., León, A. E., Pérez, G. T., & Añón, M. C. (2005). Electrophoresis studies for determining wheat-soy protein interactions in dough and bread. *European Food Research and Technology*, 221(1–2), 48–53.
- Sampson, H. A. (1999). Food allergy. Part 1: Immunopathogenesis and clinical disorders. *The Journal of Allergy and Clinical Immunology*, 103(5 II), 717–728.
- Schmitt, D. A., Nesbit, J. B., Hurlburt, B. K., Cheng, H., & Maleki, S. J. (2010). Processing can alter the properties of peanut extract preparations. *Journal of Agricultural and Food Chemistry*, 58(2), 1138–1143.
- Sicherer, S. H. (2011). Food allergy. *The Mount Sinai Journal of Medicine*, 78(5), 683–696.
- Sicherer, S. H., & Sampson, H. A. (2010). Food allergy. *The Journal of Allergy and Clinical Immunology*, 125(Supplement 2), S116–S125 (2).
- Soler-Rivas, C., & Wichers, H. J. (2001). Impact of (bio)chemical and physical procedures on food allergen stability. *Allergy*, 56, 52–55.
- Taylor, S. L. (1992). Chemistry and detection of food allergens. *Food Technology*, 46(5), 146–152.
- Taylor, S. L., Hefle, S. L., Bindslev-Jensen, C., Bock, S. A., Burks, A. W., Jr., Christie, L., et al. (2002). Factors affecting the determination of threshold doses for allergenic foods: How much is too much? *The Journal of Allergy and Clinical Immunology*, 109(1), 24–30.
- Tong, P., Gao, J., Chen, H., Li, X., Zhang, Y., Jian, S., et al. (2012). Effect of heat treatment on the potential allergenicity and conformational structure of egg allergen ovomucoid. *Food Chemistry*, 131(2), 603–610.