



Analytical Methods

Influence of baking time and matrix effects on the detection of milk allergens in cookie model food system by ELISA

Linda Monaci^{a,b,*}, Marcel Brohée^a, Virginie Tregoat^a, Arjon van Hengel^a^a European Commission, Joint Research Centre, Institute for Reference Materials and Measurements, Retieseweg 111, B-2400 Geel, Belgium^b CNR, Institute of Sciences of Food Production (ISPA), Via Amendola 122/O, 70126 Bari, Italy

ARTICLE INFO

Article history:

Received 14 July 2009

Received in revised form 2 November 2010

Accepted 30 December 2010

Available online 8 January 2011

Keywords:

Food allergens

ELISA

Allergen stability

Thermal treatments

Matrix effects

Baking

Processed food

ABSTRACT

Milk allergens are common allergens occurring in foods, therefore raising concern in allergic consumers. Enzyme-linked immunosorbent assay (ELISA) is, to date, the method of choice for the detection of food allergens by the food industry although, the performance of ELISA might be compromised when severe food processing techniques are applied to allergen-containing foods. In this paper we investigated the influence of baking time on the detection of milk allergens by using commercial ELISA kits. Baked cookies were chosen as a model food system and experiments were set up to study the impact of spiking a matrix food either before, or after the baking process. Results revealed clear analytical differences between both spiking methods, which stress the importance of choosing appropriate spiking methodologies for method validation purposes. Finally, since the narrow dynamic range of quantification of ELISA implies that dilution of samples is required, the impact of sample dilution on the quantitative results was investigated. All parameters investigated were shown to impact milk allergen detection by means of ELISA.

© 2011 Elsevier Ltd. All rights reserved.

1. Introduction

Cow milk allergy (CMA) consists of an abnormal immunological response due to the ingestion of milk or milk-derived products and is considered the most common food allergy in children below 3 years (Monaci, Tregoat, van Hengel, & Anklam, 2006). The most abundant milk proteins belong to the main classes of caseins and whey accounting for 80% and 20% of the total milk proteins, respectively. Casein (CN) represents the main fraction of milk proteins and is subdivided into a number of families (α , β , κ). They are characterised by a central hydrophobic part and a hydrophilic layer with phosphorylated sites and a hardly clear tridimensional structure suggesting the presence of preferentially linear epitopes (Wal, 2001). By contrast, lactoglobulin (LG) and lactalbumin (ALA) are the most abundant proteins of whey and together with caseins are known to be the main allergic components of milk (Natale et al., 2004; Wal, 2001). Either linear epitopes widely distributed throughout the cow milk proteins or conformational epitopes can be responsible for allergic reactions (Monaci et al., 2006). It is generally acknowledged that even minute amounts of an allergenic food, like milk, can cause severe and sometimes fatal reactions,

although insufficient knowledge is available on clinically relevant threshold levels as established by human oral challenge studies (Hourihane & Knulst, 2005). To protect sensitive individuals from the risk of allergic reactions, Directive 2007/68/EC has been issued (Commission of the European Communities, 2007) in the European Union which requires that the most allergenic ingredients, like milk, added to food products have to be declared on the respective label. Since the allergic consumer relies on the information on the label, it is of utmost importance that foods that do not declare milk, or milk-derived products as ingredients should be truly free of milk proteins. But, unfortunately contamination at any stage of food production may occur, thus introducing the hazard of hidden allergens (Kerbach et al., 2009). As far as baked products like cookies are concerned, milk/dairy products are common ingredients and therefore the risk of cross-contamination is high. As a consequence there is a need for sensitive methods to ensure sanitation of production lines and compliance with labelling legislation to improve consumer protection.

Several analytical approaches for the detection of food allergens are available to-date (Monaci & Visconti, 2009; Poms, Klein, & Anklam, 2004b). They either target the allergenic proteins or markers (e.g. specific proteins, peptides or DNA fragments) that indicate the presence of the allergenic food ingredient. Immunoassays are currently commonly used for screening purposes because antibodies can recognise specific proteins in a complex mixture of compounds of different nature (Krska, Welzig, & Baumgartner, 2004; van Hengel, 2007). They represent a good tool for screening

* Corresponding author at: CNR, Institute of Sciences of Food Production (ISPA), Via Amendola 122/O, 70126 Bari, Italy. Tel.: +39 080 5929343; fax: +39 080 5929374.

E-mail address: linda.monaci@ispa.cnr.it (L. Monaci).

purposes and generally offer a good sensitivity and specificity for detecting traces of specified allergenic ingredients. Amongst immunoassays, the enzyme-linked immunosorbent assay (ELISA) is popular because of its ease of use, fast response and low limit of detection. ELISA kits mainly employ polyclonal antibodies for the detection of food allergens, since polyclonal antibodies offer benefit of recognising a number of different epitopes in the target protein and are less sensible to small changes. ELISA tests developed for the detection of milk traces are usually directed against CN, LG or total milk and use either a sandwich configuration or a competitive detection and several commercial kits as well as in house developed ELISAs are now available (Shubert-Ullrich et al., 2009). To date only few ELISA kits have been validated by international organizations and none of these kits tailored for milk allergen detection have undergone the AOAC-RI validation test yet.

Nonetheless, the reliability of some ELISA kits was assessed in an interlaboratory evaluation study that focussed on the detection of milk protein in extracts of sausage, cookie, cereal and pasta sauce, which demonstrated that the investigated ELISA methods were reliable and reproducible (Akiyama et al., 2004). The performance of ELISA showed to be compromised when extensive food processing techniques like baking are applied to the allergen-containing food under investigation. Some consequences of thermal processing on food proteins are: loss of conformational Ig-E epitopes, Maillard reactions, decrease of protein solubility and/or extractability or stability as described by several authors (Fæste, Løvberg, Lindvik, & Egaas, 2007; Poms & Anklam, 2004). Notably, heating and technological food processing might lead to changes in the target protein structure affecting some antigenic determinants and epitope binding sites (Maleki, 2004). As a consequence, the detection of allergens by antibodies may be affected, hampering their final detection and changing the outcome of the test (Paschke & Besler, 2002). This is a matter of concern for allergic consumers since hidden milk allergens might remain undetected after analysis of food products.

This study aims to investigate the influence of the baking process on the detection of milk allergens by commercial ELISA kits in cookies chosen as model food matrix. Experiments were set up to evaluate the effect of adding milk powder to the matrix, before (incurred samples) or after (spiked) baking, on target recovery. Different baking periods were investigated and the milk allergen content was estimated before and after thermal treatment by employing four ELISA kits provided by different manufacturers and targeting CN, LG or total milk proteins (see Table 1). Since most ELISA kits have a rather narrow dynamic range (e.g. 1.6–25.6 ppm for CN), the analysis of serial dilutions of the samples is usually required. Therefore we also investigated to which extent dilution may affect the quantitative results. A better understanding of the strengths and limitations of ELISA used for the detection of milk allergens is important to monitor and control the problem of allergen cross-contamination which will ultimately protect the allergic consumer.

2. Experimental

2.1. Chemicals

Skimmed milk powder and whey powder were purchased from Sigma–Aldrich (St. Louis, MO, USA) while protein quantification kit based on copper binding was from GE Healthcare (Uppsala, Sweden).

2.2. Model food matrix

Blank cookies were prepared according to the following recipe: three medium sized eggs, 190 g wheat flour (ANCO Flour Patisserie with the following composition: proteins 10.6 g, glucids 70.6 and lipids 1.1 g per 100 g, humidity 15.5%), 90 g sugar, 20 ml extra virgin olive oil, and 50 ml of water (blank cookies) or skimmed milk powder solution (incurred cookies). The total weight for each dough prepared was approximately 500 g. Virgin olive oil was used in replacement of any milk derived product in order to avoid milk traces in the blank. In this study “incurred sample” is defined as a sample in which a known amount of food allergen (e.g. milk powder) has been incorporated and undergoes the overall processing (including baking). Alternatively, “spiked samples” where a known amount of the food allergen (e.g. milk powder) was added after their manufacturing were also prepared. Such samples are usually easier to prepare and can be representative for food products that are accidentally contaminated with allergens after their production (e.g. milk powder dust settling on baked cookies) (Diaz-Amigo & Popping, 2010).

Incurred cookies were prepared as follows: skimmed milk powder, was used to prepare aqueous solutions. Briefly, 10 g of skimmed milk powder was dissolved in 1 L of water and left stirring at ambient temperature with a magnetic bar for 15 min to have a homogeneous solution. A 1/10 dilution was also prepared from the stock solution in order to produce cookies incurred at a lower level. An aliquot of 50 ml of both solutions were added to the original dough (approximately 450 g) instead of water (used for the blank) with the aim to obtain a dough with a final milk powder concentration in cookies of 1000 mg/kg and 100 mg/kg, respectively.

Cookies incurred with whey powder were prepared as described for milk powder spiking, with the difference that the final concentration in cookies was 10,000 and 1000 mg/kg, due to the fact that these proteins are less abundant as compared to the casein fraction.

Portions of 20 g of incurred dough were spread into each open cookie tin (diameter ca. 8 cm and 1 cm high) and baked at a temperature of 180 °C for different periods of time (3, 6, 9, 12, 15, 20, 25, 30, 35 or 40 min). Care was taken that a constant and well distributed temperature was applied by checking the temperature at different locations in the conventional oven used for baking (GE, Fairfield, CT, USA). The baked cookies were allowed to cool down at ambient temperature and were subsequently ground in a mill un-

Table 1
Intended use of ELISA test kits claimed by producers and performance of the kits.

Target allergen	Type of assay	Food matrix	Specificity	Quantification range (ppm)	Limit of detection (ppm)	Supplier
Total milk allergens	S-ELISA	Juices, cake mixes, cookies, sauces, sorbets	Caseins and whey proteins	2.5–25	1	Tepnel
Caseins	C-ELISA	Cooked and uncooked food	Casein/caseinates	1.6–25.6	1	Tepnel
Lactoglobulins, whey proteins	C-ELISA	Cooked and uncooked food	β Lactoglobulin	2.5–40	2	Tepnel
Lactoglobulins	S-ELISA	Beverages and food containing whey, milk or milk powder	β Lactoglobulin	0.010–0.810	0.2	R-Biopharm

S-ELISA = direct sandwich ELISA; C-ELISA = indirect competitive ELISA.

der liquid nitrogen to obtain a fine powder. The resulting cookie powder was extracted and used in this study. An aliquot was withdrawn from each batch baked for different periods of time in order to determine the water content. According to the water content values calculated for each batch, a mass correction factor was calculated and taken into consideration to estimate the real concentration in the final cookies.

2.3. ELISA kits and methodology

The following ELISA kits as schematized in Table 1 were utilised: Veratox for Total Milk Allergen obtained from Tepnel Biokits, (Lansing, MI, USA), β -LG Ridascreen Assay Kit obtained from R-Biopharm (Darmstadt, Germany) BioKits β LG Assay Kit, and BioKits Casein Assay Kit both obtained from Tepnel BioSkits.

All ELISA analyses were performed according to the manufacturer's instructions and using the recommended buffers. While kits for total milk allergen detection used PBS at pH 7.4 as extraction buffer, those for casein detection employed a carbonate/bicarbonate buffer at pH 9.6. Quantitative results were obtained by measuring the optical density at the wavelength specified by the ELISA manufacturer using a spectrophotometer (1420 Multilabel Counter Victor ³V; Perkin Elmer, Singapore). All standard curves were constructed with the standards provided with the test kits. Calibration curves at five concentration points (three replicate for each point) were constructed for the standard curves. CVs were in the range 1–5%.

Performance of Veratox kit targeting total milk allergens was further investigated and the LOD and LOQ experimentally found in standard solutions provided along the kit, were 1.07 and 2.71 μ g/ml respectively.

Each cookie sample was analysed in triplicate and the mean of two different samples was taken into consideration. Sample extracts with signals higher than that observed for the highest point of the calibration curve obtained, were diluted in the appropriate buffer or in blank cookie extract. The homogeneity of the cookie material was assessed by using Veratox kit for total milk allergens. Two samples were taken from 10 different batches of cookies at both concentrations of 1000 and 100 mg/kg, respectively, analysed in triplicate and the average of optical density was subjected to ANOVA test.

2.4. Protein quantification in cookie extracts

To quantify proteins in cookies the same extraction protocol as described for Veratox total milk ELISA test kit was followed. Aliquots of 1 g ground cookies were extracted with 25 ml PBS and left shaking for 30 min at 60 °C since it was experimentally found that protein yields at that temperature was higher than that obtained performing the extraction at room temperature. Samples were vortexed for 1 min every 10 min of the incubation. The extracts were then subjected to analysis by ELISA test kits for total milk and their total protein content was determined by using the 2D quantification kit based on a Cu^{2+} binding assay. A calibration curve with bovine serum albumin (BSA) standard was established in the range of 0–50 μ g protein per ml, and protein quantification was performed as described before (Chassaigne, Brohé, Nørgaard, & van Hengel, 2007).

2.5. Matrix-matched calibration curves

For the construction of matrix-matched calibration curves in spiked and incurred food material, cookies baked for a standard time of 9 min were employed. As for calibration curve in spiked cookies, ground cookies were fortified with milk powder solutions at the final concentrations of 12.5, 25, 50 or 100 mg/kg (adding an equal volume of solutions at different milk powder concentrations), then left

to dry for 1 h at room temperature and carefully mixed before extraction. To prepare incurred samples, dough incurred with milk powder at 1000 mg/kg (previously described) was first diluted with blank dough in order to obtain the following concentrations 200, 100, 50, 25, 12.5, 6.25 mg/kg and then baked for 9 min. Likewise for the construction of LG calibration curves, cookies were externally spiked with whey powder at the final concentrations of 50, 25, 12.5, 5, 2.5 mg/kg while for incurred cookies, samples were incurred with whey powder (same procedure as described above) at 10,000 mg/kg and further diluted with blank dough to obtain the following concentrations: 5000, 2500, 1250, 625, 312.5 mg/kg. Each point in the calibration curve represented the mean of two different cookie samples (three replicates each sample) and CVs obtained were in the range 2–9% for both calibration curves.

3. Results and discussion

3.1. Calibration curves in baked cookies for total milk allergens and lactoglobulins

Contamination of food products with milk or milk-derived products can occur at different stages during food production (Diaz-Amigo & Popping, 2010). In order to investigate whether the stage of the production process, during which the contamination occurs, has an effect on the final detection of milk allergens by ELISA, two calibration curves were constructed, one for cookies incurred with milk powder, and one for cookies that were spiked after baking. All cookies were baked for a standard time of 9 min at 180 °C since this represented a good compromise between the protein content and the appearance of the cookie such as the final colour and its palatability (see Section 2.2 for details). Ground cookies were extracted and analysed with the ELISA kit for total milk protein detection (Veratox kit) targeting both caseins and whey proteins. Final data were corrected for the loss in water content due to baking in order to calculate the actual amount of milk proteins in the samples.

For the construction of the matrix-matched calibration curves, an aliquot of incurred cookie extract was progressively diluted with blank cookie extract to target the concentration range of the kit. As shown in Fig. 1, when milk powder was introduced in the cookie dough before thermal treatment, the calibration curve shows a slope that halves that of externally spiked samples. This gives rise to a reduced recovery of ELISA target molecules when incurred samples are analysed.

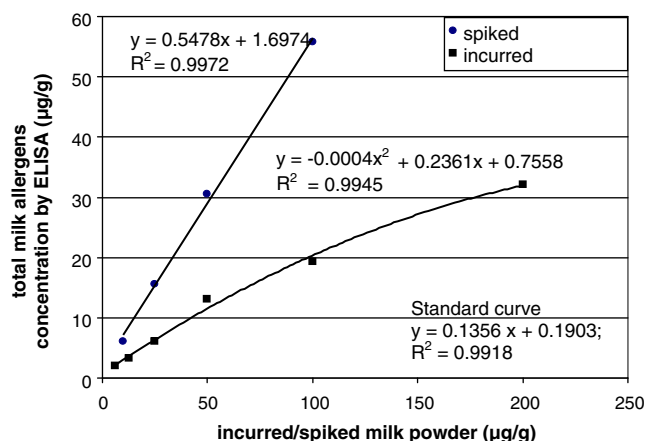


Fig. 1. Recovery determined for cookie samples incurred or spiked with milk powder. All cookies were analysed with the Tepnel ELISA kit for total milk protein detection. Equation for incurred cookies (line with squares), equation for spiked cookies (line with circles) and equation for standard solution are reported.

Since baking was found to cause a severe impact on the immunological detection of LG in the cookie extracts, we also analysed whey proteins (data not shown) containing cookies and compared incurred and spiked material by using Tepnel BioSystems ELISA kit for LG detection. Results obtained with incurred samples appear to be dramatically different from those obtained with externally spiked samples showing a reduction to nearly 1/50 of LG detected in externally spiked samples. The resulting equations were: $y = 0.027x + 0.445$ ($R^2 = 0.9942$) for spiked cookies and $y = 0.00055x + 0.276$ ($R^2 = 0.9789$) for incurred cookies, respectively.

In general, reasons for such decreased signal when baking milk allergens within the food matrix can be due to a structural and/or chemical modification that allergens undergo during baking with matrix components resulting in a decreased protein solubility/extractability. It has been already reported that denaturation of milk proteins can remove conformational epitopes as a result of the loss of tertiary protein structure (Wal, 2003). Heating may also induce formation of aggregates (Kleber, Krause, Illgner, & Hinrichs, 2004) increasing the allergenicity of heated products. The allergenicity of cow milk (CM) proteins does not only depend on the temperature and time applied but also on the possible interaction with food components. Besides, it is known that applying temperatures above 100 °C Maillard reactions can take place. These types of reactions resulting in the attachment of reducing sugars to the free amino groups of lysine on CM proteins can lead to the formation of Amadori products thus modifying the overall allergenicity of the specific food (Monaci et al., 2006).

Our findings ultimately confirm the labile behaviour of LG when submitted to thermal treatments like baking (see Section 3.2). Similar behaviour was already pointed out by other authors in different model processed matrices (de Luis, Lavilla, Sánchez, Calvo, & Pérez, 2009; Diaz-Amigo, 2010b).

For validation purposes ELISA validation studies should either validate a method by using incurred samples or opt for a validation with externally spiked samples whereby the limitations of the method to analyse incurred samples are acknowledged.

3.2. Influence of baking time on protein extraction

Dough incurred with skimmed milk powder at a level of 1000 mg/kg was prepared and baked for different periods of time. Samples were extracted with PBS (as recommended by Veratox kit) and submitted to protein quantification. Total protein content here described represented the amount of protein extracted by using PBS as extraction solution and it mirrors the protein fraction of all the ingredients used for cookie production including egg, wheat flour and milk proteins. Fig. 2 reports the relative results (expressed in %) obtained by normalising all values to the one obtained for the dough. As appearing, the extraction efficiency dropped with the increasing of the baking time. Since increasing the baking time also resulted in a water loss, the water content was determined and the values for protein extraction were corrected for the water content (water content ranging from 35% in the dough to 4% in cookies baked for 40 min). A marked drop occurs between 0 and 9 min baking hence representing the most crucial part. After 9 min baking the extractable protein dropped by nearly 80% compared to 3 min baking, and henceforth the curve started to flatten.

The decrease in protein extraction efficiency as observed for highly processed cookies is likely due to changes in protein structure due to denaturation and/or chemical complexation/modifications phenomena occurring within the food matrix that can impair protein extractability (Poms & Anklam, 2004; van Hengel, 2007). During baking protein denaturation will take place and since the solubility of denatured proteins decreases with the increase of

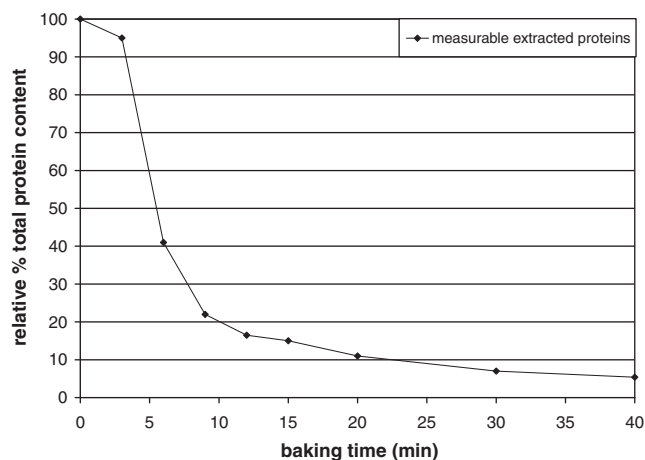


Fig. 2. Relative measurable extracted proteins determined in cookie extracts after different periods of baking. All values are expressed as a percentage of the value obtained from the dough referred to the amount of protein in unbaked cookie dough.

heating, the total protein yield is expected to be affected by the physical–chemical characteristics of the extraction solution (Poms, Capelletti, & Anklam, 2004a; Westphal, Pereira, Raybourne, & Williams, 2004). It has to be underlined that buffer composition plays an important role on protein extraction and consequently on allergen extraction. It is therefore essential when dealing with method development, to find the most suitable buffer to ensure the highest extraction efficiency of the target proteins. Previously the effect of food processing on the detectability of other allergenic proteins has been investigated and the results have shown that ELISA test kits gave significant lower estimates for peanut (Chassigne et al., 2007; Scaravelli, Broh  , Marchelli, & van Hengel, 2009), egg (Diaz-Amigo, 2010a; F  ste et al., 2007) and milk (Diaz-Amigo, 2010b) after heat treatments. This might be partially attributed to conformational changes of the epitopes that reduce antibody binding during ELISA analysis or to the Maillard reaction occurring inducing protein modification, and partly it might be due to a reduction of the extraction efficiency that is different for the individual peanut proteins.

Heating time and temperature, as well as the presence of other ingredients, such as fats and sugars also affect the patterns and kinetics of food protein denaturation and aggregation (de la Fuente, Singh, & Hemar, 2002; Wal, 2003). Moreover, a breakdown of the protein structure is also likely to occur after prolonged heat exposure. With the analysis of baked cookies we have confirmed that the duration of heat exposure has a strong effect on the protein extraction efficiency. For further experiments in the present study requiring the construction of calibration curves (incurred and spiked cookies with milk powder), a standard baking time of 9 min was set since shorter baking times resulted in cookies that were partially raw and deemed not representative for cookies destined for human consumption. In general employing baking times between 9 and 40 min resulted in still edible cookies.

3.3. Influence of baking time on immunological detection of total milk allergens, caseins and lactoglobulins by commercial ELISA kits

The same 1000 mg/kg incurred cookies (with milk powder) used to assess protein extraction efficiency were successively analysed with different commercial ELISA test kits for the detection of milk allergens. This allowed the investigation of the impact of baking time on the immunological detection of different classes of milk allergens.

Fig. 3 shows a graph reporting four curves relative to the detection of caseins (curve *a*), total milk proteins (curve *b*), and lactoglobulins (curves *c*, dotted lines) carried out with ELISA kits provided by different manufacturers. In all curves a decrease in the detection of milk proteins was observed with increasing baking time.

The curve obtained for total milk proteins (curve *b*) shows a marked drop of immunoreactive total milk proteins detected especially in the first minutes of baking, followed by a slighter decrease at the longer baking times (from 6 to 40 min). After baking the cookies for 9 min, approximately 30% of total milk allergens were still detectable.

Besides the decreased protein solubility, it is well known that food processing is likely to affect the integrity of allergenic proteins inducing chemical modifications and change in its three-dimensional conformation (Davis, Smales, & James, 2001). When a protein is denatured by heat, most of the original tertiary structure is lost and many of the three dimensional epitopes recognised by antibodies on the native molecule are destroyed (Davis et al., 2001). In addition, chemical modification of antigenic determinants is also likely to occur. This gives rise to a reduced recognition, resulting in a decreased detection by ELISA, as observed between 0 and 6 min (curve *b*). However, the relative stable signal between 6 and 15 min might be due to appearance of relatively heat stable linear epitopes bound by antibodies in the ELISA. Despite, the trend observed for caseins (curve *a*) appears smoother compared to what found for total milk proteins (curves *b*), especially in the first part of the curve (from 0 to 6 min).

Although caseins represent 80% of total milk fraction many reasons can account for the different behaviour displayed between total milk proteins and caseins. The most relevant are the antibody specificity/affinity used by the two kits, the specific extraction buffer and the protocol used. Whereas the ELISA kit for the extraction of CN employed a carbonate/bicarbonate solution at pH 9.6, the kit for total milk protein detection used PBS solution at pH 7.4. This difference in buffer composition might generate a different solubility of processed proteins. Moreover it has been demonstrated that also the format of the ELISA kits (whether competitive or sandwich) can have an influence on the performance of the kit in use (de Luis et al., 2009).

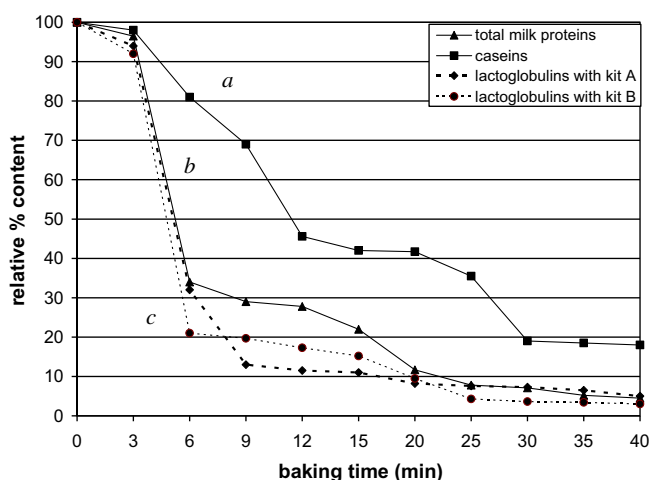


Fig. 3. ELISA analysis of incurred cookies prepared with milk powder at a level of 1000 mg/kg and baked for different periods of time. The samples were analysed with the following ELISA kits: for total milk detection (line *a*), caseins (line *b*) (from Tepnel) and lactoglobulins (lines *c*) kit A = Biokit from Tepnel and kit B = Ridascree from R-Biopharm) as explained in the legend. The relative ELISA signal is expressed as a percentage of the value obtained from the dough.

Concerning casein detection the curve obtained for this class of proteins (curve *a*) shows a smoother trend. As displayed in the figure, the CN content was halved between 0 and approximately 12 min, and dropped to nearly 20% after 30 min of baking. CNs are mainly dynamic structures and comprise an ensemble of interchanging conformations. They have been reported to be relatively unaffected by severe heat treatments (van Hengel, 2007; Wal, 2001). On the other hand, in incurred cookies we found that casein content decreased with the increase of baking time, then confirming results already shown by Diaz-Amigo (2010b). This should not surprise since the majority of studies performed in the past were carried out in a simpler matrix (like buffers) while now there are other factors involved such as the food matrix and the baking conditions.

The same samples that were analysed in the experiments described above were also analysed with LG specific ELISA kits. However, the level of residual whey proteins detected was very low. In order to investigate the effect of baking time on the detection of the whey protein LG, incurred cookies were prepared with whey powder at a level of 1000 mg/kg instead of milk powder. Two ELISA kits provided by different suppliers (R-Biopharm and Tepnel) were used to detect LG in cookies that were baked for different periods of time. As shown in Fig. 3 (dotted lines referred to curves *c*), a first steep drop of the signal occurred between 0 and 9 min, followed by a progressively smoother decrease between 9 and 25 min of baking. Finally, after 25 min both curves appear to be flattened. The trend observed confirmed more labile behaviour displayed by this class of proteins compared to CN, when exposed to thermal treatments. The results of the analyses with both ELISA test kits highlighted a high agreement of the responses obtained, where a 3- to 5-fold drop of the ELISA signal is apparent between 0 and 6 min baking time. Also in this case, the overall trend observed confirmed the obstacles encountered in protein extractability/solubility. Besides, as appearing from the figure, baking leads either to a decrease of soluble lactoglobulin available or to a loss of lactoglobulin epitopes along the proteins moiety leading to a reduced signal compared to unbaked cookie dough.

The differences in the heat induced decrease of immunologically detected milk proteins indeed points at differences in their heat stability. A comparison of the patterns observed for CN and LG (curve *a* and *c* of Fig. 3) implies a higher heat stability of CN within the cookie matrix compared to LG. In contrast to CNs, the structure of whey proteins like LG is known to be labile when submitted to thermal treatments. On a molecular level LG as well as lactalbumin possess well defined three dimensional structures. Heating causes denaturation of proteins including folding/unfolding and aggregation of the molecules as has been assessed by employing a variety of different techniques (Chen et al., 2004; Ngarize, Herman, Adams, & Howell, 2004). The heat-induced behaviour of LG has been extensively studied as well as the mechanism of its aggregation that occurs at higher temperatures (Breiteneder & Mills, 2005; de la Fuente et al., 2002; Shokker, Singh, & Creamer, 2000). Heating generally results in a loss of tertiary structure and recently MS methodology has been employed to provide more insights into the structural modifications of LGs (in standard solution as well as in milk) when submitted to thermal treatments (Monaci & van Hengel, 2007). Nonetheless, the issue of protein solubility/extraction still remains challenging for MS analysis. On the other hand, modifications of allergenic proteins (e.g. lactosylation) occurring in food matrices that might account for an increase in allergenicity, can also be highlighted by MS approach (Monaci & van Hengel, 2007). Moreover, the interaction of LG with other milk or food matrix components is likely to result in irreversible structural changes of LG. All these changes can influence the antibody recognition and then hamper the final detection by ELISA.

3.4. Effect of sample dilution method on ELISA outcome

For ELISA analysis a proper quantification of milk allergens in food products containing high levels of the analytes requires dilution of the sample. Suitable dilution buffers and/or instructions for dilution of samples are usually provided with commercial ELISA test kits for allergen detection. However, the introduction of a dilution factor might impact the quantification, since not only the allergen is diluted, but also the extraction buffer and all components that were extracted from the matrix. To investigate whether the sample dilution method might affect the detection of milk proteins we used incurred cookies (baked for 9 min) containing milk powder at a level of 10,000 mg/kg that were diluted 100-fold at the final concentration of 100 mg/kg either with blank cookie extract or with the recommended dilution buffer.

Ten samples diluted with PBS and 10 samples diluted with blank cookie extract were analysed with the total milk ELISA kit. This ELISA kit was chosen since the extraction buffer (PBS; pH 7.4) is identical to the recommended dilution buffer, and therefore the nature as well as the concentration of extraction buffer components is almost unaltered after dilution. ANOVA tests performed on the resulting datasets revealed a clear statistically significant difference at a 95% confidence level between the data obtained for the samples that were diluted with PBS, versus those diluted with blank cookie extract. This implies that at the concentration tested (=100 mg/kg), dilution of samples can affect the quantitative result and that different dilution buffers can have a different impact on this quantitative result. Interestingly, when the dilution buffers, PBS and blank cookie extract, were analysed with the ELISA kit, the results were not statistically different, which rules out any cross reactivity with matrix components.

Subsequently, we investigated whether the ELISA results obtained for the 100-fold diluted cookie samples (from 10,000 to 100 mg/kg) are comparable to those obtained by analysing undiluted cookie samples incurred with milk powder at the final level of 100 mg/kg (Diaz-Amigo & Popping 2010). Fig. 4 shows the average relative ELISA signals obtained for the diluted and undiluted samples. It is apparent from this figure that the results obtained for cookie extracts diluted 100-fold with blank cookie extract (blank dilution) at the concentration of 100 mg/kg are very similar to those obtained for cookie material incurred at 100 mg/kg (undiluted). Statistical analysis by means of *T*-tests indeed confirmed that there was no significant difference ($P > 0.05$). This implies that the extraction efficiency of the milk allergen ELISA target mole-

cules is comparable for incurred cookies prepared with 10,000 or prepared with 100 mg milk powder per kg food matrix.

On the other hand, Fig. 4 also shows that the results obtained for cookie extracts diluted 100-fold with dilution buffer (PBS dilution) to a final concentration of 100 mg/kg are significantly different from those obtained for cookie material incurred at the final concentration of 100 mg/kg ($P < 0.05$).

These results show that according to the type of dilution method applied, dilution of matrix components can occur thus altering the final results obtained by ELISA. Matrix effects are known to affect ELISA analysis (Poms et al., 2004b; van Hengel, 2007) and our findings show that dilution of matrix components can also have a negative impact on ELISA analysis. A matrix-matched calibration is therefore preferred, although it has to be recognised that for the analysis of market samples matrix matched blank controls are not available.

4. Conclusions

Immunoassays are often the method of choice for the detection of food allergens since their detection is based on antibodies that can recognise specific proteins within a complex mixture of compounds. Although the ELISA approach offers a number of benefits, general limitations encountered in allergen detection, whatever technique is being used for detection, are due to a decreased solubility/extractability of milk proteins in food undergoing processing. Besides, modification of the epitopes is also likely to occur in processed food matrix giving rise to a lack or reduction of antibody recognition thus impairing the outcome of the immunoassays.

Since the ELISA kits are used to detect milk traces in baked goods we compared their capacity to detect milk traces in incurred samples and samples that were spiked with milk proteins after baking. It can be concluded that the use of ELISA for the detection of milk proteins is severely compromised for incurred samples, especially in the case of LG detection. As far as ELISA validation studies are concerned, it is in general recommended to validate a method using either incurred samples and/or externally spiked samples.

Further studies aimed to evaluate how both protein extraction and immunoreactivity are affected by the baking process. When cookie dough is submitted to a heat treatment, the amount of proteins that can be extracted is drastically reduced with the highest drop in extraction efficiency occurring during the first 6 min of heating. The reduction of protein extraction negatively impacts the detection of milk allergens in incurred cookie samples. However, the reduction of the ELISA signal due to the baking process also depends on the nature of the targeted (milk) proteins, since the detection of CN is clearly less affected by baking compared to that of the whey protein LG. This is in line with the known differences in their thermostability.

Finally, the effect of sample dilution was investigated leading to the conclusion that sample dilution can affect the quantitative result as demonstrated by using a specific ELISA kit for total milk allergens. In conclusion, ELISA represents one of the most important analytical tools to detect allergenic ingredients in food products, but food processing and matrix effects are generally recognised to affect this detection. In this study we have investigated the extent of the influence of baking and sample dilution on the detection of milk traces in model cookies. A better knowledge of the benefits as well as the limitations of ELISA kits will assist the correct identification of milk allergen-containing food products and thereby improve the protection of allergic consumers.

Acknowledgements

The authors are grateful to Olivier De Rudder for carrying out measurements on water content, to Dr. Fernando Cordeiro for his

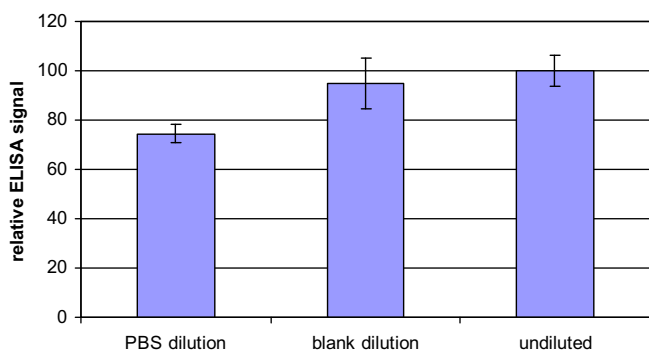


Fig. 4. Average relative ELISA signals obtained for diluted and undiluted samples. Results obtained for the ELISA analysis of incurred cookie material spiked with milk powder at a level of 10,000 mg/kg and diluted 100-fold (before analysis) with either PBS (PBS dilution) or blank cookie extract (blank dilution) (10 replicates for each) is shown in conjunction with ELISA results obtained for incurred cookies that were generated with 100-fold less milk powder (10 replicates) and for which samples were not diluted before analysis. The average ELISA signal obtained for the undiluted incurred cookies that were prepared with milk powder at a level of 100 mg/kg was set as 100%. The standard deviation is indicated.

help in statistical data treatment and to Dr. Carmen Diaz-Amigo for her critical reading and valuable comments on the manuscript.

References

- Akiyama, H., Isuzugawa, K., Harikai, N., Watanabe, H., Iijima, K., Yamakawa, H., et al. (2004). Inter-laboratory evaluation studies for development of notified ELISA methods for allergic substances (milk). *Shokuhin Eiseigaku Zasshi*, 45, 120–127.
- Breiteneder, H., & Mills, E. N. C. (2005). Molecular properties of food allergens. *Journal of Allergy and Immunology*, 115, 14–23.
- Chassaigne, H., Brohée, M., Nørgaard, J. V., & van Hengel, A. J. (2007). Investigation on sequential extraction of peanut allergens for subsequent analysis by ELISA and 2D gel electrophoresis. *Food Chemistry*, 105, 1671–1681.
- Chen, Z. L., Hwang, M. T., Liao, C. Y., Ho, J. C., Hong, K. C., & Mao, S. J. (2004). β -lactoglobulin is thermal marker in processed milk as studied by electrophoresis and circular dichroic spectra. *Journal of Dairy Science*, 87, 1158–1166.
- Commission of the European Communities (2007). *Official Journal of European Union*, L 310, 11–13.
- Davis, P. J., Smales, C. M., & James, D. C. (2001). How can thermal processing modify the antigenicity of proteins? *Allergy*, 56, 56–60.
- de la Fuente, M. A., Singh, H., & Hemar, Y. (2002). Recent advances in the characterization of heat induced aggregates and intermediates of whey proteins. *Trends in Food Science and Technology*, 13, 262–274.
- de Luis, R., Lavilla, M., Sánchez, L., Calvo, M., & Pérez, M. D. (2009). Development and evaluation of two ELISA formats for the detection of β -lactoglobulin in model processed and commercial foods. *Food Control*, 20, 643–647.
- Diaz-Amigo, C., & Popping, B. (2010). Analytical testing as a tool for the enforcement of future regulatory thresholds for food allergens. *Journal of AOAC International*, 93, 434–441.
- Diaz-Amigo, C. (2010a). Towards a comprehensive validation of ELISA kits for food allergens. Case 2 – Milk. *Food Analytical Methods*, in press. doi:10.1007/s12161-009-9095-y.
- Diaz-Amigo, C. (2010b). Towards a comprehensive validation of ELISA kits for food allergens. Case 1 – Egg. *Food Analytical Methods*, in press. doi:10.1007/s12161-009-9094-z.
- Fæste, C. K., Løvberg, 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, 427–436.
- Hourihane, J. O' B., & Knulst, A. C. (2005). Thresholds of allergic proteins in foods. *Toxicology and Applied Pharmacology*, 207, S152–S156.
- Kerbach, S., Alldrick, A. J., Crevel, R. W. R., Dörmötör, L., DunnGalvin, A., Mills, E. N. C., et al. (2009). Managing food allergens in the food supply chain-viewed from different stakeholder perspectives. *Quality Assurance and Safety of Crops and Foods*, 1, 50–60.
- Kleber, N., Krause, I., Illgner, S., & Hinrichs, J. (2004). The antigenic response of β -lactoglobulin is modulated by thermally induced aggregation. *European Food Research and Technology*, 219, 105–110.
- Krška, R., Welzig, E., & Baumgartner, S. (2004). Immunoanalytical detection of allergenic proteins in food. *Analytical and Bioanalytical Chemistry*, 378, 63–65.
- Maleki, S. J. (2004). Food processing: effects on allergenicity. *Current Opinion in Allergy and Clinical Immunology*, 4, 241–245.
- Monaci, L., Treggoat, V., van Hengel, A., & Anklam, E. (2006). Milk allergens, their characteristics and their detection in food: A review. *European Food Research and Technology*, 223, 149–179.
- Monaci, L., & van Hengel, A. J. (2007). Effect of heat treatment on the detection of intact bovine β -lactoglobulins by LC mass spectrometry. *Journal of Agricultural and Food Chemistry*, 55, 2985–2992.
- Monaci, L., & Visconti, A. (2009). Mass spectrometry-based proteomics methods for analysis of food allergens. *Trends in Analytical Chemistry*, 28, 581–591.
- Natale, M., Bisson, C., Monti, G., Peltran, G., Garoffo, L. P., Valentini, S., et al. (2004). Cow's milk allergens identification by two-dimensional immunoblotting and mass spectrometry. *Molecular Nutrition and Food Research*, 48, 363–369.
- Ngarize, S., Herman, H., Adams, A., & Howell, N. (2004). Comparison of changes in the secondary structure of unheated, heated, and high-pressure-treated β -lactoglobulin and ovalbumin proteins using Fourier Transform Raman spectroscopy and self-deconvolution. *Journal of Agricultural and Food Chemistry*, 52, 6470–6477.
- Paschke, A., & Besler, M. (2002). Stability of bovine allergens during food processing. *Annals of Allergy, Asthma and Immunology*, 89, 16–20.
- Poms, R. E., & Anklam, E. (2004). Effects of chemical, physical, and technological processes on the nature of food allergens. *Journal of AOAC International*, 87, 1466–1474.
- Poms, R. E., Capelletti, C., & Anklam, E. (2004a). Effect of roasting history and buffer composition on peanut protein extraction efficiency. *Molecular Nutrition and Food Research*, 48, 459–464.
- Poms, R. E., Klein, C. L., & Anklam, E. (2004b). Methods for allergen analysis in food: a review. *Food Additives and Contaminants*, 21, 1–31.
- Scaravelli, E., Brohée, M., Marchelli, R., & van Hengel, A. J. (2009). The effect of heat treatment on the detection of peanut allergens as determined by ELISA and real-time PCR. *Analytical and Bioanalytical Chemistry*, 395, 127–137.
- Shokker, E. P., Singh, H., & Creamer, L. K. (2000). Heat-induced aggregation of β -lactoglobulin A and B with α -lactalbumin. *International Dairy Journal*, 10, 843–853.
- Shubert-Ullrich, P., Rudolf, J., Ansari, P., Galler, B., Führer, M., Molinelli, A., et al. (2009). Commercialized rapid immunoanalytical tests for determination of allergenic food proteins: an overview. *Analytical and Bioanalytical Chemistry*, 395, 69–81.
- van Hengel, A. J. (2007). Food allergen detection methods and the challenge to protect food-allergic consumers. *Analytical and Bioanalytical Chemistry*, 389, 111–118.
- Wal, J. M. (2001). Structure and function of milk allergens. *Allergy*, 56, 35–38.
- Wal, J. M. (2003). Thermal processing and allergenicity of foods. *Allergy*, 58, 727–729.
- Westphal, C. D., Pereira, M. R., Raybourne, R. B., & Williams, K. M. (2004). Evaluation of extraction buffers using the current approach of detecting multiple allergenic and non allergenic proteins in food. *Journal of AOAC International*, 87, 1458–1465.