



Analytical Methods

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

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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.

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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

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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.

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