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Mechanisms behind matrix–protein interactions influencing receptor-based and chromatographic detection of food allergens: A case study with a fruit based snack

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ABSTRACT

Food allergies represent an important food safety problem. The only effective treatment for food allergies is the complete avoidance of the offending foods or foods containing undeclared traces of allergens. In order to verify whether foods are contaminated with allergens reliable detection methods are needed. Currently enzyme-linked immunosorbent assays (ELISA) especially commercial ones are routinely used by the food industry and enforcement agencies. However, the reliability of such receptor based methods can be affected by the matrix in several ways. In this study we showed that the proteins/allergens can strongly bind to the matrix making them inefficient for the extraction as proven by a high-performance liquid chromatography method. On the other hand, especially in the case of ELISA methods, the matrix can non-specifically bind to the antibodies leading to false positive results. In conclusion, outmost care should be taken when interpreting the results of the methods used for analysis of traces of food allergens especially when important decisions about the acceptance or removal of the products from the market should be taken.

1. Introduction

Food allergies represent an important food safety problem especially in industrialized countries affecting about 2% of the adult population and 8% of the children (Poms & Anklam, 2004). The only treatment available for food allergies is the complete avoidance of the offending foods. In order to help the allergic consumers the EU Directive 2007/68/EC specifies a number of foods and products thereof (cereals, crustaceans, eggs, fish, peanuts, soybean, milk, tree nuts, celery, mustard, sesame, lupine and molluscs) which should be declared on the label whenever deliberately added as an ingredient (European Parliament and Council, 2007). Unfortunately due to the growing complexity of food formulations and food processing, foods may be unintentionally contaminated with allergen-containing ingredients. The presence of traces of allergens might be a result of the use of contaminated raw materials, use of shared equipment, inefficient cleaning of the shared equipment, rework and inappropriate storage conditions. The only way to verify whether foods are free of traces of allergens is by using reliable analytical methods which would allow accurate detection in the raw materials and end products.

Nowadays, the enzyme-linked immunosorbent assay (ELISA) is most frequently used in routine food analysis. The ELISA methods are based on specific molecular interactions between the antibodies used in the assay and the analyte, in this case allergens/proteins. The extensive use of ELISA methods for allergen detection is based on the fact that they are relatively cheap, easy to perform, highly sensitive with a large number of commercial assays available on the market. The commercial assays are used not only by the food industry but enforcement agencies as well. This indicates that important decisions, such as allowance or withdrawal of the products from the market are taken based on results obtained using such methods (Poms & Anklam, 2004). Unfortunately, the robustness of such receptor based analytical methods depends highly on the matrix analyzed and moreover it seems to be severely affected upon processing. Previous data indicate that commercial kits used to detect food allergens in especially processed foods are prone to false negative results (Cucu, De Meulenaer, & Devreese, 2012; Cucu

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et al., 2011; Downs & Taylor, 2010; Fu, Maks, & Banaszewski, 2010; Platteau et al., 2011; Scaravelli, Brohee, Marchelli, & van Hengel, 2009). The results obtained were moreover kit dependent indicating that one ELISA kit cannot be used for all applications and that careful evaluation of the results obtained should be made. Further, the presence of the matrix can have an important impact on the analytical result as well. On the one hand because of the non-specific binding of the matrix components with the employed antibodies (Cucu, Devreese, Kerkaert, et al., 2012; Cucu, Devreese, Trashin, et al., 2012; Husain, Bretbacher, Nemes, & Cichna-Markl, 2010; Platteau et al., 2011) and on the other hand because of the interaction of the analytical target with the matrix especially during processing which makes their extraction inefficient (Schmitt, Nesbit, Hurlburt, Cheng, & Maleki, 2010). It is therefore of outmost importance that before using receptor based methods for routine analysis, a thorough evaluation of their reliability in different product matrices is performed (Abbott et al., 2010). The aim of this paper was to show how matrix can have an impact on the detection of allergens. This was performed on a fruit based snack which was tested positive for cow's milk using a commercial "RIDASCREEN β-Lactoglobulin" kit from R-Biopharm. The detected concentration of β-lactoglobulin (β-LG) was 0.27 μg/g which was slightly higher than the detection limit of the kit (0.20 μg/g β-LG corresponding to 2 μg/g milk protein). To the authors knowledge, on the whole site of the factory, there was only one milk containing ingredient present (caramel – powder) which, based on the information obtained from the producer, was not used in the production of the snack and of which it is unlikely that cross-contamination would occur.

2. Materials and methods

2.1. Materials

Chemicals and standards of analytical grade were purchased from Sigma-Aldrich (Bornem, Belgium), VWR (Leuven, Belgium) and Acros Organics (Geel, Belgium). Sodium caseinate (Miprodan 30) containing 88% protein, 1.5% fat, 0.3% sugar, 4% ash and 6% moisture were delivered by Acatris Food Belgium (Londerzeel, Belgium). Raspberry "Nature addicts fruit break" snack was obtained directly from the producer (Taura, Olen, Belgium). The fruity snack contained: concentrated apple juice (52%), concentrated apple puree (39%), concentrated raspberry puree (5%), concentrated elderberry juice, fruit fibers, fruit pectin and natural flavorings. Syringe filters with polyether sulfone (PES), polyvinylidene fluoride (PVDF) cellulose acetate (CA) membranes all of 0.45 μm were from Novolab (Belgium).

2.2. HPLC analysis of the casein

Samples were finely ground in a mortar. For extraction, 2 g of sample was incubated with 10 mL of 0.1 M potassium phosphate buffer pH 8 by shaking for 2 h at room temperature. The pH was checked and if necessary adjusted again to 8 with 1 M hydrochloric acid. The extracts were further centrifuged at 9000 *g* for 10 min at room temperature. The pH of the decanted supernatants was adjusted to 2 with 10 M hydrochloric acid and again centrifuged at 15,000 *g* for 15 min at 4 °C. The obtained pellets were washed with 0.1 M hydrochloric acid and again centrifuged at 15,000 *g* for 15 min at 4 °C. Finally the pellets were redissolved in 0.1 M potassium phosphate buffer pH 8. The pH was checked and if necessary adjusted to 8 with 1 M hydrochloric acid. The samples were filtered and analyzed by HPLC. The HPLC (1100 system, Agilent Technologies, Switzerland) was equipped with a reversed-phase polymeric column (PLRP-S 250 × 4.6 mm, 300 Å pore size, 5 μm particle size) from Varian Inc. (Belgium). The mobile phase consisted of a gradient of water and acetonitrile both containing 0.1% trifluoroacetic acid (TFA) (v/v). Eluting conditions expressed as proportion of water with 0.1% TFA were as follows: 0–10 min: 69%, 19 min: 49.3%, 20 min: 25%, 20–21 min: 25%, 22 min: 69%, 22–30 min: 69%. The flow rate was 1 mL/min, the column temperature 45 °C and the injected volume 50 μL. Detection was carried out with a fluorescence detector (FLD, G1321, Agilent Technologies, Switzerland) set at the wavelength: 280 nm excitation and 340 nm emission. An emission spectrum was taken between 300 and 500 nm in order to confirm the identity of the peak in combination with a matching eluting time and as such a guarantee of the specificity of the analysis. Several adaptations were applied on this standard HPLC method as discussed in Results and Discussion section; these and their results are further discussed.

2.3. Sandwich hazelnut ELISA

The grounded samples (1 g) were extracted in duplicate with either 10 mL of 0.1 M potassium phosphate buffer pH 8 or 10 mL water by shaking for 2 h at room temperature. The pH of the extract was checked and if necessary adjusted to 8 with 1 M NaOH. Standards of purified hazelnut protein (*Cor a 9*) in PBS (0.135 M NaCl, 1.5 mM KH_2PO_4, 8 mM $Na_2HPO_4 \cdot 12H_2O$, 2.7 mM KCl, pH 7.4) of the following concentrations were prepared: 10, 3, 1, 0.3, 0.1, 0.03 and 0.01 μg/mL. A Microlon 96 K high binding ELISA plate was coated with 100 μL chicken IgY antibodies developed towards *Cor a 9* (10 μg/mL) in the coating buffer (0.015 M Na_2CO_3, 0.035 M $NaHCO_3$), pH9.6. After overnight incubation at 4 °C the wells were washed 3 times with 200 μL of 0.05% Tween 20 in 0.9% NaCl solution and this washing step repeated after each incubation step described later, except after the substrate addition. After blocking the remaining binding sites with 200 μL per well of 4.5% sodium caseinate in PBS, 100 μL of standard *Cor a 9* or diluted/non-diluted samples were added to the wells and incubated 75 min at 37 °C in the dark. Following washing, the plates were incubated with 100 μL of HRP-anti-hazelnut IgY (diluted 1/15,000 in PBS) for 1 h at 37 °C in the dark. Finally, the wells were incubated with 100 μL of TMB substrate, containing 0.45% hydrogen peroxide for 1 h at 37 °C. To stop the color reaction 25 μL of 4 M sulfuric acid was added to each well and the absorbance was read at 450 nm using a micro plate reader (Bio-Rad, Belgium, Nazareth Eke). The obtained experimental points for the calibration curve were fitted by the standard four parameters theoretical curve:

$$\text{Absorbance} = \frac{B_{\max} - B_0}{1 + \left(\frac{C}{EC_{50}}\right)^d} + B_0,$$

where: $B_{\max}$ and B_0 – maximal and minimal value of absorbance, C – protein concentration, EC_{50} – concentration at which response is a half of the amplitude$(B_{\max} - B_0)$ (Trashin, Cucu, Devreese, Adriaens, & De Meulenaer, 2011).

2.4. Phenol content determination

The Folin Ciocalteu method was used for the total polyphenol content determination as previously described (Vandekinderen et al., 2008).

3. Results and discussion

3.1. HPLC analysis of casein

Because caseins represent 80% of the total proteins in the milk, they were used as analytical target in this study (Belitz, Grosch, &