Detection of allergens in spiked pasta by Real-time PCR

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Introduction
Food allergies nowadays represent an important health problem. Allergenic ingredients as well as undeclared allergens as contaminants in food products pose a great risk for sensitized persons (Figure 1). To ensure compliance with food labeling and protection of consumers reliable methods for detection and quantification of food allergens are required. The detection of allergens in food products can be very challenging, due to the fact that they are often present only in trace amounts or masked by the food matrix. DNA-based methods are increasingly used for the detection of foreign food constituents. The methods are specific and provide sensitive tools for the detection of specific allergenic components in food. Since reference materials (RM) are scarcely available for food allergens so far, we took an approach to imitate reference material by spiking blank pasta with different concentrations of five allergens (soy, mustard, celery, lupine, sesame). Blank pasta was made from durum wheat semolina and tested for the presence of the five selected allergens. Then spiked pasta was prepared with a starting concentration of 200 ppm for each allergen respectively. Afterwards dilutions were made using spiked pasta material and blank pasta material resulting in concentrations of allergens in spiked pasta of material 200, 50, 20, 10, 5, 1 ppm respectively. DNA extraction was performed following a modifid CTAB protocol. Real-time PCR was performed on ABI Prism 7700 or Corbett Rotorgene 6000 using commercialized test kits. Correlation between cycle threshold value and spiked amount of allergen was analyzed for each singleplex allergen. We achieved good correlations despite the fact that DNA had to be extracted from a food matrix. Limit of detection is about 10 ppm for the selected allergens in spiked pasta. Spiked food matrices are a first approach of a matrix-dependent quantification of allergens in food.

Materials and Methods

Production of blank and allergen spiked pasta: Wheat semolina was spiked with selected allergens to an initial concentration of 200 ppm (200 mg/kg) respectively. We used partially degreased soy flour (Hensler, Germany), lupine flour (L.I. Frank, Netherlands), unpeeled degreased sesame seeds (Seebberger KG, Ulm), celery tuber powder (kindly provided by CVJIA Freiburg) and mustard powder (Raoul Roussos GmbH, Greven). Blank pasta and spiked pasta were kneaded with water in a pasta maker. Pasta was formed and dried for 3 h at 80 °C. Afterwards pasta was grilled in a Thermowork (Vonwerk, Wuppertal) followed by another grinding step in a roto mill (Retsch, Haar). Serial dilutions (50, 20, 10, 5, 1 ppm) were obtained by mixing blank pasta with spiked pasta.

DNA extraction: Extractions were always performed in duplicates, extraction controls were done to exclude contaminations. DNA from spiked pasta, blank pasta and the five allergens was isolated following a modified CTAB (cetyltrimethylammonium bromide) protocol. 10 ml of CTAB extraction buffer (2 % CTAB w/v, 1.4 M NaCl, 20 mM Na2EDTA, 100 mM Tris-OH/HCl) and 30 µl of proteinase K (20 mg/ml, Merck, Darmstadt) were added to 2 g of sample material in a 50 ml falcon tube. After mixing and incubation for 1.5 h (65 °C, shaking 45 %), the samples were centrifuged for 10 min at 8000 g. 700 µl of supernatant was mixed with 400 µl of chloroform/isoamyl alcohol (24:1), followed by 15 min of centrifugation at 21000 x g. 500 µl of supernatant was mixed with 500 µl of isopropanol. 30 min of incubation at room temperature were followed by 15 min of centrifugation at 21000 x g. After removal of supernatant, the pellet was washed with 500 µl ethanol (70 % v/v) and centrifuged at 21000 x g. Supernatant was discarded, pellets were dried overnight in an exsiccator and resolved in 100 µl of 0.1 M TE buffer.

Real-time PCR Procedure: Reaction mix was prepared according to the manufacturer’s instruction. 5 µl of sample extracts have been used for Real-time PCR analysis. Reactions were carried out in duplicates unless otherwise noted. No template controls and inhibition controls have always been made. PCR reactions were carried out on ABI Prism 7700 (Applied Biosystems, USA) or Corbett Rotorgene 6000 (Corbett Research, Australia) according to the following thermal cycling program: Initial denaturation (5 min at 95 °C), cycle (45 x) denaturation (30 s at 94 °C), primer annealing, elongation and data collection (30 s at 60 °C). We used five commercialized Real-time PCR kits from Congen (Berlin) for singleplex Real-time PCR (soy, celery, mustard, lupine) and a multiplex kit (AllIAIA, kindly provided by R. Köppel, KLZH, Zurich). Analysis on Corbett Rotorgene 6000 was carried out using Dynamic Tube Normalisation and Noise Slope Correction, analysis on ABI Prism 7700 was carried out according to manufacturer’s instruction.

Results/Discussion

Each concentration was analyzed four times (2 extractions for each concentration, 2 PCRs for each extracted sample) and the resulting cycle threshold values were plotted against the respective amount of allergen. Correlations between resulting cycle thresholds and amounts of allergen were analyzed for each allergen, furthermore we determined limit of detection in this food matrix. Results for celery are exemplarily shown in figure 2-3.

Allergenic ingredients as well as undeclared allergens as contaminants in food products pose a great risk for sensitized persons. To ensure compliance with food labeling and protection of consumers reliable methods for detection and quantification of food allergens are required. Since reference materials (RM) are scarcely available for food allergens so far, we took an approach to imitate reference material by spiking blank pasta with different concentrations of five allergens (soy, mustard, celery, lupine, sesame). Spiked food matrices are a first approach of a food matrix-dependent quantification of allergens. \(C_t\)-values of unknown samples with a similar matrix can be compared to those of the spiked matrix enabling us to make semi-quantitative statements for unknown samples. We have already analyzed other spiked food matrices (e.g. spiked sausages, spiked bakery products) using single- and multiplex Real-time PCR. Currently we are producing spiked spices as another and more difficult food matrix. Further methods to quantify allergens independently of the food matrix are necessary. If reliable quantification methods are available it will be feasible to establish threshold values for allergens in food.