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. Yet, 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 material of 200, 50, 20, 10, 5, 1 ppm respectively. DNA extraction was performed following a modified 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 selected 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.

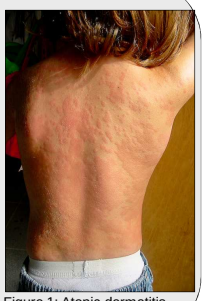
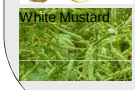


Figure 1: Atopic dermatitis caused by food allergy (milk) [1]

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, *Lupinus augustifolius*), unpeeled degreased sesame seeds (Seeberger KG, Ulm), celery tuber powder (kindly provided by CVUA Freiburg) and mustard powder (Raoul Rousso 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 grinded in a Thermomix (Vorwerk, Wuppertal) followed by another grinding step in a rotor mill (Retsch, Haan). 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 Na_2-EDTA, 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 8800x g. 700 µl of supernatant was mixed with 400 µl of chloroform/isoamyl alcohol (24:1), followed by 15 min of centrifugation at 21000x 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 21000x g. After removal of supernatant, the pellet was washed with 500 µl ethanol (70 % v/v) and centrifuged 5 min at 21000x g. Supernatant was discarded, pellets were dried overnight in an exsiccator and resolved in 100 µl of 0.1 M TE buffer. Subsequently, DNA extracts were purified using the QIAquick PCR purification Kit (Qiagen, Hilden) and eluted in 50 µl 15 mM Tris buffer. Concentration of isolated DNA was determined photometrically (Amersham Pharmacia Biotech, Sweden). DNA isolated from blank pasta or spiked pasta has not been diluted or set to a specific concentration. Genomic DNA extracted from soy, sesame, lupine, celery, mustard was diluted (5, 0.5, 0.05, 0.005 ng/µl) and used as standard.

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 (15 sec at 95 °C), primer annealing, elongation and data collection (30 sec at 60 °C). We used five commercialized Real-time PCR kits from Congen (Berlin) for singleplex Real-time PCR (soy, sesame, celery, mustard, lupine) and a multiplex kit (AllAllA, 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 PCR´s 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.

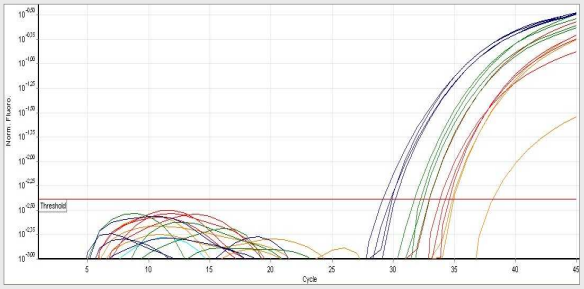


Figure 2: Singleplex celery Real-time PCR; spiked pasta, 200, 50, 20, 10 ppm allergen, 4 PCR reactions were performed for each concentration on Corbett Rotorgene using 5 µl of CTAB extracted DNA shown as relative fluorescence against cycle number

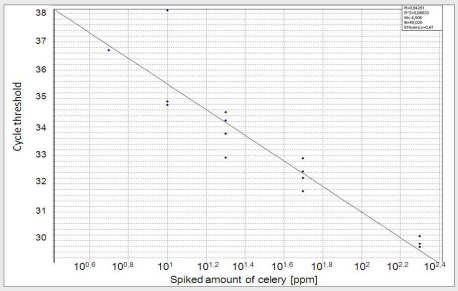


Figure 3: Singleplex celery Real-time PCR, spiked pasta, 0-200 ppm allergen, 4 PCR reactions were performed for each concentration on Corbett Rotorgene using 5 µl of CTAB extracted DNA shown as cycle threshold values against spiked amount of celery

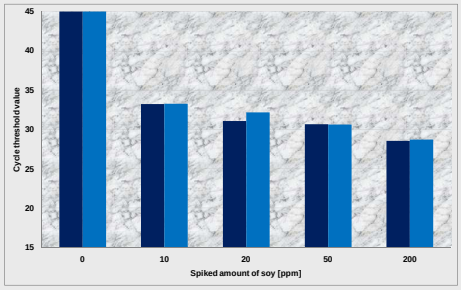


Figure 4: Multiplex AllAllA Soy Real-time PCR, spiked pasta, 0-200 ppm allergen, 2 PCR reactions were performed for each concentration on Corbett Rotorgene shown as cycle threshold value against spiked amount of soy

Blank pasta showed no signals for celery, soy, sesame and lupine but a very small portion of mustard. The source of contamination of blank pasta with mustard couldn't be ruled out so far. Figure 2 shows the results of the Real-time PCR detecting celery, figure 3 the resulting plot of relative amount of allergen (celery) expressed in cycle threshold values. Spiked amounts of 10 ppm up to 200 ppm were reliably detected by all singleplex kits; four out of four reactions were positive (except celery 10 ppm, three out of four reactions positive). Reliability of detection was significantly decreasing below 10 ppm. Therefore we determine 10 ppm allergen as limit of detection for Real-time PCR in spiked pasta. We achieved good correlations despite the fact that DNA had to be extracted from a food matrix (Correlation r of cycle threshold value and amount of allergen in spiked pasta: celery 0.943, soy 0.937, mustard 0.984, sesame 0.972, lupine 0.846). We also performed a multiplex Real-time PCR analysis using the AllAllA tetraplex kit, which is able to detect soy, celery, hazelnut and peanut. Figure 4 exemplarily shows the resulting cycle threshold values of soy in the AllAllA multiplex system. Main disadvantage of doing multiplex Real-time PCR is a decrease in sensitivity. However, the AllAllA multiplex system is able to detect 10 ppm spiked samples reliably, demonstrating the sensitivity of the provided multiplex system.

Summary/Outlook

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.

References: [1] Gary M. White & Neil H. Cox, Diseases of the skin, Elsevier Health Sciences, 2005