

Detection of Peanut Allergen Traces with a Real Time PCR Assay - The Challenge to Protect Food-Allergic Consumers

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Abstract

The aim of the present study was the implementation of molecular techniques in the detection and quantification of allergic substances of peanut in various kinds of food products, e.g., breakfast cereals, chocolates and biscuits that are frequently related to allergies. In some cases, the presence of peanuts can be due to contamination during production and are not declared on the label. A total of 152 samples were collected from supermarkets and were analysed by a Real Time PCR method. The results indicated that 125 samples (83,3%) were found positive in peanut traces but the most important finding is that from the 84 samples that had no allergen declaration for peanuts, 48 (57,1%) of them were found positive. In conclusion, Real Time PCR can be a very important tool for the rapid detection and quantification of food allergens.

Keywords: food allergen declaration, food allergy, PCR, peanut, quantification

1. Introduction

Peanuts are a well-known allergen included in the list of the 14 Major Allergens as described in EU Food Information Regulation No. 1169/2011. At the same time, studies highlighted a clear increase of the prevalence of peanut allergy in young children (Hourilane et al., 1997). Peanuts have a high abundance in allergenic proteins, containing approximately 29%. The International Union of Immunological Societies Nomenclature Subcommittee recognizes 8 allergenic proteins in peanuts, named from Ara h 1 to Ara h 8. Among these proteins, Ara h 3 and Ara h 4 are isoforms and Ara h 6 is highly homologous to Ara h 2 (Koppelman et al., 2003, De Leon et al., 2007). The major allergen Ara h 1 accounts for approximately 20% of the total protein content, while Ara h 2 accounts for about 10% (Van Hengel et al., 2007). Moreover, Ara h 1 and 2 show resistance to heat and enzymatic digestion (Burks et al., 1998). Because of the high allergenic potential of peanut, peanut allergy has become a real public health problem, attracting the attention of food control agencies and food industry. Most studies have shown that even low amounts of peanut protein (1-3 mg) are sufficient to trigger allergic symptoms (Taylor et al., 2002, Morisset et al., 2003). Particular attention should be paid to avoid cross-contamination by foodborne allergens between different food products. Therefore, the implementation of sound hygiene practices and food separation needs to be ensured. (Poms et al., 2004). Most of the times, the possibility of such a cross-contamination is optionally mentioned on the package labels with the phrase "it may contain", which is an important piece of information for consumers. Up to now, the only choice for allergic individuals, who had already experienced adverse reactions, was to avoid the consumption of offending foods, as an actual medical treatment is not yet available. In this case, prevention is of extreme importance.

Methodologies for the detection of various food allergens, which are based on diverse technologies already exist and can be applied for different purposes. Several studies report on the development of ELISA methods for the detection of peanut traces in food. Recent validation studies (Poms et al., 2004, Park et al., 2005, Whitaker et al., 2005) have shown that all ELISA test kits studied were capable of correctly identifying test samples containing 5 mg peanut per kg of food matrix. The methods reported in the above-mentioned studies were capable of detecting 2.5 pg peanut DNA (less than one copy of peanut genome equivalent) and all three assays studied were

successfully applied to detect peanut traces in a model food product where they could detect 10 mg kg^{-1} peanut. However, detection of allergenic or marker proteins is not necessarily the only way to demonstrate the presence of the allergic compounds, as the detection of another type of marker molecule, like DNA, can be an alternative method (Pedersen et al., 2008, Schoringhumer & Cichna-Markl, 2007, Holzhauser et al., 2000, Holzhauser & Vieths, 1999, Yeung & Collins, 1996). A comparison of a real-time PCR method and an ELISA method, for the detection of peanut has recently been reported, whereby the relative Ct (cycle threshold) values obtained by the real-time PCR correlated well with the amount of peanut measured by the sandwich ELISA (Stephan & Vieths, 2004). This result highlights the potential of PCR for a semi-quantitative detection of peanut traces in foods. There have been only a few studies focusing on food allergens that compare fundamentally different detection methods applied to a wide selection of food products. The aim of the present study is the implementation of a sensitive and effective Real Time PCR (RT-PCR) methodology for the detection and quantification of peanut traces in various kinds of commercial goods, in order to protect the health of allergic consumers and provide industry with a reliable detection method for food allergens.

2. Materials and Methods

2.1 Food Collection

During the study period (September 2016 to June 2017) a total of 152 widely consumed goods, which are potentially responsible for food allergies were collected from local super markets and were immediately studied. All samples were commercial products, produced in Greece without further information about the origin of the raw materials. The samples were separated in four categories namely those which contain peanuts as an ingredient (category I), samples that “may contain” “traces” of peanut (category II), samples that “may contain” “traces” of nuts without any further information (category III) as well as samples that did not have any allergen declaration or warnings on potential allergenic substances (category IV). More specifically the samples included, 30 different kinds of cereals, 13 chocolates, 30 biscuits, 24 wafers and 55 other snacks. Natural products, such as peanuts kernels (*Arachis hypogaea* from Argentina), were used as positive controls, while hazelnuts and sesame seeds were used as negative samples.

2.2 Genomic DNA Extraction and Quantification

NucleoSpin Food kit (Macherey-Nagel, GmbH & Co. KG, Germany) and Bio Scientific kit (Austin, USA) were tested for the extraction of peanuts, hazelnut and sesame kernels and commercial foods, in order to compare the extraction yield and the cleaning up of the genomic DNA. All extraction methods were applied according to the manufacturer's instructions with some modifications. The NucleoSpin Food kit was finally selected for the extraction of all samples. About 100 mg of each sample were used for the extraction, after grinding in liquid nitrogen. DNA concentration was determined spectrophotometrically (Epoch 2 Microplate spectrophotometer 100-900nm BioTek UK). All samples were tested neat and diluted 10^{-1} in dH_2O .

2.3 PCR Assay

The protocol was an in-house established RT-PCR assay (Scaravelli et al., 2008) using the primers Arah2R 5'-GAA GCT CCT CGT CGT ACA, Arah2F 5'-TGT TTT TCC CTC ACT ACA ACA and TaqManProbe: FAM-ACT TCG ACG TGA GCC CGT CCC CTC -TAMRA; RT targets the Arah 3 gene amplifying a 147 bp fragment. Reactions were performed in a 25 μL final volume, containing 12.5 μL of Master Mix (KAPA Probe Fast qPCR, KAPA BIOSYSTEMS), 0.9 μM of each primer, 0.25 μM of TaqMan Probe and 7.5 μL of eluted DNA to make up for 25 μL . Amplification conditions consisted of a 10 min initial denaturation step at 95°C , followed by 40 cycles of 15 s denaturation at 95°C , 60 s annealing and elongation at 60°C .

2.4 Standard Curves for Real-Time PCR Analysis

The Step One plus™ RT-PCR System (Applied Biosystems) was used for the PCR assay. RT fluorescence measurements were compiled in every cycle. All reactions included negative controls containing the amplification master mix and dH_2O that was used for reagent preparation.

For positive controls and DNA quantification, a standard curve was designed using known concentrations (KC) of DNA extracted from peanut seeds ($\text{KC}_1=70 \text{ ng}/100 \text{ mg}$ of food, $\text{KC}_2=7.0 \text{ ng}/100 \text{ mg}$ of food, $\text{KC}_3=0.7 \text{ ng}/100 \text{ mg}$ of food, $\text{KC}_4=70 \text{ pg}/100 \text{ mg}$ of food, $\text{KC}_5=7.0 \text{ pg}/100 \text{ mg}$ of food, $\text{KC}_6=700 \text{ fg}/100 \text{ mg}$ of food). A cycle threshold value (Ct) was defined as the cycle of the RT-PCR at which a significant increase in fluorescence was detected in comparison to the negative control and the blanks were detected; this increase is associated with an exponential growth of PCR product during the log-linear phase. RT-PCR runs were acceptable only when the negative control had an undetectable Ct, the KC_2 and KC_3 had Cts between 25 and 27, and the efficiency of the PCR was 90-100%. All samples were tested neat and diluted 10^{-1} in dH_2O for the detection of inhibition.

Inhibition was defined as a positive PCR result with a diluted specimen, while a negative PCR result was obtained with the specimen tested undiluted.

3. Results and Discussion

3.1 DNA Extraction and Quantification

The DNA extraction methods critically affect PCR sensitivity. The optimization of these methods can improve the detection and quantification ability of peanut traces in consumed foods. In order to achieve this, two different extraction methods were tested (on the quality and quantity of the extracted DNA) and the NucleoSpin Food kit was selected for peanuts kernels. About 70 ng of genomic DNA starting from 100 mg of food (peanut kernels and commercial food samples) were obtained with the NucleoSpin Food kit. RT-PCR assay did not produce any results using the extracted DNA from the hazelnut and sesame seeds, whereas a positive signal was detected using the DNA from the peanut kernels. Based on the DNA quantification performed, reproducible analytical sensitivities of RT were 700 fg/100mg of food/ Out of the 152 food products tested, a total of 38 (25%) specimens contained peanut as an ingredient (Category I), 30 (19,7 %) specimens declared to contain “traces” of peanuts (Category II), 26 (17.1%) samples were labeled with “may contain “traces” of nuts (Category III) while 58 (38,2%) specimens did not feature allergy labels. Among them using the RT-PCR, 38 (100%), 25 (83,3%), 13 (50%) and 35 (60,3%) samples were detected as positive, respectively. Results for the positive samples for RT-PCR assay of the specimens that contained no food allergy labels (Category III and IV) showed that snacks are more contaminated. More specifically from the specimens that didn't have a declaration for peanuts 48 (57,1%) were positive with the RT-PCR assay (Table 1).

Table 1. Results of the positive samples for RT-PCR assay of the specimens that contained no food allergy labels (Category III and IV)

| No | Specimen | RT-PCR | RT-PCR | |
|-------|------------|----------|----------------------------|--------------------------|
| | | | DNA yield (ng/100 mg food) | Mean Value Ct Mean Value |
| 1-5 | Wafers | Positive | 0.122 | 27.12 |
| 6-10 | Wafers | Positive | 0.656 | 26.02 |
| 11-15 | Snacks | Positive | 0.784 | 25.00 |
| 16-20 | Snacks | Positive | 0.655 | 26,00 |
| 21-25 | Snacks | Positive | 0.056 | 28.58 |
| 26-30 | Biscuits | Positive | 0.122 | 27.12 |
| 31-35 | Biscuits | Positive | 0.656 | 26.02 |
| 36-40 | Cereals | Positive | 0.056 | 28.58 |
| 41-48 | Chocolates | Positive | 0.056 | 28.58 |

When diluted specimens were examined, 10 inhibition cases were detected. In particular, in 10 samples a negative PCR result was obtained when they were tested undiluted while when diluted 10^{-1} in dH₂O they were defined as positive. RT-PCR identified all 38 specimens containing peanut as an ingredient. RT-PCR technology has been extensively evaluated for food allergens. More specifically, it has been used for the direct detection of allergenic substances in food, using technologies like SYBR Green, and hydrolysis TaqMan probes. RT-PCR is rapid, no post-PCR processing is necessary, and both amplification and detection are performed in a single closed tube, thus minimizing the risk of carry over or cross-contamination. Quantification is another potential advantage of RT-PCR protocols, which nevertheless needs to be further evaluated, in order to reach any definite conclusions regarding the improvement of detection of allergen traces. Analytical sensitivities of the RT-PCR assay tested are shown in Table 2.

Table 2. Analytical sensitivities of the RT- PCR assay studied

| <i>Food specimens</i> | <i>PCR assay</i> | <i>(%) positive samples</i> |
|---|------------------|-----------------------------|
| Specimens containing peanut (N=38) | RT-PCR | 100% |
| Specimens that may contain “traces” of peanut (N=30) | RT-PCR | 83% |
| Specimens that may contain “traces” of nuts (N=26) | RT-PCR | 50% |
| Specimens containing no food allergy labels (N=58) | RT-PCR | 60.3% |

In order to evaluate the repeatability and reproducibility of the method, 5 reference samples at a concentration of 1.5 ng/μL were randomly chosen as PCR templates and amplified in triplicate in an experiment performed 3 times. The results of the TaqMan assay showed that the coefficient of variation (CV%) values for both

intraexperimental and interexperimental data ranged from 0.45 to 0.80% and 0.23 to 0.71% respectively (Table 3). These results, as simply measures of dispersion of the variable, suggest that the method presents good repeatability and reproducibility.

Table 3. Coefficient of variation values for both intra- and interexperimental test results of 5 randomly chosen samples

| Sample | Experiment 1 | | Experiment 2 | | Experiment 3 | | Intraexperimental | |
|--------|--------------|------|--------------|------|--------------|------|-------------------|------|
| | Mean Ct | CV % | Mean Ct | CV% | Mean Ct | CV% | Mean Ct | CV% |
| 1 | 25.72 ±0.15 | 0.58 | 25.45 ±0.18 | 0.71 | 25.68 ±0.10 | 0.39 | 26.89 ±0.12 | 0.45 |
| 2 | 24.74 ±0.10 | 0.40 | 24.00 ±0.08 | 0.33 | 26.10 ±0.12 | 0.46 | 24.98 ±0.20 | 0.80 |
| 3 | 25.99 ±0.08 | 0.31 | 24.45 ±0.15 | 0.61 | 25.38 ±0.18 | 0.71 | 26.58 ±0.16 | 0.60 |
| 4 | 26.57 ±0.06 | 0.23 | 26.95 ±0.20 | 0.74 | 25.52 ±0.15 | 0.59 | 25.99 ±0.18 | 0.69 |
| 5 | 26.01 ±0.14 | 0.54 | 26.45 ±0.18 | 0.68 | 26.58 ±0.20 | 0.75 | 25.75 ±0.15 | 0.58 |

From the few reports in the literature on the detection and quantification of peanut, the first PCR method for the detection of peanut was published in 2003 (Hird et al., 2003). In this publication RT-PCR enabled the detection of peanuts as low as 2 mg kg⁻¹ in spiked biscuits. Also, two real time PCR assays have been published and the reported limits of detection were in the range of those that can be obtained by means of ELISA, around 1 or 2 mg kg⁻¹ (Stephan & Vieths, 2004, Hird et al., 2003). Commercial PCR based kits for the detection of peanuts are currently available as well. These kits are either in the format of a RT-PCR kit, a PCR combined with ELISA detection, or conventional PCR followed by gel electrophoresis analysis. Such products usually claim to be able to detect 10 mg/kg peanut in food products. Hird et al. 2003 reported a RT-PCR assay and presented data related to dilutions of pure peanut DNA extract and several real food samples, while Stephan & Vieths, 2004 have shown a comparison between samples analyzed by ELISA and a RT-PCR method. They both reported that out of the 33 products collected, 4 including no peanuts in the list of ingredients, contained peanut protein in a range of 1 to 74 ppm and that the results of both tests correlated well. The RT-PCR was able to detect one more positive sample than the sandwich ELISA. In conclusion, both assays are sensitive and specific tools for the detection of hidden allergens in processed foods. Watanabe et al., 2006 have reported a conventional PCR method combined with gel electrophoresis. In all those cases PCR was only used as a qualitative method for peanut detection in food (for RT-PCR results Ct values are given, but peanut content was not determined).

The risk of the presence of food allergens is not only associated with the use of specific foods as ingredients; allergenic residues can also be present through cross-contamination from other products or the equipment used. Food industry has a responsibility to produce foods that are safe for all consumers including food allergic people. Although labelling information such as “it may contain traces..” are not obligatory by EU Regulation 1169/2011, it is very common that food companies are using such claims as a precaution. Food labelling is very important because even though they are cases that labelling prevents susceptible customer to purchase a safe product, in most cases it helps identifying allergenic ingredients that can cause life-threatening situations, since even small traces are unsafe for very susceptible individuals. Improved allergen traceability through the food chain may aid consumers to avoid allergen consumption. As demonstrated, the presented PCR assay is highly sensitive and selective, which makes it suitable for the detection of small amounts of peanut traces in food samples. Moreover, it can also be useful for monitoring the effectiveness of the cleaning processes in the production units of the food industry.

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