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# Food Safety and Label Claims for Hazelnut Allergy Traces: Evaluation of Two PCR Assays

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**Abstract**

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The molecular techniques (C-PCR, RT-PCR) in the detection and quantification of allergic substances of hazelnut in various categories of food commodities, e.g. breakfast cereals, chocolates and biscuits, frequently involved in allergic outbreaks was implemented. For the detection of hazelnut a gene coding the major allergenic protein Cor a1 was selected. In some instances, the presence of hazelnuts is not declared on the label and the products may carry no warning for potentially allergenic substances, usually referred to as "traces". A total of 150 samples were collected from local supermarkets and analysed for the purpose of the study. From these, a total of 38 (25.3%) specimens contained hazelnut, 30 (20.0%) contained "traces" of hazelnut, 26 (17.3%) contained a label warning for the possible presence of "traces" of allergenic substances, and 56 (37.3%) specimens contained no food allergy labels. Among them, using the C-PCR, 36 (94.7%), 10 (33.3%), 5 (19.2%), and 5 (8.9%) specimens were detected as positive, respectively. Using the RT-PCR, 38 (100%), 15 (50%), 7 (26.9%) and 8 (14.3%) specimens were detected as positive, respectively. Finally, by combining both methods, 38 (100%), 17 (56.7%), 9 (34.6%), and 10 (17.9%) specimens were identified as positive, respectively.

**Keywords**: amplification; declaration; food allergy; hazelnut

Among the most serious food safety problems that raise concerns from consumers are food allergies. These are the clinical manifestation of an immunological process in which certain food ingredients (mainly proteins) or their metabolic derivatives act as antigens and stimulate the production of antibodies against them (Sampson 2004). Hazelnuts (*Corylus avellana*) are among the common tree nuts that lead to allergic reactions. More specifically, hazelnuts include allergenic seed storage proteins of the Cupin and Prolamin superfamilies (Cor a9 and Cor a8) (Murzin *et al*. 1995; Crespo *et al*. 2006): Cor a1 is the 18 kDa major hazel pollen allergen, while Cor a2 is a 14kDa profilin, known as a cross-reacting plant pan-allergen, found in both hazel pollen and hazelnuts (Hirschwehr *et al*. 1992). In a multicentre study performed in Denmark, Switzerland, and Italy, Cor a1 was identified as the major hazelnut allergen in 65 European patients sensitized to birch pollen whose food allergy to hazelnut was confirmed by a positive DBPCFC (double-blind placebo-controlled food challenge) (Pastorello *et al*. 2002). Although the risk of allergic reaction to certain proteins normally requires their presence in foods in significant quantities, there are foods in which the adverse reaction could be caused by very small concentrations of the allergenic proteins (Lidholm *et al*. 2006).

Recent studies aim to detect low concentrations of allergenic substances in foods which may have been cross-contaminated during their preparation (Steinman 1996). The implementation of immune enzymatic methods like ELISA, and the applica-

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tion of genetic mechanics (PCR and Real-time PCR) under this context represent a special challenge (Yeung & Collins 1996; Holzhauser & Vieths 1999; Koppelman *et al.*1999; Holzhauser *et al*. 2000; Poms *et al*. 2004; Schoringhumer *et al*. 2007; Pedersen *et al*. 2008). Several papers reported on the development of ELISAs to detect traces of hazelnut in food with the target molecule for the first main distinction being either a protein or DNA (Holzhauser & Vieths 1999; Rejeb *et al*. 2003). Holzhauser and Vieths (1999) developed a hazelnut-specific sandwich-type ELISA based on polyclonal antibody with a 2 ppm LOD (limit of detection). Also, Pele *et al*. (2007) reported an ELISA kit for hazelnut detection with a 1.5 ppm LOD. These immunochemical methods are sensitive, but they may suffer from cross-reactivity with other allergenic tree nuts. However, the detection of hazelnut proteins is compromised by the fact that the food industry utilises a number of different ingredients and furthermore it employs a variety of food processing methodologies like heat treatment or roasting which can modify, denature, and degrade food proteins.

Therefore, detection of allergenic or marker proteins is not necessarily the only way to demonstrate the presence of an allergic compound, and the detection of another type of marker molecule like DNA can be an alternative method (Herman *et al*. 2003; Piknová *et al*. 2008). Arlorio *et al*. (2007) proposed a method to detect hazelnut in processed foods through the use of both TaqMan and SYBR Green chemistry, achieving a limit of detection of 0.1 ng of genomic DNA.

The aim of the present study is the implementation of sensitive and conventional PCR (C-PCR) and Real-time PCR (RT-PCR) in the detection and quantification of allergic hazelnut traces in various kinds of consumer goods, in order to protect the health of allergic consumers.

## MATERIAL AND METHODS

***Food collection***. A total of 150 widely consumed goods, which are potential carriers of allergic food substances, were collected from local supermarkets and studied. More specifically, 28 cereal products, 17 chocolates, 31 biscuits, 25 wafers, and 49 snack products were collected. Out of the 150 food products tested, a total of 38 (25.3%) specimens contained hazelnut as an ingredient (Category I), 30 (20.0%) specimens declared to contain "traces" of hazelnuts (Category II), 26 (17.3%) samples were labelled with "may contain traces of nuts" (Category III) while 56 (37.3%) specimens did not feature any allergy labels (Category IV). Hazelnut seeds were also used as positive controls while peanuts, walnuts, and sesame seeds were selected as negative samples.

***Genomic DNA extraction and quantification***. The NucleoSpin Food kit (Macherey-Nagel, GmbH & Co. KG, Düren, Germany) and the Bioo Scientific (Austin, USA) were used for the extraction of hazelnut, peanut, walnut, and sesame seeds in commercial foods in order to compare the extraction yield and cleanup of the genomic DNA. All extraction methods were applied according to the manufacturer's instructions except the incubation step which was overnight at 65°C. About 200 mg of each sample were used for the extraction, after grinding under liquid nitrogen in order to have a fine powder. DNA concentration was determined spectrophotometrically. DNA was quantified by measuring the absorbance at 260 nm and 280 nm. DNA concentration was calculated according to the equation C (μg/ml) = $A_{260}$ × 50 × dilution factor. The $A_{260}/A_{280}$ ratio was used to assess the quality of isolated DNA. All samples were tested neat and diluted $10^{-1}$ in $dH_2O$

***PCR assays***. The first PCR protocol was a previously reported (Schoringumer *et al*. 2009) conventional assay (C-PCR) amplifying a 147 bp fragment coding the *Cor a1* gene. Amplification was performed in a 50 μl volume reaction containing 25 μl of MasterMix (10×) Hot Start DNA Polymerase; (PROMEGA, Wisconsin, USA), 0.4 μM of each primer Cora1F 5'-GGCTAGGCTGTTCAAGAGG-3', Cora1R 5'-AGAAATTAACCTTCATCGAAACAG-3', 15 μl of eluted DNA, and $dH_2O$ to make up for 50 μl. Amplification conditions consisted of an initial 5 min denaturation step at 95°C, followed by 40 cycles of 60 s, denaturation at 95°C, 1 min annealing at 57°C, and 1 min extension at 72°C, and finally a 10 min extension step at 72°C. PCR products were separated in a 3% agarose gel, stained with ethidium bromide (0.5 μg/ml), and documented under UV illumination.

The second protocol was also a previously reported (Schoringumer *et al*. 2009) in-house established RT-PCR assay using the primers Cora1F2 5'-ACTACATAAAGCAAAAGGTTGAAG-3', Cora1R2 5'-TCGTAATTGATTTTCTCCAGTTTG-3' and TaqMan Probe: FAM 5'-CGGACAAAGCATCGCCTTCAATCABHQ2. RT was targeting the Cora1 gene amplifying a 109 bp fragment. Reactions were performed in a 25-μl final volume, containing 12.5 μl of Master Mix (KAPA

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Probe Fast qPCR; KAPA BIOSYSTEMS, Wilmington, USA), 0.8 µM of each primer, 0.2 µM of TaqMan Probe, and 8 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 35 cycles of 15 s denaturation at 95°C and 1 min annealing at 60°C.

***Creation of standard curves for RT-PCR analysis.*** The Step One Plus™ RT-PCR System (Applied Biosystems, Waltham, USA) was used for the RT-PCR assay. RT-PCR fluorescence measurements were compiled in each 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 hazelnut seeds ($KC_1$ = 13 ng/100 mg of food, $KC_2$ = 1.3 ng/100 mg of food, $KC_3$ = 0.13 ng/100 mg of food, $KC_4$ = 13 pg/100 mg of food, $KC_5$ = 1.3 pg/100 mg of food, and $KC_6$ = 130 fg/100 mg of food). The cycle threshold value (Ct) was defined as the cycle of the RT-PCR at which a significant fluorescence increase in comparison with the negative control and the blanks was detected, an increase associated with the exponential growth of PCR product during the log-linear phase. This increase was calculated using the equation DRn = $Rn^+ – Rn^–$, where: $Rn^+$ – fluorescence of the sample of interest containing all components at any given time after the onset of the reaction; $Rn^–$ – fluorescence of the same sample detected in baseline value at the beginning of the reaction. DRn, which is the difference between $Rn^+$ and $Rn^–$, serves as an indicator of the magnitude of the signal generated by the PCR, and when plotted against the cycle numbers, it produces the amplification curves and gives the Ct. The slope of the amplification curve during the log-linear phase was used to calculate the amplification efficiency (Eff), using the formula: $Eff = 10^{(-1/slope)} – 1$. RT-PCR runs were acceptable only when the negative control had an undetectable Ct, the $KC_2$ and $KC_3$ had Ct 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.

In order to evaluate the repeatability and reproducibility of the methods according to ISO 5725-2: 1994, 5 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.

## RESULTS AND DISCUSSION

***DNA extraction and quantification.*** Optimisation of the genomic DNA extraction from a fat-rich and polyphenol-rich food such as hazelnut was the first issue of this work since DNA extraction methods critically affect PCR sensitivity. In order to achieve this, two different extraction methods were tested (data not shown) and the NucleoSpin Food kit was finally selected for hazelnut seeds.

***Dynamic range, analytical sensitivity, and specificity***. Neither C-PCR nor RT-PCR protocols produced any results using the extracted DNA from the sesame, walnut and peanut seeds, whereas a positive signal was detected with both protocols using the DNA from the hazelnut seeds. Based on the DNA quantification performed, reproducible analytical sensitivities of RT-PCR were 130 fg/100 mg of food according to the standard curve.

Out of the 150 food products tested using the C-PCR, 36 (94.7%) from Category I, 10 (33.3%) from Category II, 5 (19.2%) from Category III and 5 (8.9%) specimens from Category IV were detected as positive. Using the

Table 1. Results of positive samples for C-PCR and RT-PCR assays of the specimens labelled with "may contain traces" of hazelnut (Category II)

| No. | Specimen | C-PCR | RT-PCR | |
|---|---|---|---|---|
| | | | DNA yield (ng/100 mg food) | Ct |
| 1 | wafer 3 | positive | 0.656 | 26.02±0.11 |
| 2 | wafer 15 | positive | 0.656 | 26.02±0.12 |
| 3 | wafer 10 | positive | negative | – |
| 4 | wafer 11 | positive | negative | – |
| 5 | wafer 19 | negative | 0.122 | 27.12±0.09 |
| 6 | biscuit 21 | positive | 0.122 | 27.12±0.10 |
| 7 | biscuit 25 | positive | 0.656 | 26.02±0.10 |
| 8 | biscuit 27 | positive | 0.656 | 26.02±0.10 |
| 9 | cereal 9 | negative | 0.056 | 28.58±0.11 |
| 10 | cereal 11 | positive | 0.656 | 26.02±0.08 |
| 11 | snack 12 | positive | 1.589 | 24.12±0.09 |
| 12 | snack 13 | positive | 0.145 | 27.02±0.10 |
| 13 | snack 21 | negative | 0.122 | 27.12±0.11 |
| 14 | chocolate 1 | negative | 0.056 | 28.58±0.11 |
| 15 | chocolate 2 | negative | 0.056 | 28.58±0.10 |
| 16 | chocolate 3 | negative | 0.056 | 28.58±0.10 |
| 17 | chocolate 4 | negative | 0.056 | 28.58±0.11 |

Ct – cycle threshold value

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Table 2. Results of the positive samples for C-PCR and RT-PCR assays of the specimens labelled with "may contain traces" of nuts (Category III)

| No. | Specimen | C-PCR | RT-PCR | |
|---|---|---|---|---|
| | | | DNA yield (ng/100 mg food) | Ct |
| 1 | wafer 1 | positive | 0.656 | 26.02 ± 0.10 |
| 2 | wafer 2 | positive | 0.656 | 26.02 ± 0.10 |
| 3 | snack 1 | positive | negative | – |
| 4 | snack 2 | positive | negative | – |
| 5 | snack 3 | negative | 0.056 | 28.58 ± 0.11 |
| 6 | biscuit 1 | negative | 0.122 | 27.12 ± 0.10 |
| 7 | biscuit 2 | positive | 0.656 | 26.02 ± 0.12 |
| 8 | biscuit 3 | negative | 0.128 | 27.00 ± 0.10 |
| 9 | cereal 1 | negative | 0.122 | 27.12 ± 0.10 |

Ct – cycle threshold value

RT-PCR, 38 (100%), 15 (50%), 7 (26.9%), and 8 (14.3%) samples were detected as positive, respectively for each category. When diluted specimens were examined, 10 inhibition cases were detected (6 for C-PCR and 4 for RT-PCR). More specifically, in 10 samples that contained cocoa a negative effect on amplification result was obtained when they were tested undiluted while when diluted $10^{-1}$ in $dH_2O$ they were defined as positive.

Analytical DNA quantification of the positive specimens is shown in Tables 1–3. Both C-PCR and RT-PCR resulted in a high overall sensitivity (94.7 and 100%): RT-PCR identified all 38 specimens containing hazelnut as an ingredient and resulted in a higher specificity than C-PCR. RT-PCR technology has been extensively applied for detection purposes. More specifically, it has been used for the direct detection of allergenic substances in food, using the technology with TaqMan hydrolysis probes. Nevertheless, the overall sensitivity of the RT-PCR protocol reported in this study did not differ much from the sensitivities reported for C-PCR assays. This was also the case in this work (equal results between RT-PCR and C-PCR), indicating that increased sensitivity is not always the main advantage of RT-PCR assays over conventional ones, but rather rapid reporting as a result of faster turnaround times. In addition, no post-PCR processing is necessary since both amplification and detection are performed in a single closed tube, thus minimising the risk of carryover 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 potentially allergen traces.

Table 3. Results of the positive samples for C-PCR and RT-PCR assays of the specimens that contained no food allergy labels (Category IV)

| No. | Specimen | C-PCR | RT-PCR | |
|---|---|---|---|---|
| | | | DNA yield (ng/100 mg food) | Ct |
| 1 | wafer 2 | positive | 0.122 | 27.12 ± 0.12 |
| 2 | wafer 5 | positive | 0.656 | 26.02 ± 0.11 |
| 3 | snack 6 | positive | negative | – |
| 4 | snack 7 | positive | negative | – |
| 5 | snack 15 | negative | 0.056 | 28.58 ± 0.10 |
| 6 | biscuit 12 | negative | 0.122 | 27.12 ± 0.09 |
| 7 | biscuit 17 | positive | 0.656 | 26.02 ± 0.10 |
| 8 | biscuit 22 | negative | 0.056 | 28.58 ± 0.12 |
| 9 | cereal 5 | negative | 0.056 | 28.58 ± 0.11 |
| 10 | cereal 7 | negative | 0.056 | 28.58 ± 0.11 |

Ct – cycle threshold value

For the analysis of the combination of C-PCR+RT, specimens found positive with either of the two assays were characterised as positive. Using this approach, 38 (100%), 17 (56.7%), 9 (34.6%), and 10 (17.9%) of the specimens were identified as positive, respectively. The combination of both PCR protocols increased specificity because the RT-PCR protocol missed 6 specific samples that were detected as positive only by C-PCR. Analytical specificities of

Table 4. Analytical sensitivities of the two PCR assays studied and their combination

| Food specimens | PCR assay | Positive samples (%) |
|---|---|---|
| Specimens containing hazelnut ($N$ = 38) | C-PCR | 94.7 |
| | RT-PCR | 100 |
| | C-PCR+RT | 100 |
| Specimens that may contain "traces" of hazelnuts ($N$ = 30) | C-PCR | 33.3 |
| | RT-PCR | 50.0 |
| | C-PCR+RT | 56.7 |
| Specimens that may contain "traces" of nuts ($N$ = 26) | C-PCR | 19.2 |
| | RT-PCR | 26.9 |
| | C-PCR+RT | 34.6 |
| Specimens containing no food allergy labels ($N$ = 56) | C-PCR | 8.9 |
| | RT-PCR | 14.3 |
| | C-PCR+RT | 17.3 |

doi: 10.17221/602/2014-CJFS

Table 5. Coefficients of variation for both intra- and inter-experimental test results of 5 randomly chosen samples

| Sample | Experiment 1 | | Experiment 2 | | Experiment 3 | | Intra-experimental | |
|---|---|---|---|---|---|---|---|---|
| | 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 |

Ct – cycle threshold value; *CV*% – correlation coefficient (%)

the two assays tested as well as of their combinations are shown in Table 4.

***Repeatability and reproducibility of assays.*** The C-PCR assay showed the same positive results for both intra- and inter-experimental data. The results of the RT-PCR assay showed that the coefficient of variation values for both intra- and inter-experimental data ranged from 0.45% to 0.80% and 0.23% to 0.71%, respectively (Table 5). These results suggest that the method presents good repeatability and reproducibility. From the few reports in the literature on the detection and quantification of hazelnut, in particular, in a variety of consumer goods, the sensitivity levels of the methods developed based on conventional RT-PCR assays ranged from 1 ppm to 100 ppm for the *Cor a1* gene (Ehlert *et al*. 2008; Schoringumer *et al*. 2009; Koppel *et al*. 2010; Słowianek & Majak 2011). Therefore, the obtained LOD according to the proposed RT-PCR assay targeting the gene encoding the *Cor a1* gene allergen is 130 fg/100 mg. In addition, both proposed PCR assays for the specific detection of hazelnut did not present any cross-reactivity with other nuts tested. Finally, it was reported in another study (López-Calleja *et al*. 2013) that the analysis of PCR results, for a collection of 179 commercial food products, returned positive for 40 products that did not declare hazelnut or its traces on their label.

## CONCLUSIONS

The food industry has a responsibility to produce foods that are safe for all consumers including people with food allergy. Improved allergen traceability through the food chain may provide sufficient protection to consumers susceptible to food allergies. As demonstrated, the presented PCR methods are highly sensitive and selective, which makes them suitable for the detection of small amounts such as fg/100 mg food of hazelnut traces in food commodities. The combination of the two methods results in a higher sensitivity. The availability of multiple methods for the detection of hazelnut traces in food products is of paramount importance to protect the hazelnut allergic individuals, and has identified a multitude of chocolates and cookies for which hazelnut was not referred as ingredient and tested positive. Moreover, these methods can also be useful for inspecting the effectiveness of the production lines between different products processing in the food industry. The detection of sometimes very high levels of hazelnut in market samples stresses the need for such methods and for the analysis of food products that might endanger the health of allergic individuals.

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