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# Quantitative Detection of Hazelnut (*Corylus avellana*) in Cookies: ELISA versus Real-Time PCR

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**ABSTRACT:** Hazelnuts (*Corylus avellana*) are used widely in the food industry, especially in confectionery, where they are used raw, roasted, or in a processed formulation (e.g., praline paste and hazelnut oil). Hazelnuts contain multiple allergenic proteins, which can induce an allergic reaction associated with symptoms ranging from mild irritation to life-threatening anaphylactic shock. To date, immunochemical (e.g., ELISA or dipstick) and PCR-based analyses are the only methods available that can be applied as routine tests. The aim of this study is to make a comparative evaluation of the effectiveness of ELISA and real-time PCR in detecting and correctly quantifying hazelnut in food model systems. To this end, the performances of two commercial ELISAs were compared to those of two commercial and one in-house-developed real-time PCR assays. The results showed that although ELISA seemed to be more sensitive compared to real-time PCR, both detection techniques suffered from matrix effects and lacked robustness with regard to food processing. As these impacts were highly variable among the different evaluated assays (both ELISA and real-time PCR), no firm conclusion can be made as to which technique is suited best to detect hazelnut in (processed) food products. In this regard, the current lack of appropriate DNA calibrators to quantify an allergenic ingredient by means of real-time PCR is highlighted.

**KEYWORDS:** food allergy, hazelnut, *Corylus avellana*, ELISA, real-time PCR, food processing

## INTRODUCTION

Hazelnuts are the kernels of the hazel (*Corylus*) tree. The multiple varieties of hazel trees all produce edible nuts, but the common hazel (*Corylus avellana*) is the most extensively grown for its nuts. Hazelnuts are one of the most nutritious nuts, with a protein content of about 12%. They are also a good source of energy, with a fat content of about 60%, and an excellent source of carbohydrates, among which half are dietary fiber. Hazelnuts also contain minerals (Ca, Mg, P, K), vitamin E, B vitamins, and antioxidants. Their flavor makes them popular for use in food, especially in confectionery, where they are often found in pastries, chocolate spreads, ice cream, cereal bars, cookies, nougat, etc. Hazelnuts are consumed raw or roasted, intact, chopped, or processed into a praline paste. Hazelnuts are usually processed integrally into food products, although hazelnut oil is also often used for cooking.

The prevalence of allergy to tree nuts is estimated to be around 0.5%.[1] Between 21 and 53% of all food allergic patients in Europe suffer from hazelnut allergy in particular.[2] The minimum doses that can elicit an allergic reaction can be as low as 1 mg of hazelnut protein up to 30−100 mg, which correspond to 6.4 and 190−640 mg of hazelnut.[3]

Allergenic ingredients, such as hazelnuts, must be labeled on prepackaged food products according to Directive 2003/89/EC and amended by Directive 2007/68/EC.[4] To ensure that food production processes conform to this legislation, the food industry needs reliable allergen detection methods. The authorities also need such detection methods to check for inaccurate labeling. Routine analysis methods for food allergens presently consist of immunochemical (e.g., ELISA) and PCR-based detection techniques. These methods are expected to correctly quantify the amount of allergenic ingredient in both raw and processed food products. Moreover, analytical results obtained with both detection methodologies should be in accordance with each other. Although several of these methods are readily available on the market, profound knowledge about their performance and robustness is still lacking.

Until now, few studies have made a comparative evaluation of the different platforms for allergen detection. Stephan et al.[5] compared an in-house-developed ELISA and real-time PCR assay for the detection of peanut, but that evaluation was only qualitative. A similar qualitative comparison of commercial ELISAs, immunoblotting, MS analysis, and PCR analysis for egg detection was performed by Lee and Kim.[6] Moreover, these studies have applied retail samples for their analyses. Until now, only one study, a case study on peanut, has performed a comparative investigation of the impact of food processing on allergen detection with ELISA and real-time PCR.[7]

The aim of the current work is to study the detection of hazelnut in food model systems by means of ELISA (two commercial assays) and real-time PCR (two commercial and one in-house-developed assays) and to investigate the influence on detection of the matrix and food processing. Food processing can modify the tertiary structure or intactness of proteins/allergens or lead to polymerization or aggregation. In recent studies, we have determined the influence of these modifications on the interaction of the proteins with antibodies in ELISA.[8,9] Food production can also affect the integrity of the DNA, which could

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impair the PCR reaction. Apart from the structural changes that can be induced on the analytical molecules, there is evidence that the solubility can also be affected.[10] However, despite the myriad nature of the modifications of the target analytes induced by food processing, only a few investigations have been reported on the influence of food processing on analyte detection.

This study was designed to investigate to what extent hazelnut contamination of wheat flour, the main ingredient of cookies, can be tracked down during the cookie production process. Previous reports about the impact of food processing on the detection of allergens looked solely at the heating step, for example, the difference in detection between dough and baked cookies.[7,11] Apart from examining the influence of the baking process, we have also examined the influence of the addition of different ingredients to the wheat flour. The basic model system is wheat flour amended at various levels with defatted hazelnut powder (matrix 1). The flour was then used to prepare other food matrices. The different food samples were analyzed using ELISA and real-time PCR to evaluate the effectiveness of both detection platforms to detect and/or quantify hazelnut in food as a function of the various steps in the production process.

## MATERIALS AND METHODS

**Real-Time PCR Development.** A new real-time PCR targeting the gene encoding for the hazelnut allergen *Cor a* 8 was developed. Primers and probe were designed on the gene DNA sequence (accession no. AF329829) using the Primer 3 tool available on http://frodo.wi.mit.edu/primer3/.[12] The software parameters were set to design primers with an optimal melting temperature ($T_m$) of 60 °C, to have a GC content between 40 and 60%, and to be minimum 18 base pairs (bp) long. The probe was designed to have an optimal $T_m$ of 70 °C and a length of 18−30 bp. From the output those primers and probe were selected which contained more C's than G's, with no G at the 5′-end and which preferably contained no repetitions of more than two equal bases. This resulted in the following oligonucleotides: forward primer, 5′-TGCGTGCTCTACCTGAAGAA-3′; reverse primer, 5′-GTGGAGG-GGCTGATCTTGTA-3′; probe, 5′-FAM-ACCGCCAGTCCGCTTG-CAAC-TAMRA-3′. Primers and probe were synthesized and RP-HPLC purified by Eurogentec (Liège, Belgium).

PCR reactions were performed in a reaction volume of 25 μL containing 2.5 μL of template DNA, 1× Real-Time PCR Mastermix (Diagenode), 300 nM forward primer, 300 nM reverse primer, and 200 nM probe. PCR reactions were performed in an ABI Prism 7000 Sequence Detection System (Applied Biosystems). Each run was initiated by a decontamination reaction from dUTP-containing template at 50 °C for 2 min, followed by deactivation of the UNG and denaturation for 10 min at 95 °C. Each of the 45 cycles consisted of denaturation at 95 °C for 15 s and combined primer annealing and elongation at 60 °C for 60 s. Data were analyzed with 7000 System Sequence Detection Software, version 1.2.3 (Applied Biosystems).

**Preparation of Food Model Systems.** Nine different brands of hazelnuts, including virgin unpeeled hazelnuts and roasted peeled hazelnuts, were purchased in local Belgian supermarkets. A mixture of the hazelnuts was made by taking equal amounts of each kind. The mixture was frozen in liquid nitrogen and ground in a two-step process, first with a blender (Moulinex, France) and then with an Ultra Turrax t25 (IKA, Wilmington, NC).

To facilitate a homogeneous distribution of the ground hazelnuts in the food model systems, the ground nuts were defatted. To this end, 100 g of ground nuts was mixed with 1 L of technical hexane for 30 min, and after sedimentation of the ground nuts, the hexane was decanted. This step was repeated three times, and the residual solvent was evaporated overnight at ambient temperature. The loss of fat was determined by the weight difference of the ground nuts before and after defatting.

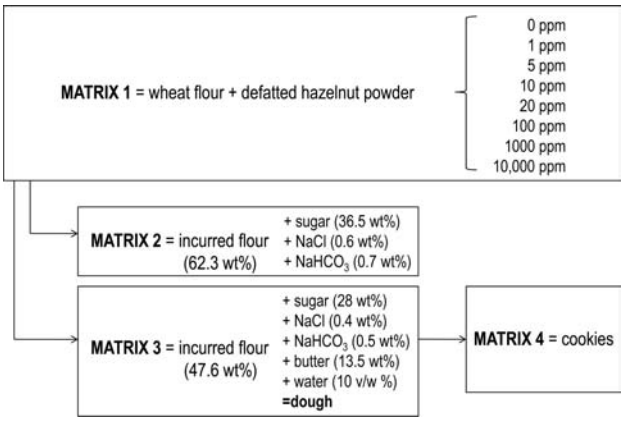


**Figure 1.** Schematic representation of the preparation of the different food model systems.

Four different food model systems containing defatted hazelnut powder were prepared. The basic model system consists of wheat flour amended with the defatted hazelnut powder (matrix 1, Figure 1). This flour was subsequently used to prepare the other matrices (Figure 1). The composition of the model systems is based on the cookie recipe of the AACC 10-50.05 method used to assess the baking quality of cookie flour.[13] The different solid components of the matrices (sugar and salts) were first ground into finer powder with a Retsch mill (ZM 200, Haan, Germany) using a sieve with an aperture size of 0.5 mm to improve homogeneous mixing of the matrix compounds.

The highest spike level of 10000 ppm was prepared by adding 10 g of defatted hazelnut powder to 990 g of wheat flour. The components were mixed thoroughly in a Kenwood kitchen mixer for 1 h at level 2, the mixing bowl being scraped every 10 min. Before dilution of the 10000 ppm flour to prepare the other spike levels, the homogeneity of the batch was evaluated. For this purpose, nine samples of 100 mg were taken from the 10000 ppm batch and DNA was extracted with the Qiagen DNeasy Plant mini kit. Samples were analyzed in triplicate with the in-house-developed real-time PCR assay targeting the gene encoding for *Cor a* 8. The real time PCR reactions were performed as described above. The other spike levels were prepared by serial dilution of the 10.000 ppm spiked flour with "blank" wheat flour through mixing as described above. To check if the applied procedure delivered an adequate dilution series of spiked hazelnut powder, three subsamples were taken of each concentration and DNA was extracted in duplicate (six samples in total) and analyzed in duplicate in the *Cor a* 8 PCR.

The batches of flour spiked with different concentrations of defatted hazelnut powder were used to prepare matrix 2 (62.3 wt % spiked wheat flour, 36.5 wt % sugar, 0.6 wt % NaCl, 0.7 wt % $NaHCO_3$) and matrix 3 (47.6 wt % spiked wheat flour, 0.4 wt % NaCl, 0.5 wt % $NaHCO_3$, 13.5 wt % butter, 10 v/w % water) (Figure 1). Matrix 2 (dry ingredients) was prepared by adding the sugar, NaCl, and $NaHCO_3$ to the flour in the bowl. The components were then mixed for 3 min at level 2, the bowl being scraped every minute. Matrix 3 (cookie dough) was prepared as described in the AACC 10-50.05 method. First, the butter was mixed with the sugar, NaCl, and $NaHCO_3$ during 3 min at level 2, the bowl being scraped every minute. Then the water was added and mixed with the other components for 1 min at level 1, the bowl was scraped and the dough mixed for another minute at level 2. Finally, the (spiked) flour was added to complete the dough and mixed with the rest for 2 min at level 1, the bowl being scraped every 30 s. The dough was divided into two parts. One part was used to analyze as such, and the other part was used to prepare matrix 4 (baked cookies). Cookies were made from each dough sample by rolling the dough on baking paper between two boards, and cookie shapes were cut out. The cookies were baked at 180 °C for 16 min, and the weight

**Table 1. Actual Concentrations (Parts per Million) of Defatted Hazelnut in Matrices 1-4 and the Corresponding Concentrations of (Undefatted) Hazelnut**

| matrix 1 | | matrix 2 | | matrix 3 | | matrix 4 | |
|---|---|---|---|---|---|---|---|
| defatted HN | HN | defatted HN | HN | defatted HN | HN | defatted HN | HN |
| 0 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| 1 | 2.58 | 0.62 | 1.61 | 0.48 | 1.23 | 0.51 | 1.31 |
| 5 | 12.90 | 3.12 | 8.04 | 2.38 | 6.14 | 2.55 | 6.57 |
| 10 | 25.80 | 6.23 | 16.07 | 4.76 | 12.28 | 5.09 | 13.14 |
| 20 | 51.60 | 12.46 | 32.15 | 9.52 | 24.56 | 10.19 | 26.29 |
| 100 | 258.00 | 62.30 | 160.73 | 47.60 | 122.81 | 50.94 | 131.43 |

was determined before and after baking to determine the moisture loss. The cookies were ground using a chopper (Philips, HR 1396 400W).

**Hazelnut Detection Using ELISA.** Samples of matrix 1 spiked with 0, 1, 5, 10, 20, and 100 ppm defatted hazelnut powder and the corresponding samples of matrices 2−4 were selected to be analyzed using ELISA. The samples were analyzed using two commercially available ELISA kits: Ridascreen FAST Hazelnut (R-Biopharm, Darmstadt, Germany) and BioKits Hazelnut Assay (Neogen Corp., Lansing, MI). Both assays are based on the sandwich ELISA principle. Three subsamples were taken of each sample, and proteins were extracted in duplicate (six samples in total) and analyzed in duplicate according to the procedure described by the manufacturer using the buffer solutions included in the kit. Absorbance measurements were made in a microtiter plate reader (Multiskan EX/355, Thermo Fisher Scientific, Aalst, Belgium). Each kit was calibrated using the respective standard protein solutions included in each kit. To fit the absorbance values to the protein concentration of the standard solutions, a four-parameter logistic dose−response curve was used.[14] Only the data obtained within the range of the respective standard curves were considered in the results.

To be able to calculate the ratio measured/spiked hazelnut, several conversions had to be made. First, the weight percentage of the spiked flour in the different matrices has to be taken into account to calculate the actual concentration of the spiked defatted hazelnut powder in the different matrices (Figure 1; Table 1) In the case of matrix 4 (cookies) the moisture loss after the dough had been baked was considered for these calculations. The measured amount of spiked hazelnut was determined using the constructed standard curves. To construct the standard curves, the calibrators included in the respective kits were used. These calibrators are expressed as a certain amount of (nondefatted) hazelnut. However, the samples prepared in this study have been spiked with different levels of defatted hazelnut powder (to facilitate spiking). Therefore, the concentrations of defatted hazelnut had to be additionally converted to the corresponding amount of (nondefatted) hazelnut, taking into account the weight difference between before and after defatting (Table 1).

**Hazelnut Detection Using Real-Time PCR.** Samples based on matrix 1 spiked with 0, 1, 10, 100, 1000, and 10.000 ppm defatted hazelnut powder were selected to be analyzed using real-time PCR. From each sample, three subsamples were taken from which DNA was extracted in duplicate (six samples in total) using the Qiagen DNeasy Plant mini kit. The samples were analyzed in duplicate using the in-house developed *Cor a* 8 PCR and two commercially available real-time PCR kits: the First Hazelnut kit (Gen-Ial, Troisdorf, Germany) and the Surefood Hazelnut kit (R-Biopharm). Analysis using the *Cor a* 8 PCR was performed as described above, and the commercial assays were performed according to the manufacturer's instructions. A calibration curve was constructed by analysing a dilution series of genomic DNA (6.4, 26.6, 64, 160, 400, and 1000 pg) obtained by DNA extraction from defatted hazelnut powder. On the basis of the obtained $C_q$ values, the amount of DNA measured in the different samples was determined using the calibration curve. The obtained values were normalized to the corresponding amount in 100% of the spiked flour, taking into account the different weight percentages of the incurred flour in the various matrices (Figure 1) (e.g., 10 pg of DNA measured in a sample of matrix 3 would correspond to 3/0.623 = 4.8 pg of DNA if the matrix consisted of 100% spiked flour).

## RESULTS AND DISCUSSION

**Preparation of Model Systems.** As it is difficult to prepare low spike levels (e.g., 1 ppm) at laboratory scale, we chose to prepare a high spike level and dilute it further with blank (hazelnut-free) matrix. In the production of spiked material, important aspects are the homogeneity of the food allergen incorporation into the food matrix and the ability to clean the processing equipment thoroughly between each batch production to exclude cross-contamination. Between each mixing process, a wet cleaning with detergent was performed. To avoid contamination of the surrounding area, the mixing bowl was covered with plastic foil during mixing, which was discarded after each mixing process. To verify homogeneity, nine subsamples of the highest spike level (10000 ppm) were taken, and DNA was extracted and analyzed using the *Cor a* 8 PCR to determine whether the hazelnut powder was indeed equally distributed. No significant differences could be found between the mean $C_q$ values of the different samples, indicating that a homogeneous spiking was obtained and that the applied mixing procedure could be used to prepare the further dilutions. After the different spike levels were prepared, the dilution procedure was verified before the preparation of the other matrices was continued. Three subsamples were taken from the flour spiked at each level, and DNA was extracted in duplicate. Each DNA sample was analyzed in duplicate using the *Cor a* 8 PCR, and the $C_q$ values were plotted against the spiked concentration. A log−linear relationship exists down to the 10 ppm spike level (Figure 2). These results indicate that the followed procedure allows to establish an adequate dilution of the incurred hazelnut flour in blank flour. At the 10 ppm level only 75% of the samples were positive; this number decreased as the concentration of hazelnut decreased further. At higher incurrence levels, all samples were positive. These data already indicate that the *Cor a* 8 PCR is able to detect hazelnut in flour to about 10 ppm.

**Detection of Hazelnut in Food by ELISA.** Baking cookies is expected to result in chemical and structural protein modifications, as we previously demonstrated in experiments on buffered model systems of hazelnut proteins where the Maillard reaction was induced in a controlled manner.[8] In addition to the effects on the hazelnut proteins by processing, additional complex interactions with the other components present in hazelnuts and the

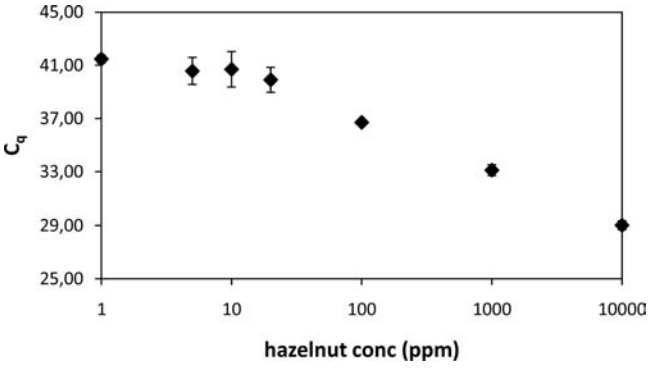


**Figure 2.** Analysis of different hazelnut spike levels in wheat flour with *Cor a* 8 PCR. Data are mean values ± SD of six samples analyzed in duplicate ($n$ = 12).

food matrix are expected. Consequently, in this experimental setup, the observed effects were a summation of physicochemical changes taking place during the different processing steps. We selected those matrices based on the flour containing 0, 1, 5, 10, 20, and 100 ppm defatted hazelnut powder, which are concentrations within or just above the calibration range of the ELISA kits used. The unit of the standards of these kits is a certain amount of hazelnut expressed in parts per million. However, our samples are spiked with defatted hazelnut powder, a more concentrated form of hazelnut due to the defatting. Therefore, to calculate the ratio measured/actual hazelnut concentration, we have converted the spiked amount of defatted hazelnut to the corresponding amount of nondefatted hazelnut (Table 1), considering the determined weight loss due to defatting (conversion factor = weight before defatting/weight after defatting = 50 g/19.39 g = 2.578). For each matrix, identical sample portions were analyzed. However, by adding other components to the spiked wheat flour to prepare the other matrices or by baking the dough, the concentration of the spiked hazelnut powder will be different in each matrix. The actual concentrations of (nondefatted) hazelnut were calculated on the basis of the weight percentage of the spiked flour in the different matrices (Table 1). These concentrations were used to calculate the ratio measured/incurred hazelnut. This is necessary to determine the influence of the matrix and the baking process on the detection. All spiked samples we analyzed returned positive results, that is, absorbance measurements higher than those obtained for the zero standard of the respective ELISA kit. The absorbance values obtained for the blank flour samples (matrix 1, 0 ppm) were comparable to those measured for the zero standard solution in each assay, indicating that the flour does not produce false-positive results. However, the blank samples of matrix 2 returned increased absorbance values compared to the noise signal of matrix 1. Quantification of the measured signal assumed that these samples contained 0.61 ± 0.27 and 0.98 ± 0.26 ppm hazelnut, as determined with the Ridascreen FAST and BioKits Hazelnut assays, respectively. The signal decreased again for the samples of matrix 3 (0.26 ± 0.02 and 0.18 ± 0.02 ppm, respectively) and 4 (0.23 ± 0.01 and 0.14 ± 0.04 ppm, respectively), possibly due to dilution with the other matrix components, but remaining higher than the noise. This indicates that the salts present in matrix 2 influenced the performance of both ELISA kits. The sugar present in matrix 2 did not contribute to the signal, as proven in another study of the authors determining the specificity of the ELISA kits (results to be published). Altered antigen−antibody binding at increasing ionic strengths has been

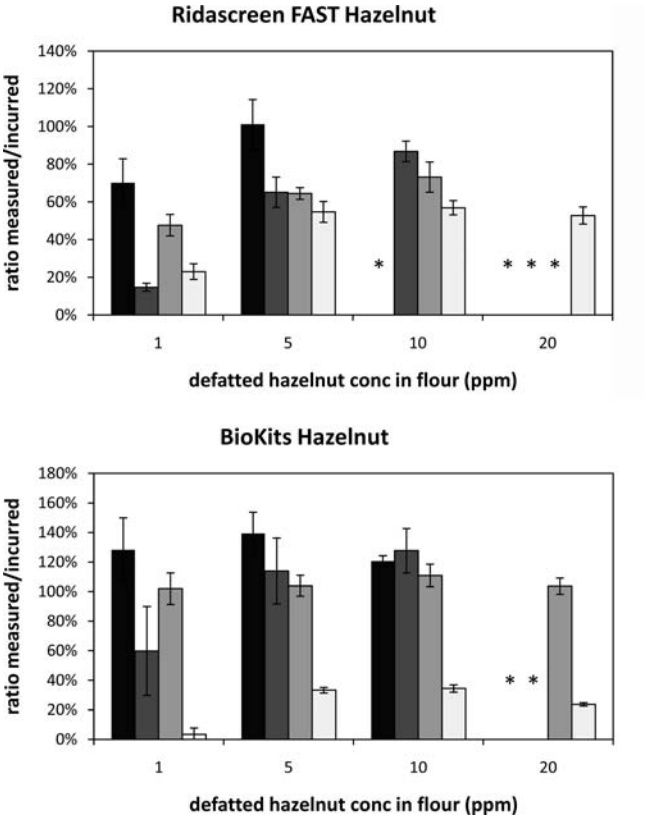


**Figure 3.** ELISA detection of hazelnut in different food model systems: (top) Ridascreen FAST Hazelnut; (bottom) BioKits Hazelnut Assay; (black bars) matrix 1 (wheat flour); (darkest gray bars) matrix 2 (wheat flour + sugar, NaCl, and $NaHCO_3$; (lighter gray bars) matrix 3 (cookie dough); (lightest gray bars) matrix 4 (cookies). Data are mean values of six samples analyzed in duplicate (∗ = absorbance value outside calibration range of the standard curve).

reported previously,[15] which could explain our observations. This background signal would, however, also be contained within the signal obtained with the incurred samples and would lead to a distorted quantification of the actual amount of hazelnut present. As this study intends to evaluate the impact of processing on the detection of the hazelnut proteins, the background signal of each matrix was subtracted from the respective sample signals to eliminate contributions not originating from the hazelnut proteins.

With the Ridascreen FAST Hazelnut kit the absorbance values measured in the flour spiked with 10 ppm and higher concentrations of defatted hazelnut flour were above the calibration range. This is not surprising, as the actual hazelnut concentration in these samples is about 26 ppm or higher, taking into account the defatting, and the highest point in the calibration curve is 20 ppm hazelnut. In matrix 1 there is a good correlation between the measured and actual hazelnut concentration, indicated by the ratio of, on average, 101 ± 13% in the samples containing 5 ppm hazelnut (Figure 3, top). In the samples containing 1 ppm defatted hazelnut it seemed more difficult to correctly quantify the hazelnut concentration, which could be due to the low incurrence level; this pushed the limits of the kit, as the manual indicated its quantification limit to be 2.5 ppm hazelnut. Upon analysing the samples of matrix 2, the detected amount of hazelnut was on average about 35% lower. This decreased detection could be explained by a negative influence of the salts, as mentioned earlier, and possibly the

**Table 2. Amount of DNA (Picograms) Detected by Analysis with the *Cor a* 8 PCR of the Samples from Matrices Prepared with Flour Spiked with Different Levels of Defatted Hazelnut Powder[a]**

| | matrix | | | |
|---|---|---|---|---|
| spike level (ppm) | 1 (flour) | 2 (flour +) | 3 (dough) | 4 (cookies) |
| 0 | 0 | 0 | 0 | 0 |
| 1 | 0 | 0 | 0.163 ± 0.026 (2) | 0 |
| 10 | 0.05 ± 0.029 (4) | 0.30 ± 0.15 (5) | 0.76 ± 0.50 (7) | 0 |
| 100 | 0.37 ± 0.17 (12) | 1.31 ± 0.55 (12) | 3.77 ± 0.88 (12) | 0.90 ± 0.86 (3) |
| 1000 | 3.40 ± 0.85 (12) | 14.53 ± 2.90 (12) | 46.07 ± 8.99 (12) | 3.52 ± 1.14 (9) |
| 10000 | 41.48 ± 6.65 (12) | 197.70 ± 39.84 (12) | 467.91 ± 68.61 (12) | 23.42 ± 6.66 (12) |

[a] Values are means ± SD of six duplicate analyses ($n$ = 12); the number of positive replicates is indicated in parentheses.

presence of sugar. Although the sugar does not contribute to the background signal as explained above, it could have a negative impact on the protein−antibody interaction. The addition of butter (matrix 3) did not seem to further influence detection. The influence of the food matrix on allergen detection has been reported earlier by Whitaker et al.,[16] who observed a difference in the amount of peanut protein that could be detected with three commercial ELISA kits in different food matrices. An additional decrease of the detected amount of hazelnut (average of about 10%) was observed when analysing the samples of matrix 4. This indicates that apart from the influence of the matrix, the baking process also influenced detection.

Similarly to the Ridascreen FAST kit, quantification of the hazelnut in the flour (matrix 1) with the BioKits Hazelnut assay was possible up to a level of 10 ppm as the values obtained for the higher concentrations were outside the calibration range. An overestimation of about 30% on average was seen for the quantification of hazelnut in the samples of matrix 1 (Figure 3, bottom). Although the background signal measured in the blank samples of matrix 2 was higher compared to the Ridascreen FAST assay, the addition of the salts and sugar did not seem to have a substantial influence on the hazelnut detection. In contrast, the baking process showed to greatly affect detection. The amount of hazelnut that was quantified in the cookies was on average only 17% of the quantity measured in the flour, whereas this was about 55% with the Ridascreen FAST kit.

**Detection of Hazelnut in Food by PCR.** On the basis of previous literature data,[17−19] we expected the baking process to result in decreased detection of the hazelnut DNA due to degradation. However, the forces induced by the mixing, applied for the preparation of the matrices, could also have a negative effect on the integrity of the DNA.[20−22] In the preliminary experiment performed to investigate the dilution process to prepare the different spike levels, it was shown that all replicates of the samples of the different concentrations analyzed were positive, starting from 20 ppm defatted hazelnut flour. At lower concentrations, several replicates did not produce an amplification curve. At 20 ppm the obtained $C_q$ values varied around 40, which is already a high value for quantification purposes. Therefore, we decided to analyze some spike levels in the real-time PCR assays higher than those analyzed with the ELISAs, namely, 0, 10, 100, 1000, and 10000 ppm defatted hazelnut powder.

After DNA extraction, the different food samples were analyzed using the *Cor a* 8, First Hazelnut, and Surefood Hazelnut PCR assays. Each sample was also tested for the presence of PCR inhibitors with the internal inhibition control DNA included in the Mastermix (First Hazelnut PCR) or the separate inhibition control Mastermix (Surefood Hazelnut PCR). The results demonstrated that no residual inhibiting compounds were present in the DNA samples. In each PCR run, a dilution series of genomic DNA of defatted hazelnut powder was analyzed in parallel to construct a calibration curve. This calibration curve was used to calculate the amount of DNA in the samples on the basis of the obtained $C_q$ values. Similarly to the ELISA-based analysis described above, the altered concentration of the defatted hazelnut powder arising from addition of the other components to the flour to prepare matrices 2 and 3 and the loss of weight due to baking of the cookies (matrix 4) have been taken into account in the interpretation of the results. This means that the amount of hazelnut DNA detected in the different samples was normalized to the corresponding amount that would be detected in 100% spiked flour, by taking into account the weight percentage of the flour in the sample (Figure 1).

Analyzing the samples of matrix 1 (spiked flour) with the *Cor a* 8, Surefood Hazelnut, and First Hazelnut PCR resulted in an amplification curve for all 12 replicates starting from 100 ppm. At 10 ppm, with these PCR assays only 4, 6, and 11 of the 12 replicates, respectively, showed amplification plots; the rest remained undetected. These results agree with the results of the preliminary experiment described to verify the dilution procedure of the incurred flour. The undetected samples were not included to calculate the mean amount of DNA detected in each matrix at the different incurrence levels. When the amounts of DNA detected in all samples with the different assays were compared, the values obtained with the *Cor a* 8 PCR were lower than those obtained with the Surefood Hazelnut and First Hazelnut PCRs (Tables 2−4). The latter two returned comparable values. Surprisingly, the amounts of DNA detected in the samples of matrix 2 were higher than the amounts in matrix 1, and a further increase in the detection was seen in the samples of matrix 3.

After baking, decreased detection was observed. For the *Cor a* 8 PCR, the amount of DNA detected in the cookie samples at 10 and 100 ppm was higher than or almost identical to, respectively, the amount detected in the flour. However, these data must be interpreted with caution, as they represent the mean values of only three highly variable data points and nine data points, respectively. The observed trend was visible for the three PCR assays, indicating that the cause is inherent to the samples and independent of the assay, although it was more pronounced in the *Cor a* 8 PCR and less in the First Hazelnut PCR.

In the search for an explanation of these remarkable observations, one hypothesis was that more DNA was extracted from the samples of matrices 2 and 3. The reasoning was that possibly the

**Table 3. Amount of DNA (Picograms) Detected by Analysis with the Surefood Hazelnut PCR of the Samples from Matrices Prepared with Flour Spiked with Different Levels of Defatted Hazelnut Powder[a]**

| | matrix | | | |
|---|---|---|---|---|
| spike level (ppm) | 1 (flour) | 2 (flour +) | 3 (dough) | 4 (cookies) |
| 0 | 0 | 0 | 0 | 0 |
| 1 | 1.08 ± 0.53 (2) | 0 | 6.37 ± 0.33 (2) | 0 |
| 10 | 3.73 ± 1.50 (6) | 4.57 ± 3.10 (7) | 6.25 ± 3.71 (7) | 0 |
| 100 | 14.67 ± 4.34 (12) | 23.93 ± 6.93 (12) | 28.72 ± 7.80 (12) | 2.16 ± 1.77 (7) |
| 1000 | 113.72 ± 23.78 (12) | 165.10 ± 35.21 (12) | 170.46 ± 36.13 (12) | 11.23 ± 5.23 (12) |
| 10000 | 808.8 ± 138.1 (12) | 1011 ± 146.4 (12) | 1083.1 ± 245.6 (12) | 71.89 ± 10.5 (12) |

[a] Values are means ± SD of six duplicate analyses ($n$ = 12); the number of positive replicates is indicated in parentheses.

**Table 4. Amount of DNA (Picograms) Detected by Analysis with the First Hazelnut PCR of the Samples from Matrices Prepared with Flour Spiked with Different Levels of Defatted Hazelnut Powder[a]**

| | matrix | | | |
|---|---|---|---|---|
| spike level (ppm) | 1 (flour) | 2 (flour +) | 3 (dough) | 4 (cookies) |
| 0 | 0 | 0 | 0 | 0 |
| 1 | 0.36 ± 0.08 (3) | 1.09 ± 0.27 (2) | 0 | 0 |
| 10 | 0.88 ± 0.53 (11) | 1.17 ± 0.50 (11) | 0.58 ± 0.22 (6) | 0 |
| 100 | 10.51 ± 2.59 (12) | 10.95 ± 3.26 (12) | 13.84 ± 3.70 (12) | 1.12 ± 1.56 (5) |
| 1000 | 95.25 ± 9.07 (12) | 76.09 ± 8.60 (12) | 135.92 ± 11.7 (12) | 12.99 ± 1.80 (12) |
| 10000 | 885.54 ± 89.6 (12) | 725.38 ± 76.9 (12) | 1100.4 ± 127.5(12) | 114.92 ± 20.9 (12) |

[a] Values are means ± SD of six duplicate analyses ($n$ = 12); the number of positive replicates is indicated in parentheses.

wheat flour alone in the samples of matrix 1 did not suspend completely in the volume of extraction buffer used, although attention was paid to this during the extraction procedure. In the other matrices, the weight percentage of the wheat flour is lower, making it easier to suspend and promoting DNA extraction. To confirm this hypothesis, the DNA content of the samples was measured. The obtained DNA concentration comprised the amount of DNA from the defatted hazelnut flour and wheat flour, as it is not possible to make a distinction between both. However, as only minor amounts of defatted hazelnut powder are present compared to the majority of wheat flour, no great differences in the DNA content between samples of the different spike levels are expected. The other components used to prepare the food samples do not contain DNA. The concentrations were normalized by accounting for the different weight percentages of the (spiked) wheat flour in the different matrices. The proposed hypothesis proved to be unvalid (Figure 4), as the DNA concentration in the samples of matrices 2 and 3 was slightly lower than that in the matrix 1 samples. On the basis of these observations, it could be concluded that some components present in matrices 2 and 3 that have not been removed during DNA extraction positively influenced the PCR reaction, leading to an increased detection. In contrast, it seems more plausible that components present in the wheat flour inhibit the PCR reaction, with the concentration of inhibitors being lower in matrices 2 and 3 compared to matrix 1.

The lower DNA content in the cookie samples compared to the dough samples (Figure 4) demonstrates a reduced DNA extractability due to the baking process and is consistent with the data obtained by PCR. Apart from the reduced DNA extractability, DNA denaturation or modification additionally contributed to the decreased detection of the hazelnut DNA target in the

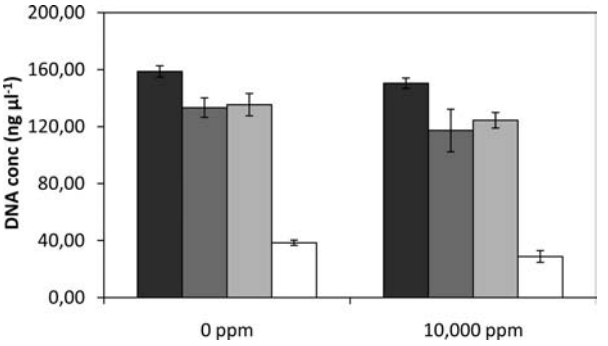


**Figure 4.** DNA concentration (normalized as a function of flour weight percent) in the samples of the different matrices prepared with blank wheat flour (0 ppm) and spiked wheat flour (10000 ppm): (black bars) matrix 1; (dark gray bars) matrix 2; (light gray bars) matrix 3; (white bars) matrix 4. Data are mean values of duplicate measurements of six replicates ($n$ = 12).

cookies. This was proven by comparing the relative changes in extracted total DNA and quantified hazelnut DNA in the dough and cookies to each other. At 10.000 ppm only 40% of the total DNA measured in the dough was present in the cookie samples (Figure 4). At the same level the *Cor a* 8, Surefood Hazelnut, and First Hazelnut PCR tests could in the cookies detect, respectively, 6, 7, and 10% of the amount of hazelnut DNA the tests had quantified in the dough. The size of the *Cor a* 8 target (218 bp) being bigger compared to the sizes of the amplicons obtained with the commercial kits (between 100 and 150 bp as estimated from agarose gel electrophoresis) could explain why lower amounts of hazelnut DNA are detected after baking with this

PCR assay. Longer fragments are more prone to degradation than shorter ones, leading to fewer template molecules available of the former that can be amplified.

The *Cor a* 8 PCR assay was in general the least sensitive, producing the lowest number of positive amplification reactions at the lower concentrations, and the amount of quantified hazelnut DNA was also lower compared to the other two PCRs (Tables 2−4). Again, the longer amplicon size could explain these observations. The intensive mixing to prepare the spiked flour and the other matrices could also have caused DNA degradation and reduced part of the DNA fragments to sizes smaller than the PCR targets.

**ELISA versus Real-Time PCR.** It can be concluded that the investigated ELISA procedures were more sensitive with respect to detecting hazelnut in the different matrices than the investigated real-time PCR procedures; the defatted hazelnut powder could be detected down to the lowest spike level of 1 ppm with the ELISAs, whereas the PCR assays produced a positive signal in all 12 replicates only starting from 100 ppm. This means that the detection limit of the PCR assays lies somewhere between 10 and 100 ppm defatted hazelnut. The lower sensitivity of the real-time PCR detection platform is probably related to the lower abundance of DNA as target analyte compared to the proteins targeted in ELISA, rather than being inherent to the PCR assay itself.

A negative matrix effect on the hazelnut detection was more pronounced in one of the two ELISAs. The matrix also seemed to have a negative influence on DNA amplification, a trend independent of the type of PCR assay investigated. A common characteristic of both detection platforms is their susceptibility to food processing on the detection of hazelnut (DNA) in the cookies. This study hence illustrates that both the food matrix and processing can lead to erroneous quantification of the hazelnut (DNA) content in the food sample. This is problematic and may present a risk for the allergic consumer in cases when quantitative results are applied in risk assessment procedures. The obtained analytical results are therefore only useful if the amount of detected analyte can be converted to the corresponding amount of the allergenic ingredient.

In the case of ELISA, the amount of proteins that is detected can be correlated to the amount of food, on the basis of the protein content of the latter. However, a similar conversion is not possible for PCR assays applying calibrators consisting of pure hazelnut DNA, as the DNA content of hazelnuts is not known. Despite knowing the genome size of hazelnut, the intended calculations require knowledge of the amount of DNA for a certain amount of the allergenic ingredient. The genome size gives information only on the amount of DNA present in the nucleus of a single cell and does not take into account the DNA that is available in other organelles, such as mitochondria and chloroplasts. Moreover, for the calculations the number of cells in a certain amount of the food should also be known. Estimates of the DNA content based on the average DNA yield extracted from a particular amount of the food are not correct, as losses during the extraction procedure are not taken into account. This could lead to a serious underestimation of the average DNA content. Moreover, the obtained yield will also depend on the DNA extraction protocol used. This means that with the calibrators applied in this study it was not possible to quantify the amount of allergenic ingredient. The obtained data thus give only qualitative information on the presence of the allergenic ingredient. To be able to use real-time PCR quantitatively in allergen detection, suitable calibrators will have to be defined, which reflects the urgent need for allergen reference materials.

Reference materials for allergens should be able to provide a link between the detected analyte and the allergenic potential. The fundamental goal of allergen detection in food is to determine the potential risk of a product for the allergic patient. From this viewpoint, protein-based detection methods are said to be more representative for the allergenic potential of the product, as they detect the allergic reactors themselves, that is, (epitopes of) proteins. This is because the presence of a certain gene, as determined in DNA-based methods, does not necessarily guarantee actual expression of the corresponding protein nor give information on the expression level. However, neither do protein-based methods per se detect allergens. Instead, a species-specific marker protein that is not an allergen can be the target of the analytical method as well. This has a direct impact on the determination and/or quantification of the allergenic potential. Even if the target protein is an allergen, antibodies raised in animals (IgG) will not necessarily bind to the epitopes of the IgE antibodies from patients, which are the actual motifs responsible for the allergic reaction. This means that such tests cannot prove that the actual allergenic structures (epitopes) are indeed present and intact and, hence, cannot justify the allergenic potential of the sample. None of the currently used routine methodologies for allergen detection can actually verify the true allergenicity of a food product.

Finally, from this study it can be concluded that food processing has an impact on hazelnut detection in cookies and cookie ingredients as evidenced with both investigated allergen detection platforms, that is, (sandwich) ELISA and real-time PCR. From the obtained data, we cannot conclude which analyte (protein or DNA) is most susceptible to food processing in the case of hazelnut and this particular case study. This would require a quantitative comparison of the residual amount of hazelnut detected after processing. A semiquantitative comparison between both detection platforms can be made based on the recovery of the amount of hazelnut protein or DNA detected in the spiked flour and cookies, respectively. However, as the recovered amount varies considerably between the different assays for both types of detection methods, no unequivocal conclusion can be made.

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