FOOD COMPOSITION AND ADDITIVES

Detection of Cashew Nut DNA in Spiked Baked Goods Using a Real-Time Polymerase Chain Reaction Method

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The detection of potentially allergenic foods, such as tree nuts, in food products is a major concern for the food processing industry. A real-time polymerase chain reaction (PCR) method was designed to determine the presence of cashew DNA in food products. The PCR amplifies a 67 bp fragment of the cashew 2S albumin gene, which is detected with a cashew-specific, dual-labeled TaqMan probe. This reaction will not amplify DNA derived from other tree nut species, such as almond, Brazil nut, hazelnut, and walnut, as well as 4 varieties of peanut. This assay was sensitive enough to detect 5 pg purified cashew DNA as well as cashew DNA in a spiked chocolate cookie sample containing 0.01% (100 mg/kg) cashew.

Allergy to tree nuts affects approximately 0.4% of the population (1). Nut allergies are a significant public health concern, affecting approximately 1.4 million Americans. Prevention of allergic reactions can be achieved only by avoiding the ingestion of the allergen. Symptoms following exposure to tree nuts (e.g., walnut, hazelnut, pecan, Brazil nut, and cashew) can range from generalized skin inflammation to life-threatening anaphylaxis (2). Even with dietary restrictions in place, 30% of tree nut-allergic patients will have an incident of accidental ingestion within a 5-year period; several of these reactions will be the result of hidden or undeclared ingredients or cross-contamination (3).

Cashew nut allergy is the second most common tree nut allergy, after walnut, accounting for approximately 20% of tree nut-allergy sufferers, based on a national registry of tree nut-allergy sufferers, as well as a random telephone survey (1, 4). Sensitivity to cashew nuts can be manifested by 2 hypersensitivity reactions. Cashew nut shell oil contains compounds that produce a poison ivy-like contact dermatitis; ingestion of cashew proteins results in an IgE-mediated food allergy (5). The clinical manifestation of cashew allergy is largely found in atopic individuals and is consistent with other food allergens (6, 7). Most of the known cashew allergens are classified as seed storage proteins and include members of the vicillin (8), 13S globulin (9), and 2S albumin families of proteins (5).

The 2S albumin proteins are small water-soluble proteins that are rich in sulfur-containing amino acids such as cysteine and methionine and consist of 2 subunits held together via a disulfide linkage (10). The 2S albumins are highly resistant to proteolytic digestion, as well as thermal and chemical denaturation (11, 12). Due to the high methionine content of the 2S albumin proteins, the gene encoding the Brazil nut 2S albumin protein initially showed promise as a potential transgene that could be used to generate food legumes and grains with increased nutritional content (13, 14). However, further study demonstrated that not only was the Brazil nut 2S albumin protein a major human allergen (15), but that allergenicity is transferred to transgenic soybeans (16). The 2S albumins have also been determined to be an allergen of sesame seed (17), hazelnut (18), walnut (19), and mustard seed (20).

The recently passed American Food Allergen and Consumer Protection Act (FALCPA) is designed to protect the public from "hidden" food allergens. This law requires that the labels of food products manufactured after January 1, 2006, indicate the presence of any of the 8 identified major food allergens, including tree nuts. Even with increased regulation, errors in food labeling and cross-contamination can still occur. For example, of the food products recalled in fiscal year 1999, 36% was recalled because of the presence of undeclared antigens. Half of these recalls were based on labeling omissions or errors, another 40% was recalled because of cross-contamination (21). Therefore, the development of rapid and sensitive methods for the detection of allergens in food is an important goal, which may serve to minimize future allergen-related food recalls.

The current study describes the development of a real-time polymerase chain reaction (PCR) method designed to detect cashew nut in food products. In this assay, amplification primers are directed against the cashew 2S albumin gene sequence. The amplification product is hybridized with a dual-labeled probe, which is cleaved via the exonuclease activity of *Taq* polymerase, releasing a fluorescent molecule that is correlated with the amount of starting template. The instrument records the amplification using the cycle threshold (C_T) value, which is the amplification cycle number wherein the fluorescence reaches a specified threshold level; this is measured in real time during the amplification process, and

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therefore an inverse relationship exists between the amount of starting template and the C_T value (22).

Experimental

DNA Sources

Locally purchased almonds, Brazil nuts, cashews, hazelnuts, walnuts, and several species of peanut (including Runner, Virginia, Valencia, and Spanish) were used in the preparation of genomic DNA templates.

DNA Extraction

Genomic DNA was extracted from 100 mg of each nut/legume (ground) using Genomic-tip 20/G columns (Qiagen, Valencia, CA), following the instructions for tissue samples. The protocol for the DNA extraction was modified to extend the Proteinase K digestion from 2 to 16 h. After purification, DNA pellets were suspended in 100 μL 1X TE (10 mM Tris, 1 mM EDTA, pH 8.0). After quantification, the DNA sample was adjusted to a concentration of 10 ng/μL in 1X TE (10 mM Tris, 1 mM EDTA, pH 8.0).

Sample Preparation

Samples of locally purchased prepared chocolate chip cookies (containing enriched flour, chocolate, sugar, partially hydrogenated soybean and/or cottonseed oil, molasses, salt, egg, baking soda, natural and artificial flavor, and nonfat milk), fruit and nut bars, cereal, crackers, chocolate, and granola were ground to a fine powder in a food processor. DNA was extracted from 200 mg of these preparations using the NucleoSpin Food Kit (BD Biosciences, Palo Alto, CA). After quantification, the DNA samples were adjusted to a concentration of 25 or 50 ng/μL in 1X TE.

Sample Spiking

For detection limit analysis, 200 g ground chocolate chip cookie was spiked with 20 mg ground raw cashew nut (100 ppm) and combined in a food processor. A 5 g portion of the 100 ppm spike was combined with an equal amount of ground chocolate chip cookie (50 ppm), and 1 g of the 100 ppm preparation was combined with 9 g ground chocolate chip cookie and mixed in a food processor (10 ppm). This procedure was repeated with the 10 ppm cookie–cashew mixture to generate a 1 ppm spiked preparation. Additionally, 10 g ground chocolate chip cookie was mixed with 10 mg ground raw cashew (1000 ppm), and 2 g of this mixture was combined with 2 g ground chocolate chip cookie (500 ppm). Equal portions of the 500 ppm mixture and ground chocolate chip cookie were combined to generate a 250 ppm spiked mixture.

DNA was extracted from 200 mg of all these preparations using the NucleoSpin Food Kit (BD Biosciences). After quantification, the DNA samples were adjusted to a concentration of 50 ng/μL in 1X TE.

2S Albumin Real-Time PCR

Cashew nut DNA was amplified in a 25 μL reaction containing 1X TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA), 120 nM of each of the forward and reverse primers (5′TGCCAGGAGTTGCAGGAAGT3′ and 5′GCTGCCTCACCATTTGCTCTA3′, respectively); and 200 nM probe (5′FAM-ACAGAAGGTGCCGCTGCCAGAA-TAMRA3′), all from Synthegen, LLC (Houston, TX). GenBank accession No. AY081853 was used in the design of primers/probes. Thermal cycling conditions were as follows: 50°C 2 min, 95°C 10 min followed by 50 cycles of 95°C 15 s and 58°C 1 min. All data were acquired using the Smart Cycler II (Cepheid, Sunnyvale, CA).

Results and Discussion

Assay Sensitivity

Figure 1 demonstrates the sensitivity of the assay to detect purified raw cashew nut DNA. The limit of detection (LOD) of the assay was approximately < 5 pg >1 pg. As little as 5 pg cashew DNA was consistently amplified with this assay, in a concentration-dependent manner. As shown in Figure 1A, a 10-fold reduction in sample concentration resulted in the expected 3-cycle increase in C_T values. Samples containing 1 pg raw cashew nut DNA amplified, but did not consistently reach, the fluorescent threshold (data not shown). In Figure 1B the standard curve derived from the data in Figure 1A shows good linearity. DNA was also extracted from

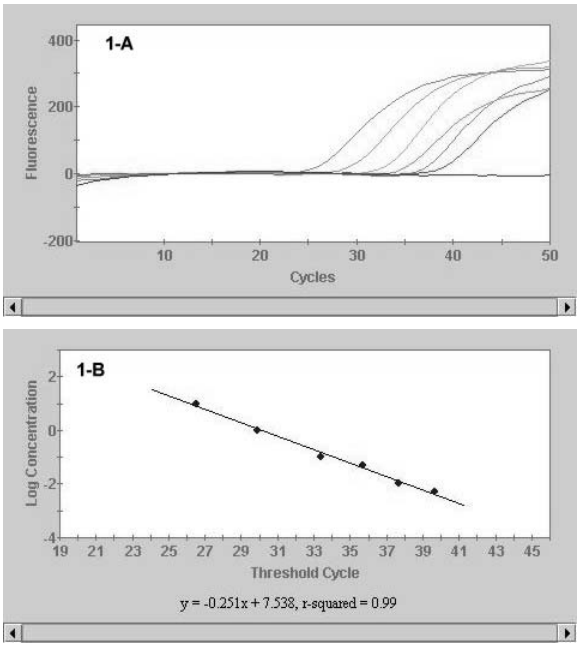

Figure 1. (A) Sensitivity of the cashew nut real-time PCR assay using various concentrations of purified raw cashew genomic DNA. Amounts of DNA/rxn and C_T values appear from left to right as follows: 10 ng (26.5); 1 ng (29.86); 100 pg (33.38); 50 pg (35.70); 10 pg (37.66); 5 pg (39.69); NTC (ND). (B) Standard curve generated from the data present in Figure 1A. NTC = No template control; ND = not detected.

Table 1. Representative C_T values for raw and roasted cashew DNA samples

Amount of DNA/ reaction	Raw cashew, C_T	Roasted cashew, C_T
10 ng	26.50	27.16
1 ng	29.86	30.91
100 pg	33.38	34.70
50 pg	35.70	35.98
10 pg	37.66	37.32
5 pg	39.69	40.68
1 pg	ND[a]	ND

[a] ND = Not detectable.

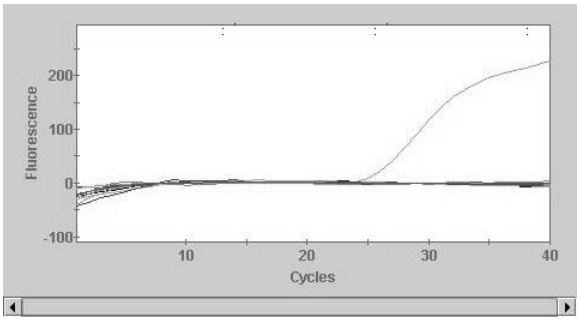

Figure 2. Specificity of the cashew nut real-time PCR. Data represent the amplification of 10 ng each of DNA from the following species: almond (ND); Brazil nut (ND); cashew (26.43); hazelnut (ND); Runner peanut (ND); Spanish peanut (ND); Valencia peanut (ND); Virginia peanut (ND); walnut (ND); NTC (ND). NTC = No template control; ND = not detected.

roasted cashew nuts to determine whether the heat applied to the nuts during processing would negatively affect the sensitivity of the assay. Table 1 lists the C_T values that were obtained with equivalent amounts of DNA extracted from either raw or roasted cashews. The LOD in reactions containing roasted or raw cashew nut DNA is the same, approximately 5 pg/reaction. The C_T values from either raw or roasted cashew samples are in good agreement, indicating that no significant loss of assay sensitivity occurred as a result of roasting.

Assay Specificity

As shown in Figure 2, amplification of several species of tree nuts (almond, Brazil nut, hazelnut, and walnut), as well as several types of peanut (Runner, Virginia, Valencia, and Spanish), were tested with the cashew nut-specific primers to determine cross-reactivity. Only the cashew DNA sample amplified in the presence of specific primers. Based on the supposition that contamination of a food product would likely take place where baked goods are produced, several baked goods, including crackers, cereals, granolas, and nut-containing chocolate bars, were tested for the presence of cashew DNA. Additionally, 2 fruit and nut bars, purchased from a health food store, both of which were labeled as containing cashews, were analyzed as positive controls. Only the products labeled as containing cashews were positive for this assay when 100 ng DNA was amplified for 50 cycles (data not shown).

Limit of Detection and Assay Reproducibility

In order to determine the amount of cashew nut DNA that could be detected in a complex food matrix, ground cashew was added to ground chocolate chip cookies at concentrations of 0.1, 0.025, and 0.01% (100 ppm). DNA was extracted from 200 mg of each of these mixtures. A 100 ng amount of DNA from each of the mixtures was amplified, using 5 replicates each. Table 2 shows the average C_T for each of the spiked mixtures tested. Table 2 also compares the predicted amount of cashew DNA that should be recovered in the assay based on the spike concentration (pg/rxn). The values were estimated by the Smart Cycler software when assay values were compared to a standard curve. The values obtained were similar to the quantity of cashew DNA present in each reaction vessel, demonstrating that cashew DNA can be detected in a sample containing as little as 100 mg/kg cashew. However, it should be noted that extreme care is necessary to reduce pipetting error, which, given the sensitivity of the assay, can result in poor quantification of target DNA. DNA from unspiked chocolate chip cookies did not amplify using the cashew-specific primers (data not shown). Repeated attempts to amplify cashew DNA from a 1 mg/kg cookie/cashew mixture were negative (data not shown). Given that this assay can detect 5 pg purified cashew DNA, the LOD for a spiked cookie sample is likely to be 50 ppm, when 100 ng of DNA is tested.

Table 2. Quantification of cashew DNA from chocolate chip cookies spiked with various amounts of cashew nut

Quantity of DNA analyzed from sample	Mean C_T	Predicted, pg/rxn	Mean, pg/rxn	RSD, %[a]
100 ng/0.1% cashew	35.3 ± 0.61	100	88.2 ± 26.6	30.1
100 ng/0.025% cashew	38.0 ± 1.2	25	21.6 ± 13.7	63.4
100 ng/0.01% cashew	39.3 ± 0.43	10	9.2 ± 2.8	30.4

[a] RSD = relative standard deviation.

The sensitivity of the assay may be improved by analyzing more DNA per reaction or increasing the probe concentration in the reaction. The DNA used in all the assays was purified; however, the high protein and fat content in both the target and the matrix may result in the carryover of PCR inhibitors after purification. The food matrix to be analyzed should, therefore, be considered carefully, and appropriate measures should be taken to minimize these effects.

The current study describes the development of a real-time PCR assay capable of detecting cashew nut DNA in food matrixes. The use of real-time PCR technology has several advantages: the use of fluorescent (TaqMan) probes allows for the quantification of cashew nut DNA as compared to conventional PCR, when the results are compared to a standard curve. Secondly, because digestion of the hybridized probe produces the detected signal, interferences such as primer dimers and nonspecific products are eliminated, increasing specificity.

A criticism of DNA-based methods used for allergen testing is that this method does not detect the allergen in question and, therefore, may not coincide with allergen exposure (23). Although this may be a valid criticism, the amount of cashew nut (or any tree nut) that needs to be ingested to bring about a clinical reaction has never been determined. A recent study attempted to determine the threshold dose for several allergenic foods based on clinical data. Although the study did not specifically address tree nuts, analysis of the data for peanuts suggests that the lowest threshold dose for peanuts is <1 mg (24). Because no "safe level" of nuts has been established, the presence of nut DNA may indicate that a food product has the potential to be a health hazard. It is anticipated that this assay will be most useful in the rapid identification of a potential contaminant and used in conjunction with a secondary, more sensitive method, such as the enzyme-linked immunosorbent assay (ELISA) for allergen protein quantification. A recently published ELISA assay for the detection of cashew major protein had a sensitivity of 1 mg/kg when tested in food matrixes (25). The identification of undeclared nut DNA in a food product represents a violation of U.S. and European ComMission food labeling laws and may alert a food manufacturer to potential cross-contamination issues.

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