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Are nut ingredients accurately labeled on granola bars?

Abstract

Individuals with allergies can have serious health complications after consuming foods with certain allergens. It can be difficult to ensure that packaged food will be allergen-free and can be safely consumed. Peanut and tree nut (Almond, Brazil Nut, Cashew, Hazelnut, Macadamia, Pecan, Pistachio, Walnut) allergies are some of the main food-induced reasons of anaphylaxis. We sought to determine whether we could detect all tree nut ingredients as labeled on granola bar packaging and predicted that all (100%) of the granola bars will be accurately labelled. We used 5 types of granola bars and isolated DNA from them. We conducted a polymerase chain reaction to detect 8 types of nuts in our samples. We ran the samples on a gel and analyzed the presence of tree nuts. We successfully detected nut products as labeled on the granola bar packaging in 4/5 (80%) of the samples. However, one granola bar sample labeled as "contains almonds" and almond powder (positive control) were not detected by the primer set leading to 1/5 (20%) false negative in the granola bar samples. Granola bars contain ingredients as listed on their packaging, suggesting that they can be trusted by consumers. Based on our findings, people should be cautious as there may be cross-contamination between tree nut allergens and peanut-free products.

Introduction

With the prevalence of food allergies increasing around the world (McWilliam et al., 2022), individuals must allocate more of their attention to ensure no cross-contamination has occurred with their allergen during the manufacturing process of foods they consume. Peanuts and tree nuts are common allergens that can cause life-threatening reactions, including anaphylaxis (Brough, 2014; Lomas, 2015). Patients are usually advised to be cautious to strictly avoid allergens to prevent health risks (Marra, 2017). Food allergen labeling is used internationally and can be utilized by individuals to prevent exposure to allergens. Notably, tree nuts are commonly used in foods and there can be high risk of cross-contact in processing facilities among different types of foods (Taylor, 2010).

We chose to focus our research project on granola bars, as peanuts and tree nuts tend to be common ingredients in these commonly processed snacks. Additionally, these packaged

granola bars frequently include a precautionary warning stating that they “may contain tree nuts” or are “not produced in a facility that is completely peanut-free”. Interestingly, these advisory labels are included voluntarily by the manufacturer, thus some foods may have traces of tree nuts even if it is not indicated on the label (Gagné, 2022).

We hypothesize that all labels on the processed granola bars we test are accurate and that we will be able to detect presence of tree nuts if mentioned as one of the main ingredients in the food. This project does not aim to quantify the amount of tree nuts or traces of ingredients in each sample, but rather seeks to confirm that the ingredients listed on the packaging of each sample are certainly present in it.

In order to learn more about the composition of each granola bar, we used DNA isolation and Polymerase chain reaction (PCR) using two sets of tetrameric primers to detect unique nucleotide sequences expressed by the tree nuts. The amplicon of each tree is listed in Table 3 below. Each set of tetrameric primers is able to detect four tree nuts. Thus, we conducted our procedure twice in order to use both primer sets. Overall, this paper seeks to provide evidence to the question that some individuals with allergies have, which is if product labeling can be trusted enough for safe consumption of the product.

Methods

First we labeled 16 sterile 1.5mL Eppendorf tubes (3 replicates of 5 granola bar samples and one for almond powder (positive control) to prepare for DNA Isolation (Table 1). We used an ethanol flame to ensure the tweezers and scissors were sterilized before we collected small samples from granola bars into the Eppendorf tubes. We crushed each sample using a toothpick to ensure proper DNA isolation occurs. For the 17th sample, we used almond powder as our positive control for the experiment and put it into an Eppendorf tube using the procedure

outlined above. We added 300 uL of Cell Lysis solution with Proteinase K to each tube. Next we placed the tubes in a water bath set at 65°C for 15 minutes and vortexed each tube for ten seconds after every 5 minute interval. The tubes were then placed on ice for 5 minutes. Protein Precipitate Reagent (150 uL) was then added to each tube and all the tubes were vortexed. Tubes were then placed in the centrifuge for 10 minutes at maximum speed. While the samples were in the centrifuge, we labeled 16 new Eppendorf tubes with the previous labels we had already made (Table 1). We collected the supernatant into the new tubes. The tubes holding the pellets and fat layers were thrown out. 500 uL of ice cold isopropanol was then added to the supernatant in each new tube. All tubes were tilted up and down 30-40 times to ensure proper mixing. The tubes were put into the centrifuge at maximum speed for 10 minutes. We then poured the isopropanol from each tube into a liquid discard beaker, without the pellets being disrupted. 500 uL of ethanol was added to each pellet and then we poured the ethanol out of each tube into a waste beaker. The tubes were left open, on their sides overnight on a paper towel.

The next morning we added 30uL of TE buffer to each DNA pellet. We then labeled 34 PCR tubes in preparation for the PCR protocol. Half of the tubes were labeled with a red circle indicating master mix 1 (which detects almonds, pistachios, macadamias and brazil nuts) and the other half were labeled with a green star indicating master mix 2 (detects cashews, hazelnuts, pecans, walnuts). Additionally, the labeling included the labels from table 1 for the granola bars and positive control and the remaining PCR tube for each master mix was used as the negative control (distilled water, shown as a minus). Next, we labeled two Eppendorf tubes as master mix 1 (red dot) or 2 (green star). We then made two master mixes, with each being tailored to one of the tetraplex primer sets. Both recipes are further outlined in Table 2 and all ingredients were kept on ice during this step. 19 uL of master mix 1 and master mix 2 were added to their

respective set of tubes. Then 1 uL of the corresponding DNA was added to each sample's tube and 1 uL sterile distilled water was added to the negative controls. All PCR tubes were kept on ice until they were placed in the PCR machine. Tubes were then removed from the PCR machine and put in the freezer.

In preparation for gel electrophoresis, we used a 3% agarose gel to ensure proper separation of the bands. We then performed gel electrophoresis by adding 5 uL of 6X loading dye to each PCR tube. We loaded 10 uL of each sample into the matrix of the gel. We used two separate gels, each corresponding to one of the master mix sets. Initially, the gels were run at 80V for 15 minutes. Then, they were run at 150V for 45 minutes.

Sample	**Nut Status**	Replicate 1 (a)	Replicate 2 (b)	Replicate 3 (c)
1. Baked Oatmeal Bar	Does not contain nuts but label says "not all our are manufactured in a peanut free facility"	1a	1b	1c
2. Cashew Bar	Contains cashew ingredients	2a	2b	2c
3. Almond Bar	Contains almond and peanut ingredients, may contact other tree nuts	3a	3b	3c
4. Coconut Cream Bar	Contains almond and cashew ingredients	4a	4b	4c
5. Granola Bar	May contain tree nuts	5a	5b	5c
Almond Powder (only 1 replicate)	Positive control for almonds	+		

Table 1: Ingredient advisory warning present on each granola bar packaging and sample labeling system used during experiment for DNA Isolation.
*identifying brand names of samples have been omitted

Ingredients for master mix 1 (almond, brazil nut, macadamia nut, and pistachio)	**Amount (µL)**	**Master Mix (X17) for samples (µL)**	**Ingredients for master mix 2 (cashew, hazelnut, pecan, and walnut)**	**Amount (µL)**	**Master Mix (X17) for samples (µL)**
10X PCR Buffer	2.5 µL	42.5 µL	10X PCR Buffer	2.5 µL	42.5 µL
dNTP (10 mM)	0.5 µL	8.5 µL	dNTP (10 mM)	0.5 µL	8.5 µL
Taq	0.5 µL	8.5 µL	Taq	0.5 µL	8.5 µL
$MgCl_2$ (25 mM)	2.0 µL	34 µL	$MgCl_2$ (25 mM)	2.0 µL	34 µL
Almond (10uM) F	1.0 µL	17 µL	Cashew (10uM) F	0.5 µL	8.5 µL
Almond (10uM) R	1.0 µL	17 µL	Cashew (10uM) R	0.5 µL	8.5 µL
Brazil nut (10uM) F	0.2 µL	3.4 µL	Hazelnut (10uM) F	0.5 µL	8.5 µL
Brazil nut (10uM) R	0.2 µL	3.4 µL	Hazelnut (10uM) R	0.5 µL	8.5 µL
Macadamia nut (10uM) F	0.2 µL	3.4 µL	Pecan (10uM) F	0.2 µL	3.4 µL
Macadamia nut (10uM) R	0.2 µL	3.4 µL	Pecan (10uM) R	0.2 µL	3.4 µL
Pistachio (10 uM) F	0.2 µL	3.4 µL	Walnut (10 uM) F	0.5 µL	8.5 µL
Pistachio (10 uM) R	0.2 µL	3.4 µL	Walnut (10 uM) R	0.5 µL	8.5 µL
50% glycerol	5.0 µL	85 µL	50% glycerol	5.0 µL	85 µL
dH_2O	5.3 µL	90.1 µL	dH_2O	5.1 µL	86.7 µL
Final Volume	19 uL	323 µL	**Final Volume**	19 uL	323 µL
Sample DNA or distilled water (not part of master mix, added last)	1 uL		**Sample DNA or distilled water (not part of master mix, added last)**	1 uL	

Table 2: Master mix 1 and 2 recipes for polymerase chain reaction.

Common Name	Scientific Name	Primer Name	Nucleotide Sequence (5′–3′)	Amplicon (base pairs)
Almond	*Prunus dulcis*	AL-F	AGTTCTAGTTTC AAAGCGGGGGC	515
		AL-R	ACGACGGGCAA CCGAGGTC	
Brazil nut	*Bertholletia excelsa*	BR-F	GACGAGTGGTG GATCACGACACG	91
		BR-R	TCGATGCCTTGC CGCTTCG	
Cashew	*Anacardium occidentale*	CS-F	TGGCGTTCGGAA CGAACCCG	102
		CS-R	GCGATGCGGGC GGGCATAG	
Hazelnut	*Corylus spp.*	HZ-F	ATGCCAGGGGC GAATCTTGTG	361
		HZ-R	GCTACAGGGTCA CAGAGCACAAG AC	
Macadamia nut	*Macadamia spp.*	MC-F	GCACCCCGTGTC TCTGTTCG	111
		MC-R	CGATGTCCGAAC AATGGCAAAG	
Pecan	*Carya illinoinensis*	PC-F	TGGGAGGGCAC GATGAAAGC	543
		PC-R	CGACGCAATAGG GTCGAGGAGAG	
Pistachio	*Pistacia vera*	PS-F	GGTGTCGGTCGT ATGCTTCTGCAT	163
		PS-R	GTCGTTATTGAT AATGAAAGAAG GCTACC	
Walnut	*Juglans spp.*	WL-F	GGTTGGGAGGG CACGTTGAG	501
		WL-R	CGACGGGTCAC GAGGGTTTC	

Table 3: Tree nuts of interest and corresponding sequence amplified by the tetrameric primer set.

Results

Tree nut products as labeled on granola bar packaging were correctly detected in 4 out of 5 (80%) of the granola bars. Bar 1 was labeled peanut-free and no tree nut traces were found in all three replicates. Bar 2 was labeled as containing cashew ingredients. We detected cashews in 3/3 replicates. Bar 3 contained almonds; however, almonds were not detected in all 3 replicates. Bar 4 contained both almonds and cashews. Both tree nuts were detected separately in each tetraplex set. Almonds were detected in 3/3 replicates in Set A and cashews were detected in 3/3 replicates in Set B. Bar 5 was labeled as "may contain tree nuts" but we did not detect any nut presence. Lastly, both the positive control (almond powder) and negative control (distilled water) had no tree nuts detected. In total, we had 5/7 (71%) correct detections of tree nut presence or absence if the sample was nut free or a negative control. 2/7 (29%) of the samples were false negatives even though tree nuts were present, labeled on the processed granola bar and the unprocessed almond powder.

Granola Bar Sample	**Almond**	**Brazil Nut**	**Cashew**	**Macadamia**	**Pistachio**	**Hazelnut**	**Walnut**	**Pecans**
1	-	-	-	-	-	-	-	-
2	-	-	+	-	-	-	-	-
3	-	-	-	-	-	-	-	-
4	+	-	+	-	-	-	-	-
5	-	-	-	-	-	-	-	-

Distilled Water (- control)	-	-	-	-	-	-	-	-
Almond Powder (+ control)	-	-	-	-	-	-	-	-

Table 4: Gel Electrophoresis Results ((PCR positive (+) is presence of nut) , (PCR negative (-) is absence of nut))
- control: negative control; + control: positive control
* Refer to Table 1 for details on the granola bar samples

Discussion

From our experiment, we were able to determine if the tree nuts labeled on our granola bar packaging could be detected and if there were any traces of nut contamination. We successfully detected the labeled tree nut ingredients in the granola bars in 71% of the samples. Although we did not detect any nut contamination in the samples, there could be a possibility that there were small amounts of nut contamination that were not detected by our primers. Therefore, individuals with an allergy should still be careful with the products they consume, as some foods may not be heavily regulated and thus advisory warnings may not be as accurate.

We were unable to detect any almonds in the positive control (almond powder) or sample 3 (contained almonds as labeled on packaging), and this could be due to experimental error, secondary metabolites present in almonds, or the primer sets not being effective at detecting almonds. Our errors could have come from RNA or DNA contamination or insufficient mixing of PCR reagents. However, this would be unlikely as the experimental methods used for the other samples was the same. Furthermore, we did not measure the concentration of DNA extracted or its quality, which are factors that can alter DNA amplification (Gryson, 2010). Granola bars are processed foods that contain preservatives and other ingredients that might interfere with DNA extraction (Nurhayatie et al, 2018). Moreover, food processing that involves temperature changes can degrade plant DNA and reduce the fragment length of the DNA

extracted (Gryson, 2010). The initial DNA extraction process can impede downstream applications. High quality DNA is required to be appropriately amplified and allow for accurate molecular identification. Tree nuts contain a high quantity of secondary metabolites that can interfere with the DNA extraction and subsequent steps, including PCR and electrophoresis (Bashalkhanov, 2008; Dean, 2018). Due to the positive control and sample 3 almonds not being detected, further research should be done to determine the efficiency of primer sets at detecting almonds.

In the experiment conducted by Ito et al (2018), they used PCR to detect 8 tree nuts (Almond, Brazil Nut, Cashew, Hazelnut, Macadamia, Pecan, Pistachio, Walnut) by using 8 different primer pairs in processed and unprocessed food. Similarly, our experiment tested different granola bars for the presence of tree nuts. Their results, including processed and unprocessed foods, showed that only the labeled tree nuts were detected and no other tree nuts were present (Ito et al, 2018). Similar to our results, there was no nut contamination in their samples. However, Ito et al (2018) detected nut contamination in their processed food samples. They detected walnuts in bread; cashew and hazelnut were found in cereal; pecan and cashews were detected in chocolate, and cashews were detected in curry and dressings. Although we did not test for nut traces in other foods, Ito. et. al. found tree nut traces in various types of processed foods supporting the occurrence of cross-contamination. This information is important in determining if food products are accurately labeled for concerned individuals with tree nut allergies.

Conclusion

We did not detect any additional nuts contaminating the 5 granola bar samples. The granola bars only contained the tree nuts that were specified on the label of the packaging, if

there were any precautionary warnings. We were not able to detect almond in the positive control which was almond powder and sample 3 which was a granola bar containing almonds, which may indicate that the primer set did not detect almonds well, or it may be a source of experimental error. However, since the disease mechanism of food allergies is complicated, individuals should be careful because allergens can go undetected if present in small amounts and the severity of allergies may differ across individuals.

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Appendix

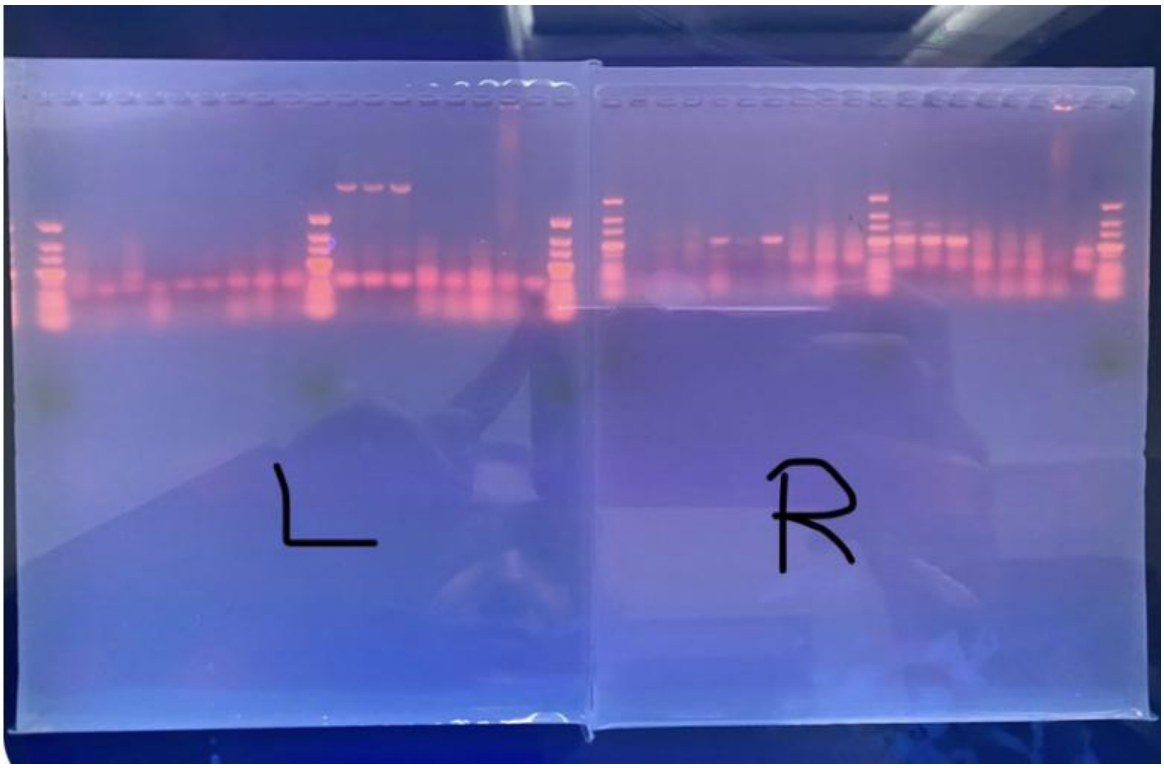

Figure 1: Detection of tree nut samples by two sets of tetraplex primers (L: Set A - Almond, Brazil Nut, Macadamia, Pistachio; R: Set B - Cashew, Hazelnut, Pecan, Walnut)
The 1st, 10th and 19th columns are ladders in both sets. Three contiguous rows are three replicates of each sample. The 17th and 18th rows are the positive and negative controls, respectively on both sets.