Pilot Study: Evaluating the Risk of Allergen Cross-Contact in Ice Cream Scoop Shop Dipper Wells

Lindsey Lake
Clemson University, lkeatin@g.clemson.edu

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PILOT STUDY: EVALUATING THE RISK OF ALLERGEN CROSS-CONTACT IN ICE CREAM SCOOP SHOP DIPPER WELLS

A Thesis
Presented to
the Graduate School of
Clemson University

In Partial Fulfillment
of the Requirements for the Degree
Master of Science
Food, Nutrition, and Culinary Sciences

by
Lindsey Lake
August 2017

Accepted by:
Dr. John McGregor, Committee Chair
Dr. Xiuping Jiang
Dr. Julie Northcutt
Mrs. Sara Cothran
ABSTRACT

Food allergies are a serious and growing problem in developed countries. Allergen cross-contact at foodservice establishments is a common cause of food allergic reactions. Therefore, this study sought to determine if dipper wells used in ice cream scoop shops pose a relevant risk to food allergy sufferers. First, a matrix study was conducted to evaluate if peanut detection by real-time PCR was inhibited by the ice cream matrix, as fat and proteins are known PCR inhibitors. Frozen ice cream, liquid ice cream mix, and water matrices were tested. Second, a controlled time trial was conducted to evaluate the efficacy of allergen removal in ice cream dipper well water. Peanut butter ice cream was added to a dipper well and water samples were collected at various rinse times. A continuous use scenario and two dipper well basin cleaning techniques were also evaluated. Finally, a survey of ice cream scoop shop owners was conducted to collect relevant information regarding current dipper well practices and policies. Results of the matrix study showed low peanut recovery in all matrices, with recovery rates of 23.9%, 17.7%, and 6.2% in frozen ice cream, liquid ice cream mix, and water matrices, respectively. The recovery rate of plain peanut butter was 5.6%. PCR inhibitors, the physio-chemical properties of ice cream, and the PCR extraction and quantification kit were all believed to be factors in the recovery rate. Based on these results, we recommend using a DNA extraction technique designed specifically for fatty food matrices for future peanut butter sample analysis, and either a matrix-calibrated or a matrix-independent PCR system for future ice cream sample analysis. Results of the controlled time trial showed that peanut removal followed an exponential decay pattern.
Quantitative results showed that while it is possible for peanut levels to be above the threshold dose, it is extremely unlikely. Dipper well basin cleaning techniques were not able to remove all traces of allergens, so more robust cleaning procedures are necessary to deal with high loads of allergens. Results of the survey showed that while most ice cream scoop shop owners had a good understanding of allergen cross-contact, advisory allergen signs were not prevalent in ice cream scoop shops. We conclude that ice cream dipper wells do not pose a significant risk to food-allergic consumers, but as a precaution for a worst case scenario, we recommend that ice cream scoop shops post allergen advisory signs and avoid using scoops from the dipper well to serve customers with a food allergy.
DEDICATION

I would like to dedicate this manuscript to my husband, Hunter Lake, for his constant support and patience throughout this process. Thank you for always making me laugh and for keeping me sane in the midst of stress. I couldn’t have done it without you.
ACKNOWLEDGMENTS

I would like to thank God for giving me the opportunity and perseverance to earn my Master’s degree. I would like to thank my family for supporting and encouraging me throughout this process.

I would like to thank my committee members, Dr. John McGregor, Dr. Jiang, Dr. Northcutt, and Mrs. Cothran for working with me on this research project. Dr. McGregor envisioned the idea for this research and provided valuable insight into the study design. Dr. Jiang generously allowed me to use her lab and lab equipment throughout my research. Dr. Northcutt kindly loaned me equipment to use for the duration of my research. Mrs. Cothran was always available for questions and encouragement. I would like to thank Dr. Rieck for his help with the statistical analysis portion of the research. I would also like to thank BIOTECON Diagnostics for providing the equipment, training, and support necessary for my research project. Christina Harzman was an invaluable resource.

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CHAPTER 1
LITERATURE REVIEW

Introduction

Food allergies are not just an individual problem anymore, but a serious public health concern. In fact, the World Health Organization classified allergens as the fourth most important public health issue (Kirsch et al., 2009). Food allergies affect a significant proportion of the population, up to 10% of young children and 2-3% of adults in industrialized countries, and several studies have shown that the prevalence is on the rise (Jackson et al., 2013; Husain and Schwartz, 2013; Carrard et al., 2015). It is estimated that food allergy affects 15 million Americans, and up to 1 in 13 children (Food Allergy Research & Education, undated). A food allergy is an adverse immune response to a normally tolerated food protein. Symptoms can affect the cutaneous, cardiovascular, gastrointestinal, and respiratory systems (Husain and Schwartz, 2013). Allergic reactions range from mild reactions to potentially fatal anaphylactic reactions. The potential for fatality has a considerable detrimental effect on the quality of life for consumers with food allergy and their loved ones. Additionally, food allergies burden the health care system (Walker et al., 2016). Though advances have been made in food allergy diagnostic tools and therapeutic treatments, no cure has been found (Carrard et al., 2015). The main management technique for people with food allergy is complete avoidance of the trigger food. Consequently, clear and correct labeling is of upmost importance. The
presence of undeclared allergens is a serious health risk for individuals with food allergy because they cannot make informed decisions.

Food Allergen Labeling in the U.S.

1. Food Allergen Labeling and Consumer Protection Act

In 2004, the FDA estimated that 2% of adults and 5% of infants and young children in the U.S. had food allergies. Approximately 30,000 individuals required emergency room treatment each year and 150 individuals died each year due to allergic reactions to food. Additionally, recalls due to unlabeled allergens were on the rise, from 35 recalls in 1990 to 121 recalls in 2000. A study conducted by the FDA in 1999 in Minnesota and Wisconsin found that 25% of randomly selected baked goods, ice cream, and candy failed to list peanuts or eggs as ingredients on food labels. In response to these statistics, the FDA developed the Food Allergen Labeling and Consumer Protection Act of 2004 (FALCPA). FALCPA mandated that major allergens had to be declared in plain language on prepackaged food labels if, “it is, or it contains an ingredient that bears or contains, a major food allergen” (FDA, 2004). Major food allergens account for over 90% of food allergies in the U.S. and are defined as milk, eggs, fish, crustacean shellfish, tree nuts, peanuts, wheat, and soybeans. The use of plain language ensures that food allergens are not hidden in a processed food. Food manufacturers have three labeling options to declare major food allergens in plain language. The first option is to declare the allergen in the ingredient list, for example “Ingredients: peanuts, wheat starch, and soy lecithin.” The second option is to declare the allergen in parenthesis following the
common or usual name of the food in the ingredient list, for example, “Ingredients: natural flavor (milk), lecithin (soy), and casein (milk).” The third option is to declare the allergens in a ‘contains’ statement immediately after or adjacent to the ingredient list, for example, “Contains Wheat, Milk, Egg, and Soy” (FDA, 2004; Taylor and Hefle, 2006). FALCPA focuses on informing consumers of the intentional addition of allergen-containing ingredients to prepackaged food through clear allergen labeling. However, two shortcomings of FALCPA is that it does not address labeling of allergen cross-contact or allergens in non-prepackaged foods.

2. Food Safety Modernization Act

In 2011 the FDA passed the Food Safety and Modernization Act (FSMA). In accordance with the FDA’s longstanding position that Current Good Manufacturing Practices (cGMPs) address allergen cross-contact, FSMA explicitly states that covered establishments must now have a food safety plan in place that addresses allergens, which are considered a chemical hazard. A hazard analysis must be conducted and preventive controls must be put in place to significantly minimize or prevent the occurrence of allergens. Prepackaged foods with unidentified allergens will now be considered misbranded (FDA, 2011). Within FSMA, the FDA specified that ‘allergen cross-contact’ will now be used in place of ‘allergen cross-contamination’ and ‘allergen contamination’ because an allergen is a normal component of food, and not itself a contaminant (FDA, 2011). Though FSMA requires that food manufacturers carefully analyze and control for allergens, there is still considerable ambiguity surrounding this concept. The FDA has yet
to release any regulations regarding the use of advisory allergen statements and there are no defined allergen threshold levels above which allergens must be labeled.

3. *Precautionary Allergen Labeling*

Precautionary allergen labeling (PAL), such as “may contain [allergen]” and “processed in a facility that also processes [allergen]” is used in many countries. In the U.S., PAL is completely voluntary and its usage is not defined by any federal regulation. The FDA states that PAL must be truthful and not misleading. In reference to similar guidelines in the United Kingdom (U.K.), Brough et al. (2015) states that although PAL “is often based upon a thorough risk assessment by a manufacturer with adherence to Good Manufacturing Practice (GMP), it is suspected that some manufacturers use PAL as an alternative to allergen risk management, circumventing the process of an actual allergen risk assessment.” It is reasonable to believe that some manufacturers in the U.S. take similar shortcuts, although the new FSMA regulations should cut down on this behavior. Regardless, there is a tendency of PAL overuse among food manufacturers, which in turn leads to unnecessarily restrictive food choices for consumers with food allergy. In addition to PAL usage, its terminology is not regulated. A survey of 1,016 food products in the U.S. found nineteen different types of PAL terminology (Chung et al., 2008). Likewise, a separate survey of 20,241 food products in the U.S. found twenty five different types of PAL terminology (Pieretti et al., 2009). Many consumers incorrectly believe that different terminology carries different degrees of risk and then make decisions accordingly (Sheth et al., 2008; Verrill and Choiniere, 2009; Noimark et al., 2009). Studies have found that inconsistencies in both PAL usage and terminology
among food manufacturers leads many consumers to believe that PAL is not credible and therefore they ignore it completely (Hefle et al., 2007; Noimark et al., 2009; Hourihane et al., 2011; Zurzolo et al., 2013). It is clear that the current PAL scheme is not working. Though initially designed to be helpful, many consumers find PAL to be frustrating and confusing.

**Recommendations for Advancing Allergen Labeling in the U.S.**

1. **Allergen Thresholds**

   The U.S. has made significant advances in allergen labeling over the years, but there are still many concerns and problems that need to be addressed. First, defining allergen thresholds, or limits below which only the most sensitive allergic subjects might react, would drastically improve the credibility of PAL. “Thresholds,” are also sometimes referred to as “action levels,” “reference doses,” or “minimum eliciting doses” (Taylor and Hefle, 2006; Walker et al., 2016). As stated by Allen et al. (2014a), “establishment of a reliable labeling system that is informed by evidence and practical to use will not only enhance the safety and credibility of precautionary labeling but also enable manufacturers to minimize its overuse through a formal risk assessment tool. This will in turn provide increased consumer confidence in their validity and reliability and enable allergic consumers to eat a wider variety of food with safety and confidence.” The Allergen Bureau of Australia and New Zealand has created such a labeling system, called the Voluntary Incidental Trace Allergen Labeling (VITAL) scheme. The VITAL scheme was developed as a formal, transparent, research-based risk assessment tool for the application of PAL. The most recent version of VITAL, VITAL 2.0 which was launched
in 2012, defines two action levels. Food manufacturers can calculate action levels for a specific product using an online interactive tool that takes into account research-based reference doses as well as reference amount per serving size. “Action Level One” does not require PAL. Food products that fall into Action Level One have a concentration of allergens below the threshold, and therefore have a low chance of adverse reaction.

“Action Level Two” requires a “May be present” allergen statement. Food products that fall into Action Level Two have a concentration of allergens at or above the threshold, and therefore have a high chance of adverse reaction. The thresholds provided by VITAL 2.0 vary by allergen (Table 1.1).

<table>
<thead>
<tr>
<th>Allergen</th>
<th>Proposed ED</th>
<th>Protein Level (mg)</th>
<th>Suggested clinically relevant(^1) RM allergen protein concentrations (mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peanut</td>
<td>ED01</td>
<td>0.2</td>
<td>2-10</td>
</tr>
<tr>
<td>Cow’s milk</td>
<td>ED01</td>
<td>0.1</td>
<td>1-10</td>
</tr>
<tr>
<td>Egg</td>
<td>ED01</td>
<td>0.03</td>
<td>0.3-5</td>
</tr>
<tr>
<td>Hazelnut</td>
<td>ED01</td>
<td>0.1</td>
<td>1-10</td>
</tr>
<tr>
<td>Soy</td>
<td>ED05</td>
<td>1.0</td>
<td>10-100</td>
</tr>
<tr>
<td>Wheat</td>
<td>ED05</td>
<td>1.0</td>
<td>10-100</td>
</tr>
<tr>
<td>Cashew</td>
<td>ED05</td>
<td>2.0(^2)</td>
<td>20-100</td>
</tr>
<tr>
<td>Mustard</td>
<td>ED05</td>
<td>0.05</td>
<td>0.5-5</td>
</tr>
<tr>
<td>Lupin</td>
<td>ED05</td>
<td>4</td>
<td>40-200</td>
</tr>
<tr>
<td>Sesame</td>
<td>ED05</td>
<td>0.2</td>
<td>2-10</td>
</tr>
<tr>
<td>Shrimp</td>
<td>ED05</td>
<td>10</td>
<td>100-1000</td>
</tr>
<tr>
<td>Fish</td>
<td>ED05</td>
<td>0.1(^2)</td>
<td>1-10</td>
</tr>
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Where EDxx is the eliciting dose for xx% of the allergic population

\(^1\)Assuming a minimum portion size of 100 g

\(^2\)Provisional

(Walker et al., 2016)
Three advantages of the VITAL labeling system are: (1) thresholds are defined, (2) thresholds are calculated based on reference doses and serving size, and (3) PAL terminology is consistent. The VITAL scheme promotes uniformity, transparency, and clarity for food manufacturers and consumers alike. However, the VITAL scheme also has some disadvantages. At this time, participation in the VITAL scheme is voluntary, as the name implies, and therefore industry implementation has been limited (Allen et al., 2014a). Additionally, thresholds only protect the majority of consumers with food allergy. Crevel et al. (2008), explains that, “Individual experimental thresholds in a study lie between the No Observed Adverse Effect Level (NOAEL), the highest dose observed not to produce any adverse effect, and the Lowest Observed Adverse Effect Level (LOAEL), the lowest dose that is observed to produce an adverse effect.” These individual patient thresholds must then be translated into population thresholds for food safety regulation purposes. Economic and experimental design limitations prevent allergen thresholds from being established in absolute terms. That being said, the vast majority of stakeholders believe the benefits of defining thresholds outweigh the drawbacks (Crevel et al., 2008).

Some countries have already defined mandatory allergen labeling thresholds. In Japan, the threshold dose is 10 mg/kg. The following allergens require labeling if they are present at levels at or above the threshold: eggs, milk, wheat, buckwheat, peanuts, shrimp/prawn, and crab. Labeling is strongly recommended, though not required, for the following allergens: abalone, squid, salmon roe, orange, kiwifruit, beef, walnut, salmon,
mackerel, soybean, chicken, banana, pork, Matsutake mushroom, peach, yam, apple, gelatin, sesame, and cashew nut (Akiyama et al., 2011; University of Nebraska - Lincoln, 2013). In Switzerland, the “big eight” allergens must be labeled if their concentration exceeds 1000 mg/kg (100 mg/kg gluten for cereals), even if they are not part of the recipe (Stephan et al., 2004; Allen et al., 2014b). The “big eight” allergens are the same as the “major food allergens” in the U.S.: milk, eggs, fish, crustacean shellfish, tree nuts, peanuts, wheat, and soybeans. It is highly recommended that the U.S. follow these examples and define threshold doses for the major food allergens.

One caveat of these examples is that the threshold doses used in Japan and Switzerland need to be updated to protect larger proportions of the allergic population. Allen et al. (2014b) discusses how the current threshold in Japan, equivalent to 10 mg/kg, might not protect consumers with hazelnut or milk allergy. For the majority of allergens, “an allergic consumer would need to eat 1 kg of a food product to be exposed to 10 mg of allergen, a serving size greater than that which would normally be expected.” However, the eliciting dose is much smaller for hazelnut and milk allergy. Consuming a realistic 10 g serving of a food with less than 10 mg/kg hazelnut allergen (and thus, no allergen label would be required) could contain sufficient allergen to trigger an allergic reaction in 1 in 100 hazelnut-allergic individuals. Similarly, the authors expressed concern that a threshold dose of 10 mg/kg for cow’s milk might not protect up to 1 in 10 milk-allergic children (Allen et al., 2014b). Switzerland’s allergen threshold, which equates to 1000 mg/kg, is even higher than that of Japan. Consequently, it is protective of an even smaller proportion of the food-allergic population. In the case of peanut, 1000 mg/kg is predicted
to cause an allergic reaction in up to 50% of peanut-allergic individuals (Allen et al., 2014b), which is significant, particularly since peanut allergy is oftentimes life-threatening.

At this time, it appears that the VITAL reference doses are the most reliable threshold doses available. According to Allen et al. (2014a), “because the [VITAL] reference doses are based on the ED01 value (or the 95% lower CI of the ED05 value for the less common foods), these doses would provide a level of protection of 99% for the allergic population represented in various challenges. The dose-distribution models predict that only 1% of the allergic population would not be fully protected when consuming foods with the reference doses of a particular allergen.” Accordingly, it is recommended that the U.S. adopt the VITAL scheme for defining threshold doses and analyzing the need for PAL. Defining allergen thresholds would greatly increase the accuracy and credibility of PAL, and would ultimately aid consumers with food allergy in making more informed decisions.

2. Allergen Labeling of Non-Prepackaged Foods

Another shortcoming of the allergen labeling system in the U.S. is that it does not regulate the labeling of allergens in non-prepackaged foods, such as those sold in foodservice establishments. The Food Standards Agency of the U.K. identified unclear labeling and incorrect allergen information provided at a point of sale as a point of weakness in the food chain (Walker et al., 2016). Furthermore, Brough et al. (2015) found that the majority of fatal allergic reactions to peanuts and tree nuts occur outside of the home, following exposure to allergens in non-prepackaged foods. It is clear that
allergen labeling of non-prepackaged foods is a serious issue that needs to be addressed. The Food Standards Code of Australia and New Zealand was first published in December 2000 and most recently revised in March 2016. The Code requires that if food is not in a package or if it is not required to have a label, allergen information must be displayed in connection with the food or provided to the purchaser if requested (Food Standards Australia New Zealand, 2016). Though this requirement is good in theory, the actual implementation has been less than ideal. The Food Standards Australia New Zealand (FSANZ) conducted a study from 2008 to 2009 to examine the perspectives, attitudes, and behaviors of consumers with (or who have dependents with) food allergy in regards to allergen labeling. The results showed that the majority of respondents encountered problems when eating out. Around half of respondents reported that information provided by vendors of unpackaged food was not satisfactory. The main reasons listed for why information was not considered adequate by respondents included: staff not knowing what was in the food or being unable to find the ingredients (42%), incorrect or incomplete information leading to the allergen being consumed (23%), staff unaware of allergen presence/cross-contact (23%), staff not aware of the consequences/’uneducated’ about seriousness (14%), staff reluctant to commit that the food does not contain an allergen (11%), and cannot trust the information given (10%) (Food Standards Australia New Zealand, 2009). It seems that this requirement needs to be more closely regulated, possibly in conjunction with allergen education for vendors and retailers, in order to be more effective.
The European Union (E.U.) passed a similar law: the E.U. Food Information for Consumers Regulation (EU FIC), effective December 2014. Non-prepackaged foods made with intentionally added allergen-containing ingredients will now require allergen labeling. Regarding EU FIC, a Guide to Compliance document supplied by the Department for Environment Food & Rural Affairs states that allergen information can be “supplied on the menu, on chalk boards, tickets or provided verbally by an appropriate member of staff as well as in other formats made available to the consumer. It must be clear and conspicuous, not hidden away, easily visible, and legible. If the information is to be provided verbally by a member of staff then it is necessary to make it clear that the information can be obtained by asking a member of staff by means of a notice, menu, ticket or label that can easily be seen by customers. It is no longer enough for [a food business operator] to say that they do not know whether or not a food contains an allergen listed in Annex II and deny any knowledge, nor is it enough to say that all their foods may contain allergens. Allergen information must be specific to the food, complete and accurate” (Department for Environment Food & Rural Affairs, 2012). The allergens listed in Annex II of EU FIC are: cereals containing gluten (namely wheat, rye, barley, oats, spelt, kamut or their hybridized strains), eggs, milk, fish, crustaceans, molluscs, peanuts, tree nuts (namely almonds, hazelnuts, walnuts, cashews, pecans, Brazil nuts, pistachios, and macadamia nuts/Queensland nuts), sesame seeds, soya, celery and celeriac, mustard, lupin and Sulphur dioxide and sulphites at concentrations of more than 10 mg/kg (Food Standards Agency, undated). At this time, EU FIC only applies to intentionally added allergen-containing ingredients and not to allergens present through
allergen cross-contact. However, mandatory allergen labeling of non-prepackaged food in the E.U. is a huge victory for consumers with food allergy. The more transparency there is in allergen usage, the more food-allergic consumers are able to make informed decisions about food consumption. The adoption of a similar law in the U.S. could lead to increased dietary freedom for Americans with food allergy as well. Of note, more comprehensive labeling and regulations would require additional funding and manpower.

3. **Official Detection Methods**

U.S. allergen legislation has not identified an official detection method (or methods) to aid manufacturers and regulators in detecting allergens accurately and consistently. Official methods are needed because analytical results can vary significantly between detection methods and between manufacturers. Official methods are especially needed when there are defined thresholds, as small differences in detection could be the difference between PAL being required for a food product or not. Japan is the only country that has released official methods of detection to regulate and quantify allergens in processed foods. Japan’s official methods include allergen screening using two kinds of ELISA immunoassay kits. If the ELISA result is positive, Western blotting analysis is used to confirm egg and milk allergens, while PCR analysis is used to confirm wheat, buckwheat, peanut, shrimp/prawn, and crab allergens. Specifications and standardization of extraction buffers, reference materials, and standard solutions for testing allergenic ingredients have also been developed (Akiyama et al., 2011). Releasing official methods of detection along with allergen thresholds in the U.S. could vastly improve the credibility and objectivity of PAL.
4. A Global Allergen Framework

Many countries have defined common allergens that require mandatory labeling on prepackaged foods. However, these allergens differ from country to country (Table 1.2). Furthermore, the specific species included in different allergen categories differs from country to country (Table 1.3). For instance, the E.U. considers pine nuts to be seeds, whereas the U.S. and Canada consider pine nuts to be tree nuts. These differences can make it difficult for consumers with food allergy to make informed decisions. If an American is allergic to pine nuts, and usually avoids prepackaged food products containing tree nuts, exhibiting the same behavior in a country that is part of the E.U. could lead to a potentially life threatening anaphylactic reaction, because in the E.U., pine nuts might be generically labeled as ‘seeds’ on the label. Another source of confusion is the classification of coconut and lychee as tree nuts in the U.S., when in fact, coconut palms are not trees but ferns and lychee is a fruit (Allen et al., 2014b). Inconsistencies in which allergens are required to be labeled, which species fall into which allergen categories, and how these allergens are declared on food labels can make traveling to foreign countries a difficult task for consumers with food allergy.

Allen et al. (2014b) explains that, “the global nature of food production and manufacturing makes harmonization of allergen labeling regulations across the world a matter of increasing importance.” The need for a global allergen framework is exemplified in the following example: Six consumers with peanut allergy in various regions of Australia experienced significant allergic reactions after consuming seafood products with a crumb coating provided by a Chinese food supplier. The soy flour in the
<table>
<thead>
<tr>
<th>Country</th>
<th>Milk</th>
<th>Eggs</th>
<th>Fish</th>
<th>Crustacean</th>
<th>Tree nuts</th>
<th>Peanuts</th>
<th>Soybeans</th>
<th>Wheat</th>
<th>gluten-containing cereals</th>
<th>Other</th>
<th>Maltese</th>
<th>Celery</th>
<th>Mustard</th>
<th>Sesame</th>
<th>Lupin</th>
<th>Sulphur dioxide³</th>
<th>Other</th>
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<td>X</td>
<td>X</td>
<td>X</td>
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<td>X</td>
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<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
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<td>X</td>
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<td>O⁵</td>
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<td>O⁵</td>
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<tr>
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<td>X</td>
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<td>X</td>
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<td>Malaysia</td>
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<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
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<td>X</td>
<td>X</td>
<td>X</td>
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<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
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<tr>
<td>South Africa</td>
<td>X</td>
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<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>South Korea</td>
<td>X</td>
<td>X</td>
<td>X⁸</td>
<td>X⁶</td>
<td>X</td>
<td>X</td>
<td>X⁷</td>
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<td>United States</td>
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<td></td>
</tr>
</tbody>
</table>

Table 1.2: Allergen labeling by country

The “Big-8”

- Milk
- Eggs
- Fish
- Tree nuts
- Peanuts
- Soybeans
- Wheat
- Other gluten-containing cereals
- Maltese
- Celery
- Mustard
- Sesame
- Lupin
- Sulphur dioxide³
- Other
The 28 constituent member states of the European Union (EU) are: Austria, Belgium, Bulgaria, Croatia, Cyprus, Czech Republic, Denmark, Estonia, Finland, France, Germany, Greece, Hungary, Ireland, Italy, Latvia, Lithuania, Luxembourg, Malta, Netherlands, Poland, Portugal, Romania, Slovakia, Slovenia, Sweden, and the United Kingdom.

The following countries use Codex wording in their regulatory frameworks: Barbados, Chile, Papua New Guinea, Philippines, St. Vincent, and the Grenadines. The wording for the Papua New Guinea uses the term “shellfish” instead of “Crustacean.” It is not clear if this is intended to include Molluscan shellfish. Mongolia cites the CODEX standard by reference.

1 Not an actual food allergen, but a food intolerance
2 Tartrazine
3 Fish includes salmon roe, salmon, and mackerel. Mollusk includes abalone and squid. Other includes oranges, cashew nut, kiwifruit, beef, walnuts, soybeans, chicken, bananas, pork, “matsutake” mushrooms, peaches, yams, apples, and gelatin (University of Nebraska - Lincoln, 2013)
4 Only shrimp/prawn and crab
5 Only buckwheat
6 Only mackerel
7 Only mackerel
8 Pork, peaches, and tomatoes

Where “X” signifies mandatory labeling and “O” signifies recommended but optional labeling.

Table edited from Gendel (2012) and Allen et al. (2014b).
### Table 1.3: Allergen category definitions by country

<table>
<thead>
<tr>
<th>Country</th>
<th>Cereals</th>
<th>Fish</th>
<th>Crustaceans</th>
<th>Tree Nuts</th>
</tr>
</thead>
<tbody>
<tr>
<td>Australia/New Zealand</td>
<td>Same as Codex</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Canada</td>
<td>Wheat or triticale, plus</td>
<td>“gluten” as protein from</td>
<td></td>
<td>Almonds, Brazil nuts,</td>
</tr>
<tr>
<td></td>
<td>“gluten” as protein from</td>
<td>barley, oats, rye,</td>
<td></td>
<td>hazelnuts, macadamia nuts,</td>
</tr>
<tr>
<td></td>
<td>hybridized strain</td>
<td>triticale, wheat or a</td>
<td></td>
<td>pecans, pine nuts, pistachios,</td>
</tr>
<tr>
<td></td>
<td></td>
<td>hybridized strain</td>
<td></td>
<td>walnuts</td>
</tr>
<tr>
<td>China</td>
<td>Grain and products</td>
<td></td>
<td>Examples:</td>
<td></td>
</tr>
<tr>
<td></td>
<td>containing gluten</td>
<td></td>
<td>shrimp,</td>
<td></td>
</tr>
<tr>
<td></td>
<td>protein (for example</td>
<td></td>
<td>lobster,</td>
<td></td>
</tr>
<tr>
<td></td>
<td>wheat, rye, barley,</td>
<td></td>
<td>crab</td>
<td></td>
</tr>
<tr>
<td></td>
<td>spelt, or cross-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>breeding products)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>European Union(^1)</td>
<td>Cereals containing</td>
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<td></td>
<td>Almonds, Brazil nuts,</td>
</tr>
<tr>
<td></td>
<td>gluten; i.e., wheat, rye</td>
<td></td>
<td></td>
<td>cashews, hazelnuts,</td>
</tr>
<tr>
<td></td>
<td>barley, oats, spelt,</td>
<td></td>
<td></td>
<td>macadamia nuts,</td>
</tr>
<tr>
<td></td>
<td>kamut or their</td>
<td></td>
<td></td>
<td>pecans, pistachio</td>
</tr>
<tr>
<td></td>
<td>hybridized strains</td>
<td></td>
<td></td>
<td>nuts, walnuts</td>
</tr>
<tr>
<td>Hong Kong</td>
<td>Same as Codex</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Japan</td>
<td>Wheat, buckwheat</td>
<td></td>
<td>Shrimp, crab</td>
<td></td>
</tr>
<tr>
<td>South Korea</td>
<td>Wheat, buckwheat</td>
<td>Mackerel</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mexico</td>
<td>Cereals containing</td>
<td></td>
<td>Shrimp, crab</td>
<td></td>
</tr>
<tr>
<td></td>
<td>gluten</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>United States</td>
<td>Wheat</td>
<td>Examples: bass,</td>
<td>Examples:</td>
<td>Examples(^3):</td>
</tr>
<tr>
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<td></td>
<td>flounder, cod</td>
<td>shrimp,</td>
<td>almonds, pecans,</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>crab, lobster</td>
<td>walnuts</td>
</tr>
<tr>
<td>Codex(^2)</td>
<td>Cereals containing</td>
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</tr>
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<td></td>
<td>gluten; i.e., wheat, rye</td>
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</tr>
<tr>
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<td>barley, oats, spelt or</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>their hybridized strains</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

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\(^1\)The 28 constituent member states of the European Union (EU) are: Austria, Belgium, Bulgaria, Croatia, Cyprus, Czech Republic, Denmark, Estonia, Finland, France, Germany, Greece, Hungary, Ireland, Italy, Latvia, Lithuania, Luxembourg, Malta, Netherlands, Poland, Portugal, Romania, Slovakia, Slovenia, Sweden, and the United Kingdom.

\(^2\)The following countries use Codex wording in their regulatory frameworks: Barbados, Chile, Papau New Guinea, Philippines, St. Vincent, and The Grenadines.

\(^3\)Examples given in FALCPA. Full list includes: Almond, beech nut, Brazil nut, butternut, cashew, chestnut (Chinese, American, European, Seguin), chinquapin, coconut, filbert/hazelnut, ginko nut, hickory nut, lichee (lychee) nut, macadamia nut/bush nut, pecan, pine nut/pinon nut, pili nut, pistachio, sheanut, walnut (English, Persian, Black, Japanese, California), and heartnut (FDA, 2016b).

Tabled edited from Gendel (2012).
crumb coating had been contaminated with peanut flour somewhere along the supply chain (Allen et al., 2014b). Allergen tracing can be difficult when supply chains stretch across countries and when allergen labeling differs by country. Furthermore, a study conducted by FSANZ found that consumers had problems identifying the allergen risk of imported foods due to different labeling systems (Food Standards Australia New Zealand, 2009). Though it would be difficult, moving towards a more cohesive global allergen labeling system would benefit consumers, regulators, and manufacturers alike. The U.S. should work with other countries to develop more consistency in labeled allergens, allergen categories, PAL (and thresholds), and allergen labeling format.

**Risk Factors for Allergic Reactions to Food**

Several studies have shown that serious anaphylactic reactions to food are difficult to predict. A study of 83 children diagnosed with peanut allergy found that the severity of the initial reaction to peanut did not predict the severity of subsequent reactions to peanut. The results of the study showed that in patients whose initial reaction was mild, subsequent reactions were life-threatening in 44% of patients, while in patients whose initial reaction was life-threatening, subsequent reactions were life-threatening in 71% of patients (Vander Leek et al., 2000). Similarly, in a study conducted in the UK from 1999 to 2006, over half of food allergy-related deaths were in patients whose previous reactions were considered mild. Epinephrine auto-injector pens were provided in 19 out of the 48 cases (40%), but the patients still died. Of note, only nine pens were used correctly, two of which had expired (Pumphrey and Gowland, 2007). Consequently,
it would be wise to focus on preventing a reaction from occurring in the first place rather than relying on proper treatment of the individual after a reaction has already started.

Brough et al (2015) identified the following factors to be associated with increased risk of life-threatening reactions to peanut: prior anaphylaxis to the same food, teenagers and younger adults, comorbidities (asthma, cardiovascular disease, mastocytosis), concurrent use of medications, and exercise. Although several factors have been associated with a higher risk of severe reaction, no factors have been associated with a lower risk of severe reaction, and thus, severity of allergic reactions is still difficult to predict (Brough et al., 2015). Turner et al (2016) reviewed the evidence regarding factors that might be used to identify those at more risk of severe allergic reactions to food. However, it was concluded that healthcare professionals are unable to reliably identify allergic individuals most at risk of severe anaphylaxis at this time. The authors explained that, “A previous anaphylactic episode and asthma are risk factors, but both are limited in terms of predictive value in clinical practice. Further research is required to understand the interplay of factors that result in severe life-threatening or fatal anaphylaxis, in order to improve risk stratification of allergic individuals” (Turner et al., 2016). These studies are in agreement with the conclusions reached by the National Institute of Allergy and Infectious Diseases-sponsored expert panel, who worked to develop the “Guidelines for the Diagnosis and Management of Food Allergy in the United States.” The Guidelines are a harmonized guide to the best clinical practices related to food allergy across numerous medical specialties. The panel identified the following as one of the current gaps in the published literature: the factors that may cause
higher morbidity and mortality from food allergy (aside from the association with asthma) (Panel, 2010). Though serious anaphylactic reactions to food are unpredictable in nature, some circumstances are associated with an increased risk of allergic reaction. Therefore, more precautions, such as proper allergen labeling, should be taken in these circumstances. More comprehensive labeling would allow food-allergic consumers to make safer food choices and prevent many reactions from occurring in the first place.

1. Foodservice Establishments

Consumption of food prepared away from home has significantly increased in the last several decades. According to the USDA, “In 1970, 25.9 percent of all food spending was on food away from home; by 2012, that share rose to its highest level of 43.1 percent” (USDA Economic Research Service, 2016). The rise in food consumed away from home is concerning because a significant proportion of fatal food allergic reactions occur at foodservice establishments. A study by Bock et al. (2001) investigated 32 cases of fatality due to anaphylactic reactions to food. The cases were voluntarily reported to a national registry between 1994 and 1999. The results showed that 84% of cases occurred outside of the home, with 31% occurring in restaurants and similar establishments (i.e., country club, university cafeteria, banquet, and hotel bar). In all cases, individuals were not aware that the food about to be ingested contained life-threatening allergens. A follow-up study by the same authors analyzed 31 additional cases reported to the national registry between 2001 and 2006. Out of the 29 cases with known locations, the results showed that, 72% of cases occurred outside of the home, with 28% occurring in restaurants and similar establishments (i.e., fast food establishment and carnival booth).
(Bock et al., 2007). The results of these two studies show that about 1 in 4 fatal food allergic reactions are caused by consuming food from a foodservice establishment. More recent studies have found similar patterns. In 2007, a survey conducted at the Food Allergy & Anaphylaxis Network conference found that out of 294 respondents, 34% had experienced at least one food allergic reaction at a restaurant, and of those, 36% had experienced at least three reactions (Wanich et al., 2008). In a literature review of 24 articles regarding frequency, severity, and causes of unexpected allergic reactions to food, it was reported that unexpected reactions took place in restaurants 21-31% of the time (Versluis et al., 2015). The frequency of allergic reactions, particularly fatal ones, at foodservice establishments highlights the need for more stringent allergen labeling in these environments. Consumers have the right to know what is in their food so they can make informed decisions about which foods to consume. A study conducted in the U.K. of 73 “take-away” (fast food) establishments found that only two of the sixty-two premises (3%) visited displayed allergy warning stickers, but peanut was found in 21% of meals requested to be peanut-free. The authors stated that, “in a worst case scenario, the findings from this study indicate that one in five times a peanut allergic consumer visits a take-away, they are putting their life at risk” (Leitch et al., 2005). A study conducted by the Environmental Health Specialists Network (EHS-Net) found that allergen information was only available on menus 22% of the time, available in the front of the restaurant 23% of the time, and available in the kitchen 36% of the time. Several limitations of the study (i.e., only English-speaking managers and staff were included, interviewed workers were chosen by managers rather than randomly selected, and the low participation rate
(32.6%) led the authors to believe that these results might actually be an overrepresentation of better and safer restaurants (Radke et al., 2016). Accordingly, the availability of allergen information in restaurants might be even lower than the percentages reported by this study. Availability of allergen information is necessary to allow consumers to make informed decisions and to aid preparation staff in providing allergen-free meals when requested.

Proper training of restaurant staff has been proposed as a possible solution to reducing the number of food-induced anaphylactic reactions in foodservice establishments, but it is believed that allergen labeling might be a more reliable, objective method of informing consumers of allergens. The efficacy of training could be diminished by factors such as high employee turnover rates, poor knowledge retention, and time constraints of the restaurant, just to name a few. Several studies have shown major shortcomings in current foodservice workers’ knowledge of food allergens. The EHS-Net study collected data from interviews with restaurant managers and staff. Twelve percent of managers and staff incorrectly believed that a person with food allergy can safely consume a small amount of that allergen, and less than half of respondents had received food allergy training while working at their current restaurant (45% of managers, 44% of food workers, and 34% of servers). Though food allergy training was associated with a positive attitude towards serving customers with allergens, the trainings were not found to be effective. The authors stated that, “either [the trainings] do not impart enough food allergy knowledge or do not result in retention of that knowledge […] Further research could explore which training techniques are most effective and
result in long-term retention of important food allergy information” (Radke et al., 2016). A related study on foodservice workers conducted in the Philadelphia area found the following: 11.7% incorrectly believed that customers with food allergy can safely consume a small amount of that food, 10.7% incorrectly believed that removing an allergen from a finished meal (e.g., removing the nuts) may be all that is necessary to provide a safe meal for a food-allergic customer, and 6.7% incorrectly believed that cooking (for example, frying) can stop food from causing allergies. These seemingly innocent misconceptions can have fatal consequences for sensitive consumers. The majority of participants could only name zero or one preventive measures out of seven “best practices” to reduce the risk of food allergy adverse events in restaurants. The workers also expressed low participation levels when asked if they would follow each of the seven practices in an effort to reduce the risk of food allergy adverse events (Table 1.4).

Table 1.4: Foodservice worker participation in allergy management best practices

<table>
<thead>
<tr>
<th>Step</th>
<th>Description</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Step 1</td>
<td>Communicate with patron to clarify allergy and make recommendations</td>
<td>28.0%</td>
</tr>
<tr>
<td>Step 2</td>
<td>Record allergy/communicate with staff</td>
<td>11.3%</td>
</tr>
<tr>
<td>Step 3</td>
<td>Check ingredients</td>
<td>21.5%</td>
</tr>
<tr>
<td>Step 4</td>
<td>Use fresh (uncontaminated) ingredients/avoid cross-contact</td>
<td>23.1%</td>
</tr>
<tr>
<td>Step 5</td>
<td>Sanitize equipment/surfaces and use new instruments</td>
<td>37.1%</td>
</tr>
<tr>
<td>Step 6</td>
<td>Clean hands or change gloves</td>
<td>19.9%</td>
</tr>
<tr>
<td>Step 7</td>
<td>Verify order and/or deliver separately</td>
<td>4.8%</td>
</tr>
</tbody>
</table>

The proportion of workers (n = 186) who said they would follow each of the outlined preventive measures to reduce the risk of food allergy adverse events (Dupuis et al., 2016).
Foodservice worker misconceptions regarding allergies are further exemplified in a study conducted in the U.K. on take-away meals. Only 16% of the orders that were requested to be peanut-free prompted any response in the staff, for example consulting with the chef. Just over 11% of the staff assured the customer that the meal was peanut-free when analytical testing found otherwise. The authors proposed that the inaccurate allergen information provided by staff was due to lack of knowledge regarding trace peanut cross-contact (as opposed to visible peanut content) (Leitch et al., 2005).

Although consumers with food allergy are advised to inquire about the preparation and ingredients of the food they are planning to eat when eating outside of the home, these studies show that many times both inaccurate information can be provided by staff and insufficient cross-contact prevention measures can be taken by the kitchen. These shortcomings can result in a dangerous perception by the consumer that the food is safe to eat and, in a worst case scenario, end in death. Comprehensive allergen labeling in foodservice establishments, including potential for cross-contact, would add a level of objectivity to allergen information provided by foodservice workers. Proper allergen labeling based on a hazard analysis would be a more reliable method of identifying which foods are safe for food-allergic consumers, compared to relying on the accuracy of information provided by staff or the adequacy of cross-contact prevention measures taken by the kitchen. Though allergen labeling would not eliminate all allergic reactions at foodservice establishments, it is believed that it could significantly reduce their occurrences.
Food-allergic consumers face many challenges when eating at foodservice establishments. Leftwich et al. (2011) found that nut-allergic consumers face the following challenges when eating out: restricted food and restaurant choices, uncertainty and anxiety regarding unfamiliar foods and restaurants, language barriers with staff, and social embarrassment. Lack of desire to communicate allergies with staff led to risk-taking behavior. The inconvenience of communicating allergen information to staff at every dining occasion leads many consumers to develop a strategy of sticking to foods that have been safe for them to consume in the past. Unfortunately, slight changes in ingredients or formulation can result in a serious food-induced allergic reaction. Weiss and Munoz-Furlong (2008) report such an incident: “An 18-year old female university student ordered an apple dessert, which she had eaten safely in the past, at a university dining hall. However, the dining hall had recently changed the ingredients by adding nuts to the dessert. Not being aware of this change resulted in her death.” To avoid such risks, many consumers with food allergy avoid dining out altogether. Wanich et al. (2008) reported that rate of “never eat” was 20% for fast food, 25% for informal dining, and 19% for formal dining establishments in adults with food allergy. Such restrictions have a significant negative impact on the quality of life for families with a food-allergic individual. The inconvenience and embarrassment of conveying food allergies to foodservice staff could be circumvented by providing allergen information to customers in an easily accessible format. Allergen labeling in foodservice establishments could also increase the dining freedom of families with a food-allergic individual. Overall, eating out is a difficult and sometimes risky task for consumers with food allergy, but with more
stringent allergen labeling in foodservice establishments, consumers would be able to make more informed decisions about food consumption outside of the home. Of note, it would still be recommended that consumers mention their allergy to the restaurant staff so that the staff could make an effort to avoid allergen cross-contact.

2. Dessert Foods

In addition to foodservice establishments, several studies have found that dessert foods pose an increased risk to food-allergic consumers. Gern et al. (1991) evaluated six case reports of patients with milk allergy who had adverse reactions after eating a product that was labeled as dairy-free. The results showed that 50% of cases were caused by frozen dessert products. A study conducted to determine the factors and patterns associated with food allergic reactions in restaurants and other food establishments analyzed 156 episodes from 129 distinct subjects/parental surrogates. Ice cream shops and bakeries/donut shops were commonly cited establishments, at 14% and 13%, respectively, and desserts were found to be the most commonly cited meal course at 43%. The authors noted that baked goods and ice cream appear to pose particular risk to food-allergic consumers (Furlong et al., 2001). Two studies investigated cases of fatal food-induced anaphylaxis in different time frames. The results showed that when the culprit food was known, nine of twenty-one fatalities (43%) and twelve of thirty-one fatalities (39%), were caused by dessert foods (including baked goods, candy, and ice cream) (Bock et al., 2001, 2007). Based on this data, the authors suggested that food-allergic consumers should avoid eating desserts and bakery goods, especially when consumed away from home (Bock et al., 2007). As dessert foods appear to pose an increased risk to
food-allergic consumers, an allergen hazard analysis would be particularly advisable in bakeries, ice cream shops, and similar establishments.

3. **Cross-Contact**

Cross-contact is another commonly cited source of food allergic reactions. Cross-contact at food establishments can be due to shared utensils, preparation areas, cooking oil, etc. (Weiss and Munoz-Furlong, 2008). A study entitled “Food allergy: Gambling your life away on a take-away meal” highlights the potentially life-threatening consequences of the lack of knowledge regarding cross-contact in the food industry. The sampling protocol for the study was as follows: first, the sampling officer ordered a meal containing peanuts. Second, the officer ordered another meal, stressing that he/she wanted a meal suitable for a peanut allergy sufferer and therefore without any peanut ingredients. The meals were kept separate and later analyzed using a commercial enzyme immunoassay. Conditions surrounding the purchases (e.g., the server seeking clarification of the request or the server seeking further information from the chef) were also noted. Results showed that of the 62 sampling pairs that were collected, 21% were positive for peanut protein, with 10% containing more than 1000 µg of peanut protein. Staff reassured the sampling officer of the safety of the peanut-free meal in 11% of the cases. Furthermore, only 2 of the establishments had an allergy warning sticker posted. The authors concluded that, “in a worst case scenario, the findings from this study indicate that one in five times a peanut allergic consumer visits a take-away, they are putting their life at risk. Very few caterers currently provide any prior warnings about potential allergens. Useful awareness of allergy issues is also significantly lacking. Staff were
insufficiently aware in 83.6% of instances to remark on the request for a peanut-free meal and concerning that where positives were found, they gave reassurance that was not warranted” (Leitch et al., 2005). The results of this study demonstrate the high potential for allergen cross-contact in the foodservice industry, which is supported by other studies.

Furlong et al. (2001) investigated peanut and tree nut reactions in restaurants and similar establishments and found that cross-contact was found to be a significant risk for food-allergic consumers. Even when the food establishment was specifically warned of the consumer’s allergy, cross-contact occurred in the following ways: jelly sandwich/jelly jar was contaminated with peanut by shared utensil, ice cream scoop was previously used for nut ice cream, ice cream was contaminated with one peanut candy mixed from toppings bar, and server sprinkled nuts on another dessert, then handled the cookie. Shared cooking or serving supplies were cited as the source of allergen cross-contact in 22% of the 106 cases investigated. It is apparent from these studies as well as several other studies that investigated food service workers’ knowledge regarding food allergens (Abbot et al., 2007; Radke et al., 2016; Dupuis et al., 2016) that cross-contact is a poorly understood concept in the foodservice industry.

Allergen cross-contact can be particularly confusing to untrained foodservice workers because cross-contact is not usually visible, even when it is present in amounts sufficient to cause an allergic reaction. Leitch et al. (2005) validates this reasoning by explaining that the servers’ unwarranted reassurance about the peanut-free meals was, “almost certainly due to the confusion that exists between visible peanut content and trace peanut cross contamination.” Additionally, Abbot et al. (2007) reported confusion
among foodservice employees about the differences in procedures for preventing bacterial cross contamination versus allergen cross-contact. Specifically, the authors explained that, “prevention of pathogen cross contamination has similar elements, in that raw food must not touch cooked food, but sanitation measures include cooking foods thoroughly, which does not work for contamination by allergens.” These reasons, among others, are why allergen cross-contact is still a major issue for allergy sufferers when eating outside the home. A thorough allergen hazard analysis would allow foodservice establishments to better understand and control for allergens. In cases where allergens can’t be controlled for, proper allergen labeling is highly recommended to protect food-allergic consumers.

**Peanut Allergy**

Peanuts (*Arachis hypogaea*) are a popular food due to their low cost, impressive nutrient profile, and taste. Peanuts are high in heart-healthy, monounsaturated fat and are considered a good source of protein, vitamin E, niacin, folate, phosphorus, and magnesium (King et al., 2008). Over one billion pounds of peanuts are produced annually in the U.S., most of which remain in the country for human consumption. A majority (63%) of the peanuts used for human consumption are processed into peanut butter (Chang et al., 2013). Although peanuts are an enormously popular food, particularly in the form of peanut butter, they also pose a substantial threat to peanut-allergic individuals.
1. Prevalence and Impact

Peanut allergy is significant because it typically presents early in life, is severe, has quick onset of symptoms, and does not resolve with age (Hourihane, 1997; Husain and Schwartz, 2013). Peanut allergies affect approximately 0.6-1% of people in developed counties (Husain and Schwartz, 2013), and it has been proposed that the incidence of peanut allergy is increasing (Sicherer et al., 2003; Brough et al., 2015). The estimated prevalence of peanut allergy in the U.S. is 0.4-1.9% among children (Kotz et al., 2011) and 0.7% among adults (Husain and Schwartz, 2013). In the U.S., an estimated 30,000 emergency room visits each year are due to food-induced anaphylaxis, and approximately one third of those visits are peanut-induced (Wen et al., 2007). Unsurprisingly, peanut allergy accounts for the majority of food-induced anaphylaxis incidents (Husain and Schwartz, 2013) and fatalities (Bock et al., 2001; Bock et al., 2007). It is estimated that 50 to 100 deaths each year in the U.S. are due to peanut-induced anaphylaxis (Wen et al., 2007). Bock et al. (2001) reported that out of 32 cases of fatal food-induced anaphylaxis reported to a national registry from 1994 to 1999, 63% were caused by peanut. Out of 31 additional cases reported to the registry from 2001 to 2006, 55% were caused by peanut (Bock et al., 2007).

2. Threshold Dose

Peanut allergy has a low threshold dose compared to other allergens, and therefore peanut-allergic individuals can react to smaller doses of food (Kotz et al., 2011). The threshold dose for peanut using the VITAL scheme (ED01) is 0.2 mg of peanut protein (Allen et al., 2014a). According to Lexmaulová et al. (2013), one peanut
contains about 200 mg protein, so 0.2 mg protein would be equivalent to one one-thousandth (1/1000) of a peanut. Notably, clinical trials have found that LOAEL doses for peanut range from 0.5 to 10,000 mg of whole peanut (Chang et al., 2013). The large degree of variability in LOAEL doses can be explained by the following factors: (1) variability in study design, such as the lowest dose tested, dose increments, time between doses, and form of peanut used; (2) interpersonal factors, such as weight, metabolism, and sensitivity; and (3) intrapersonal factors, such as daily activity level, previous food eaten that day, and stress level (Taylor et al., 2002; Wensing et al., 2002; Taylor et al., 2009; Ballmer-Weber et al., 2015). The wide variety of terminology used to define and measure the threshold dose for peanut is shown in Table 1.5.
Table 1.5: Allergen threshold terminology

<table>
<thead>
<tr>
<th>Terminology</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>No Observed Adverse Effect Level (NOAEL)</td>
<td>The highest dose of peanut or peanut protein observed not to produce any adverse effect (Crevel et al., 2008).</td>
</tr>
<tr>
<td>Threshold Dose</td>
<td>The lowest dose of peanut or peanut protein eliciting a positive reaction (Wensing et al., 2002). The lowest dose of peanut or peanut protein that would elicit mild, objective symptoms in the most sensitive individuals (Taylor et al. 2002). Lies between NOAEL and LOAEL (Crevel et al., 2008).</td>
</tr>
<tr>
<td>Lowest Observed Adverse Effect Level (LOAEL) or Minimum eliciting dose</td>
<td>The lowest dose of peanut or peanut protein observed to produce an adverse effect (Crevel et al., 2008).</td>
</tr>
<tr>
<td>Eliciting Dose (e.g., ED05)</td>
<td>The dose of peanut or peanut protein predicted to provoke a reaction in the specified percentage (e.g., 5%) of the peanut-allergic population (Allen et al., 2014a; Ballmer-Weber et al., 2015)</td>
</tr>
<tr>
<td>Action Level</td>
<td>A defined dose of peanut or peanut protein, based on the reference dose, below which PAL is not required (Allergen Bureau, 2012; Campden BRI, Undated).</td>
</tr>
<tr>
<td>Reference Dose</td>
<td>The dose considered safe for the vast majority (95-99%) of the peanut-allergic population (Allergen Bureau, 2012; Campden BRI, Undated).</td>
</tr>
</tbody>
</table>

3. Allergenic Proteins

Peanuts contain 24-30% protein (Lexmaulová et al., 2013), although only some proteins elicit an allergic reaction. As of December 2016, the World Health Organization Allergen Nomenclature Database had identified seventeen peanut-derived food allergens, Ara h1-Ara h17 (World Health Organization, 2016). Ara h1 and Ara h2 peanut proteins are considered to be the “major peanut allergens” because they cause the majority of adverse reactions (Lexmaulová et al., 2013). Over 90% of peanut-allergic patients have
IgE antibodies exclusively to these proteins, while 45-95% have IgE antibodies to Ara h3. Food processing techniques, such as heating, can affect allergenicity by altering protein structure. A study by Beyer et al. (2001) found that the amount of Ara h1 protein was reduced in fried and boiled peanuts compared to roasted peanuts, resulting in significantly less IgE binding. Furthermore, there was significantly less IgE binding to Ara h2 and Ara h3 in boiled and fried peanuts compared to roasted peanuts, even though there was not less Ara h2 and Ara h3 protein present. It was concluded that boiling and frying peanuts reduces peanut allergenicity compared with dry roasting. A related study found that peanuts roasted 10 to 15 min (which emulates conventional oven roasting) contained 22-fold higher extractable Ara h1 compared to raw peanuts (Pomés et al. 2006). These findings are significant because roasting is one of the most commonly used peanut processing methods in the U.S. Higher allergenicity of roasted peanuts and peanut products, like peanut butter, would lead to lower provoking doses of these products compared to unroasted ones.

Allergen Hazard Analysis

1. FSMA Allergen Control Programs

Allergen cross-contact can occur at many points during the manufacturing and selling of food. Even small amounts of allergenic proteins can cause allergic reactions in sensitive individuals, which is why allergen cross-contact is a serious issue in the food manufacturing and foodservice industries. Consequently, part of the new FSMA Preventive Controls for Human Food rule requires covered establishments to have a food
allergen control program (ACP) in place. The first step of the program is conducting a hazard analysis, including hazards that may occur naturally, may happen unintentionally, or may be intentionally introduced for economic gain. Food allergens are classified as chemical hazards. The second step of the program is to specify controls that will minimize or prevent the identified hazards. Preventive controls can include controls at critical control points and other controls which are necessary for food safety. Food allergen controls are procedures for preventing allergen cross-contact during storage, handling, and use as well as correctly labeling the finished food if it contains any of the eight major food allergens. Sanitation controls ensure that the facility’s sanitation practices are adequate to significantly minimize or prevent hazards, including food allergens. Management of preventive controls includes: monitoring, corrective actions and corrections, verification and validation, product testing and environmental monitoring, and record keeping. Allergen controls also apply to various aspects of cGMPs, such as personnel, plant and grounds, sanitary operations, equipment and utensils, processes and controls, and warehousing and distribution (FDA, 2016c). At this time, ACPs are only required for covered facilities, as specified by FSMA, and do not apply to retail foodservice establishments.

2. Validation of Cleaning Procedures

Allergen cross-contact during food manufacturing can occur from a variety of sources: improper storage of raw materials, carry-over food allergen residue on shared equipment, inadequate facility design (e.g., inadequate air handling), improper handling of rework, and ineffective equipment cleaning and sanitation procedures. Sharing
equipment between allergen-containing and non-allergen-containing products is a common practice in the food industry, mainly because dedicated equipment is not feasible for most manufacturers. Gendel et al. (2013) reports that out of 463 facilities that were inspected by the FDA for allergen control practices in 2010, 77% of all facilities used shared equipment. When broken down by size, 70% of small, 79% of medium, 80% of large, and 100% of unknown size facilities used shared equipment. When shared equipment is used, validation of cleaning and sanitation procedures for allergen removal is of upmost importance. Many facilities rely on cleaning protocols and production scheduling to control allergen cross-contact on shared equipment and processing lines (Taylor et al., 2006; Jackson et al., 2008). However, if the cleaning and sanitation procedures do not adequately remove allergens, cross-contact is likely to occur, regardless of scheduling controls. Accordingly, adequate cleaning procedures are oftentimes considered the first line of defense against allergen cross-contact (Jackson et al., 2008). The importance of cleaning validation for allergen removal is exemplified in the following studies, where inadequately cleaned equipment caused allergic reactions in susceptible individuals: two different instances of milk allergen in sorbet (Jones et al., 1992; Laoprasert et al., 1998) and peanut allergen in sunflower butter (Yunginger et al., 1983). A 2005 survey conducted by the Institute of Food Technologists investigated strategies used by food manufacturers to address allergen concerns. Representatives from 59 food companies were interviewed, representing small, medium, and large companies, as well as 14 different food product categories. Product carry-over (i.e., cross-contact) from shared equipment was identified as a potential allergen source by 69% of small,
95% of medium, and 93% of large companies. “Clean-up” was addressed by company ACPs in 77% of small, 100% of medium, and 94% of large companies. Water and detergents/chemicals were the most commonly used cleaning methods, but the method utilized depended on the food product produced and the manufacturing environment (wet or dry). Overall, the majority of manufacturers (≥ 80%) used written Sanitation Standard Operating Procedures (SSOPs) for allergen control (Table 1.6).

Table 1.6: Cleaning practices used to control allergens in the food manufacturing industry

<table>
<thead>
<tr>
<th>Cleaning Practices Used</th>
<th>SSOPs</th>
<th>Equipment disassembly</th>
<th>Water</th>
<th>Detergents, chemicals</th>
<th>COP (clean-out-of-place)</th>
<th>CIP (clean-in-place)</th>
<th>Cleaning methods validated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Company Size</td>
<td>Percent</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Small, &lt; 100 employees (n = 13)</td>
<td>100</td>
<td>85</td>
<td>69</td>
<td>100</td>
<td>38</td>
<td>31</td>
<td>85</td>
</tr>
<tr>
<td>Medium, 100 - 500 employees (n = 21)</td>
<td>90</td>
<td>90</td>
<td>100</td>
<td>86</td>
<td>62</td>
<td>81</td>
<td>86</td>
</tr>
<tr>
<td>Large, &gt; 500 employees (n = 86)</td>
<td>80</td>
<td>92</td>
<td>93</td>
<td>93</td>
<td>84</td>
<td>83</td>
<td>85</td>
</tr>
</tbody>
</table>

1Sanitation Standard Operating Procedures

(Taylor et al., 2006)

The majority of manufacturers (≥ 85%) also validated their cleaning methods (Table 1.7). However, the authors noted that the cleaning validation question was open-ended and the responses ranged from “visually clean” to outsourcing samples for ELISA
testing. The use of analytical testing as part of ACPs was investigated. Overall, more than 71% of companies conducted testing within their ACPs. Components of sanitation (equipment surfaces, water rinse, and push-through) were tested most frequently, but most companies admitted that they only conducted testing periodically rather than routinely.

Table 1.7: Cleaning verification practices used to control allergens in the food manufacturing industry

<table>
<thead>
<tr>
<th>Verification Procedures Used</th>
<th>Visual inspections</th>
<th>ELISA</th>
<th>Protein detection</th>
<th>Bioluminescence/ATP</th>
<th>Lateral flow devices (dipsticks)</th>
<th>Other</th>
</tr>
</thead>
<tbody>
<tr>
<td>Company Size</td>
<td>Percent (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Small, &lt; 100 employees (n = 13)</td>
<td>100</td>
<td>15</td>
<td>0</td>
<td>38</td>
<td>15</td>
<td>31</td>
</tr>
<tr>
<td>Medium, 100 - 500 employees (n = 21)</td>
<td>90</td>
<td>38</td>
<td>0</td>
<td>43</td>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td>Large, &gt; 500 employees (n = 86)</td>
<td>93</td>
<td>52</td>
<td>14</td>
<td>44</td>
<td>10</td>
<td>13</td>
</tr>
</tbody>
</table>

Table edited from Taylor et al. (2006).

Several studies have evaluated cleaning procedures for allergen control. Stephen et al. (2004) evaluated a cleaning method used for industrial slurry preparation equipment to analyze the risk of peanut and celery allergen cross-contact. The slurry preparation equipment was used to produce worst case scenario recipes with a high content of peanut (30 and 20%) and celery (40%). Following recipe preparation, the slurry equipment was cleaned using the following steps: wash with water (pre-wash), wash with alkaline
solution, wash with water, wash with acidic solution, and wash with water. Wash water was analyzed for peanut and celery allergens using sandwich ELISA and real-time PCR, respectively. A commercial Bradford assay was used to verify the protein content of all samples. The results showed that only rinsing with water (pre-wash step) did not effectively remove allergens. However, following the full cleaning procedure, allergens were not detected in the wash water by any method. From these results, the authors concluded that allergen cross-contact from equipment was unlikely to occur using the prescribed cleaning procedure. A related study evaluated the risks of celery allergen cross-contact via carry-over during fresh-cut vegetable processing (Kerkaert et al., 2012). Various fresh-cut vegetables (leek, celeriac, celery, lettuce, carrots, and soup greens) were washed and samples were taken from the wash water at several time intervals. Crude and net protein content of wash water was analyzed by Kjeldahl analysis. Allergen carry-over to vegetables in subsequent batches was measured using lysozyme as an allergen indicator. The authors stated that, “all industrial wash waters contained a significant amount of protein which illustrates that protein carry-over from the vegetables to the wash water occurs” (Kerkaert et al., 2012). Furthermore, the authors calculated that the degree of carry-over would be sufficient to cause an allergic reaction in celery-allergic individuals, making reuse of wash water during vegetable processing a relevant food safety risk. The methodologies used in these two studies can be used as models to evaluate other cleaning and processing procedures for risk of allergen cross-contact. According to Taylor et al. (2006), analytical testing of water rinse for allergens as a method of sanitation validation is a relatively common practice in the food manufacturing
industry. Though sanitation validation is mainly used in the food manufacturing and processing industries, it would be wise for foodservice retailers to implement similar validation and allergen control procedures. Performing an allergen risk assessment can help companies better understand and control for allergens by identifying potential cross-contact points. Furthermore, cleaning procedure validation could help better inform the need for PAL so that it is not used flippantly (Stephan et al., 2004).

3. Validation of Dipper Wells as a Cleaning Method for Allergen Control

Dipper wells are small, continuously running countertop sinks that are found in many ice cream scoop shops and coffee shops. In the U.S., dipper wells have been approved by the FDA for storage of in-use utensils such as ice cream scoops and barista thermometers. However, Section 3-304.12 D the 2013 Food Code is designed around pathogen control, not allergen control (FDA, 2013). There are several reasons why dipper wells might pose a significant allergen cross-contact risk: (1) water has been shown to be a viable source of allergen cross-contact (Stephan et al., 2004; Kerkaert et al., 2012), (2) ice cream has been deemed a high-risk food (Brough et al., 2015) and ice cream shops have been identified as a high-risk environment for food-allergic consumers (Furlong et al., 2001), (3) unlike microorganisms, allergens do not need time to multiply nor do they need nutrients to survive, and (4) very low doses of allergens can cause immediate and potentially fatal allergic reactions in sensitive individuals. Furthermore, Furlong et al. (2001) reported that an allergic reaction occurred in a nut-allergic individual after the individual’s ice cream was served using an ice cream scoop that had previously been used to serve ice cream which contained nuts. There was no information provided as to
whether the ice cream scoop was rinsed in a dipper well between uses, but this anecdote highlights the need to investigate this matter further. In light of these reasons, it is believed that the use of ice cream dipper wells for utensil storage should be evaluated in regards to allergen control.

To date, studies involving dipper wells have only examined their ability to control microorganisms. In particular, recent studies have evaluated reduced water (RW) dipper well systems compared to traditional, continuous flow (CF) dipper well systems due to water usage concerns. Gibson and Almedia (2015) compared a CF dipper well to a RW dipper well combined with ultraviolet radiation. The experimental design was two-fold. First, a sterile ice cream scoop was inoculated with *E. coli* (in either water or milk medium) and then placed in either the CF dipper well or the RW dipper well for rinse times of 5, 10, or 30 s. Second, the inoculated utensil was evaluated over a 2 h period with inoculations every 5 min to model a continuous use scenario. According to the authors, the treatment times were selected to cover a wide range of acceptable times for cleaning an in-use utensil during periods of high customer volumes. The results showed that the CF dipper well had greater variability in the reduction of *E. coli* compared to the RW dipper well, especially in the water medium. The authors attributed the differences in variability to the distinct dipper well designs. The RW dipper well had a very precise amount of water sprayed for programmed amounts of time, while the CF dipper well was modeled with a sink that was constantly filling and overflowing in an inexact manner. The results also showed that the RW dipper well performed better than the CF dipper well for all rinse times in the milk medium. However, after 2 h of continuous use, the CF
dipper well performed the same or better than the RW dipper well. Overall, the authors concluded that, “a reduced water dipper well that uses significantly less water than a continuous flow dipper well—1.55 L/treatment vs. 8.3 L/treatment, respectively—is capable of controlling and preventing microbial contamination of stainless steel utensils” (Gibson and Almeida, 2015). The same authors compared a recirculating dipper well ozone sanitation system (DWOSS) to a CF dipper well for the control of microbes. The microorganisms tested were: *Escherichia coli*, *Listeria innocua*, PRD1 bacteriophage, and *Staphylococcus aureus*. Inocula was prepared by adding $10^7$-$10^8$ CFUs or PFUs of prepared culture or phage per ml to dechlorinated tap water or 10% skim milk medium.

To evaluate the DWOSS unit, a sterile stainless steel ice cream scoop was placed into the water inoculum and then placed into the dipper well reservoir for 30, 180, or 600 s. To compare the DWOSS unit to the CF system, the same procedure was used but with milk inoculum. Results showed that the DWOSS unit achieved a 5-log reduction in CFU for all rinse times. Comparison of the two systems showed that the DWOSS unit was significantly better at controlling microorganisms for all rinse times compared to the CF unit. Additionally, the CF dipper well treatment resulted in significantly more microorganisms remaining on the basin surface compared to the DWOSS unit. The highest microbe concentrations on the basin surface were found after the 30 s rinse time. The authors found that the DWOSS unit more effectively removed microbes from inoculated water compared to inoculated milk. The authors attributed this phenomenon to the protective effect of fats and proteins found in milk. Overall, the DWOSS unit achieved a greater reduction of all microorganisms on the ice cream scoop, in both high
and low ozone demand media, compared to the conventional CF dipper well. The authors proposed that integrating ozone into a dipper well system could be a potential critical control point for reducing the incidence of microbial contamination during retail food service (Almeida and Gibson, 2016). Though these studies examined microbial control of dipper wells, a similar study design can be used to evaluate allergen control of dipper wells.

**Allergen Detection Methods**

Allergen detection serves many purposes: validation and verification of cleaning procedures, investigation of recalls and incidents, and surveillance and enforcement of labeling requirements. Accurate detection supports consumer safety, business integrity and responsibility, and traceability (Walker et al., 2016). To detect small amounts of allergen cross-contact in food, highly sensitive and specific methods have been developed. Several studies have provided an overview of allergen detection methods (Poms et al., 2004a; Kirsch et al., 2009; Monaci and Visconti, 2010; Prado et al., 2016). Although numerous detection methods are available, Kirsch et al. (2009) explains that, “only ELISA- and PCR-based tests are currently convenient for routine screening and quantification in the catering and food industry, whereas certain other methods are nowadays only applicable in the research field.” ELISA and PCR are both considered to be indirect methods of detection because ELISA measures antibody-antigen complexes and PCR measures allergen coding genes, rather than the allergen itself (Kirsch et al., 2009). However, ELISA and PCR offer several advantages over direct methods (e.g., mass spectrometry), such as cost, ease of use, and availability of commercial kits.
1. **ELISA**

ELISA is the most commonly used method for routine allergen analysis by the food industry and regulatory agencies due to its low cost, relatively simple and quick procedure, and target analyte: protein. ELISA is an immunological technique that utilizes IgG antibodies from immunized animals to detect proteins, referred to as antigens. The method can detect one, several, or total species proteins (allergenic or not) (Poms et al., 2004a; Cucu et al., 2013). Quantification is based on a colorimetric reaction produced by an enzyme-labeled antibody complex. Antigen capture can be direct (by direct adsorption to the plate surface) or indirect (by adsorption via a pre-coated capture antibody on the plate). However, it is mainly the detection step that determines the sensitivity of the ELISA method. Antigen detection can be direct or indirect as well. In direct detection, the enzyme-labeled antibody directly attaches to the antigen and causes a color change directly proportional to the concentration of antigen in the sample. In indirect detection, the enzyme-labeled antibody indirectly detects the antigen. First, an unlabeled primary antibody attaches to the antigen. Then a secondary, enzyme-labeled antibody attaches to the primary antibody and causes a color change directly proportional to the amount of antigen in the sample. Indirect antigen detection has higher sensitivity than direct detection because multiple secondary antibodies can attach to the primary antibody, resulting in greater signal intensity. In sandwich ELISA, the antigen is “sandwiched” between antibodies. Indirect antigen capture is used in combination with either direct or indirect detection. Competitive ELISA is usually used when the antigen only has one antibody binding site. Generally, an unlabeled secondary antibody is immobilized on the
plate. The unlabeled sample antigen (target antigen) is added to a solution with labeled antigen and primary antibodies. The labeled and unlabeled antigens compete for binding sites on the primary antibodies. The antigen-primary antibody complexes then bind to the secondary antibodies on the plate. After washing, the labeled antigen is detected via a colorimetric reaction. The amount of color change is inversely proportional to the amount of target antigen in the sample (ThermoFisher Scientific, undated a). Sandwich ELISA is the most common assay for food allergen detection kits. Commercially available ELISA kits are available for the detection or quantification of wheat, crustaceans, egg, peanut soybeans, milk, almond, hazelnut, mollusks, lupin, sesame, mustard, and buckwheat in complex food matrices. Limit of detection (LOD) of these kits ranges from 0.1 to 20 mg/kg (Prado et al., 2016).

2. *PCR*

 PCR is commonly used to detect genetically modified crop material or microbial pathogens in food (Stephan and Vieths, 2004; Poms et al., 2004a). In recent years, PCR techniques have been developed to detect food allergens due to the method’s high specificity, sensitivity, and precise quantification. The target sequence is usually a DNA segment on the allergenic protein of interest (e.g., Ara h2 for peanut allergen detection), but can be any specific DNA marker (Prado et al., 2016). There are three main types of PCR: traditional PCR, real-time PCR, and PCR-ELISA (Table 1.8).
Table 1.8: Types of PCR

<table>
<thead>
<tr>
<th>Type of PCR</th>
<th>Synonyms</th>
<th>Type of Analysis</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Traditional PCR</td>
<td>PCR, conventional PCR, end-point PCR, ordinary PCR</td>
<td>Qualitative or semi-quantitative</td>
<td>(Poms et al., 2004a)</td>
</tr>
<tr>
<td>Real-time PCR</td>
<td>Quantitative PCR, qPCR</td>
<td>Quantitative or semi-quantitative</td>
<td>(Monaci and Visconti, 2010)</td>
</tr>
<tr>
<td>PCR-ELISA</td>
<td>PCR-ELOSA (enzyme-linked oligosorbent assay)</td>
<td>Qualitative or semi-quantitative</td>
<td>(Prado et al., 2016; Poms et al., 2004a)</td>
</tr>
</tbody>
</table>

In traditional PCR, the target DNA is amplified to a detectable level and then visualized either by staining with a fluorescent dye or by southern blotting following gel electrophoresis (Poms et al., 2004a). DNA amplification consists of three steps: denaturation, annealing, and extension, each determined by a different temperature. In denaturation, the double-stranded DNA template is separated with heat into two single-stranded DNA templates. The mixture is cooled to facilitate annealing, or the attaching of primers to the single-stranded DNA templates. Primers are short, single-stranded sequences that are selected to be complementary to the DNA target region. After the primers are attached, the temperature is adjusted based on the optimum activity temperature for the DNA polymerase used (e.g., Taq polymerase). Extension occurs as DNA polymerase binds to the primer-template hybrids and begins to elongate the DNA strands by adding complementary nucleotides to the template strands. The three steps (denaturation, annealing, and extension) make up one cycle. PCR is an exponential process because the number of target DNA strands doubles after each cycle. By using a
thermocycler to automate the process, a single DNA fragment can be amplified to millions of copies in just a few hours (Goodwin, 2004). DNA can typically be detected after 25-45 cycles (Poms et al., 2004a). Traditional PCR can be used for qualitative or semi-quantitative (if internal standards are used) analysis.

In real-time PCR, DNA copies are measured in real time, unlike in traditional PCR where they are measured at the end-point. Real-time PCR relies on the same amplification technique as traditional PCR, but utilizes a fluorescent dye, such as SYBR Green, or a fluorogenic probe, such as TaqMan, to quantify the DNA. When SYBR Green or other double-stranded DNA binding dyes are used, the dye immediately binds to all double-stranded DNA present in the sample. During amplification, the dye binds to each new copy of double-stranded DNA, so there is an increase in fluorescence proportional to the amount of PCR product produced. In TaqMan-based detection, a reporter dye and quencher dye are attached to the probe. Initially, the quencher suppresses the reporter because they are close in proximity, but during amplification, the polymerase enzyme separates the dyes by cleaving the hybridized probe if the target sequence is present. The free reporter dye creates fluorescence proportional to the amount of amplified PCR product produced, and can be used to quantify the DNA in the original sample (Poms et al., 2004a; ThermoFisher Scientific, undated b). TaqMan-based detection has higher specificity and reproducibility compared to SYBR Green-based detection (ThermoFisher Scientific, undated b). Real-time PCR can be used for relative or absolute (using a calibration curve) quantification. Additionally, an internal standard can be used to compensate for the variability in DNA extraction and amplification.
efficiencies (Kirsch et al., 2009). Real-time PCR is considered less labor-intensive than traditional PCR, but generally requires more expensive equipment. Prado et al. (2016) cites that real-time PCR also offers advantages such as the possibility of using shorter fragments and the ability to more reliably detect highly fragmented DNA over traditional PCR. In PCR-ELISA, the amplified DNA fragments are hybridized to a protein probe and detected by ELISA (Kirsch et al., 2009).

3. **PCR vs. ELISA**

ELISA and PCR both have their own merits and drawbacks. The main advantages of ELISA over PCR are the cost, ease of use, and target analyte (i.e., protein vs. DNA). The major drawbacks of using ELISA for allergen detection are cross-reactivity between similar species (leading to false positives), changes in protein detection due to processing methods, seasonal and geographical variations in protein levels, and the inhibitory matrix effect exhibited by some foods (Monaci and Visconti, 2010; Prado et al., 2016). Conversely, DNA is more specific, more stable to processing methods, exhibits less seasonal and geographical variation, and has reduced matrix effect due to extraction efficiency, making PCR a good alternative to ELISA (Poms et al., 2004a; Monaci and Visconti, 2010; Prado et al., 2016). A major drawback of PCR is that DNA detection does not necessarily indicate the presence of allergenic proteins. Disparity between protein and DNA detection would be particularly important in highly processed matrices such as vegetable oils, pickled products, and canned foods, or in cases where isolated protein is used as an ingredient (Stephan and Vieths, 2004). Still, in the majority of cases, PCR can be a useful screening method to determine if further allergen analysis is needed.
A direct method, such as mass spectrometry, should be used to verify results from both ELISA and PCR when absolute quantification is needed (Kirsch et al., 2009; Monaci and Visconti, 2010). Some other drawbacks of the PCR method are that it is generally more expensive, requires more equipment, and requires more technical skills compared to ELISA. Overall, choice of method depends on numerous factors and should be evaluated on a case by case basis.

Several studies have compared results from various ELISA and PCR methods. Holzhauser et al. (2002) compared a PCR-ELISA to a sandwich-ELISA for the detection of hazelnut in food products. Both techniques were highly specific for hazelnuts, but the sandwich-ELISA showed some cross-reactivity with non-hazelnut foods, while the PCR-ELISA showed no cross-reactivity. Both methods were also highly sensitive, allowing for detection of less than 10 mg/kg of hazelnut in complex food matrices. An analysis of commercial food products showed that the two methods were in good agreement. The sandwich-ELISA had two false negatives samples (both dairy products). The authors hypothesized that the false negatives were due to the acidic conditions or microbial enzymatic activity in the milk products, which may have degraded or denatured the hazelnut protein so that it was not accessible for detection with antibodies. Overall, it was found that both methods were useful tools for trace hazelnut allergen detection in food. Stephan and Vieths (2004) compared a real-time PCR method to a sandwich ELISA method for the detection of peanut in processed food. Both assays were able to detect the lowest level of spiked peanut tested (10 mg/kg) in whole milk chocolate and semisweet chocolate matrices. Furthermore, neither assay showed cross-reactivity with any of the
nuts, legumes, or cereals tested. An analysis of prepackaged food showed that the real-
time PCR assay might have been slightly more sensitive than the ELISA assay, but
overall, the authors stated that, “Although the results of both assays are not in complete
concordance, our data indicate that DNA-based and immunological assays give
comparable results for the detection of peanut traces in processed foods, and that both
assay types are suitable for analyzing foods for the presence of hidden allergens.” In
general, these studies show that ELISA- and PCR-based assays tend to have similar
results.

4. **PCR for Peanut Allergen Detection**

Numerous real-time PCR systems for peanut allergen detection have been
developed, including several multiplex real-time PCR methods which detect multiple
allergens at once (Table 1.9). The detection limits ranged from 0.1 to 100 mg/kg. Several
target genes were utilized, but *Arah2* was the most common.

There are several commercially available PCR kits for peanut detection with
detection limits of 0.1 to 10 mg/kg (Table 1.10). PCR kits are a convenient option for
highly sensitive and reliable detection of allergen DNA. Most kits include a master mix
(typically with an internal amplification control), a positive control, and a negative
control. Several manufacturers also offer a corresponding DNA extraction kit.
Table 1.9: Real-time PCR methods for peanut detection

<table>
<thead>
<tr>
<th>Target Gene</th>
<th>Detection Limit</th>
<th>PCR Method</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arah1</td>
<td>50 mg/kg</td>
<td>Real-time PCR with internal amplification control</td>
<td>(Zhang et al., 2015)</td>
</tr>
<tr>
<td>Arah1</td>
<td>100 mg/kg</td>
<td>Multiplex real-time PCR</td>
<td>(Pafundo et al., 2010)</td>
</tr>
<tr>
<td>Arah2</td>
<td>2 mg/kg</td>
<td>Real-time PCR</td>
<td>(Hird et al., 2003)</td>
</tr>
<tr>
<td>Arah2</td>
<td>2 mg/kg</td>
<td>Real-time PCR</td>
<td>(Stephan and Vieths, 2004)</td>
</tr>
<tr>
<td>Arah2 and ITS1</td>
<td>10 and 0.1 mg/kg, respectively</td>
<td>Real-time PCR</td>
<td>(López-Calleja et al., 2013)</td>
</tr>
<tr>
<td>Arah2</td>
<td>10 mg/kg</td>
<td>Real-time PCR with internal competitive standard</td>
<td>(Holzhauser et al., 2014)</td>
</tr>
<tr>
<td>Arah2</td>
<td>10-50 mg/kg</td>
<td>Multiplex real-time PCR</td>
<td>(Köppel et al., 2010)</td>
</tr>
<tr>
<td>Arah2</td>
<td>100 mg/kg</td>
<td>Multiplex real-time PCR</td>
<td>(Wang et al., 2014)</td>
</tr>
<tr>
<td>Arah3</td>
<td>10 mg/kg</td>
<td>Real-time PCR</td>
<td>(Scaravelli et al., 2008)</td>
</tr>
</tbody>
</table>

Table 1.10: Commercially available PCR kits for peanut detection

<table>
<thead>
<tr>
<th>Brand</th>
<th>Product Name</th>
<th>Detection Limit</th>
<th>Type of Analysis</th>
<th>DNA Extraction Kit</th>
</tr>
</thead>
<tbody>
<tr>
<td>BIOTECON Diagnostics</td>
<td>foodproof® Peanut Detection Kit</td>
<td>≥0.1 mg/kg peanut allergen reference material</td>
<td>Real-time PCR</td>
<td>Yes</td>
</tr>
<tr>
<td>Neogen</td>
<td>BioKits Allergen Selection Module</td>
<td>10 mg/kg peanut</td>
<td>Conventional PCR</td>
<td>Yes</td>
</tr>
<tr>
<td>r-biopharm</td>
<td>SureFood® ALLERGEN Peanut</td>
<td>1 mg/kg peanut</td>
<td>Real-time PCR</td>
<td>Yes</td>
</tr>
<tr>
<td>4LAB Diagnostics</td>
<td>PeanutKit Real-Time PCR</td>
<td>1 copy of peanut haploid genome, approximately 2.87 pg <em>Arachis hypoagea</em> DNA</td>
<td>Real-time PCR</td>
<td>No</td>
</tr>
</tbody>
</table>
5. **PCR Inhibitors**

PCR inhibitors are a heterogeneous group of chemical compounds known to have a negative effect on PCR. The occurrence, properties, and removal of common PCR inhibitors is discussed in depth by Schrader et al. (2012). Poms et al. (2004b) explain that inhibitors, “pose a special challenge to the extraction procedure for obtaining sufficient amplifiable DNA and they may, in some instances, totally (negative results) or partially (impaired sensitivity) inhibit the DNA amplification.” Due to the exponential nature of DNA amplification, differences in extraction and amplification efficiencies as a result of inhibition can result in significant variation between replicates and sample types (Holzhauser et al., 2014).

Many PCR inhibitors have been identified in food: fats, proteins, polysaccharides, minerals, and enzymes (Poms et al., 2004b; Schrader et al., 2012). Thus, several studies have examined the matrix effect of food products on allergen detection by PCR. Siegel et al. (2013) examined the matrix effect of sausage, cookie, and hollandaise sauce powder matrices on allergen detection. The authors concluded that the food matrix affects the quantification of allergenic ingredients by real-time PCR. However, they mentioned that the matrix effect might be within an acceptable range to use results as an estimation of magnitude of food contamination by an allergenic ingredient. Martín-Fernández et al. (2016) investigated the influence of soybean, maize, and rice matrices on wheat flour detection as a way to monitor gluten content in processed foods. The results showed that the assay’s sensitivity was considerably affected by both the food matrix and the target gene. The authors concluded that the assay could be used to verify labeling compliance,
but different standard curves based on the food sample would be required for accurate quantification. To minimize the matrix effect, food matrix standards similar to the food sample being analyzed can be used for calibration, but this is unfeasible in many cases. Therefore, matrix-independent assays have been developed. Kenk et al. (2012) evaluated a matrix-independent approach based on magnetic particles (MCH) compared to two commercially available real-time PCR kits for hazelnut allergen detection in zwieback and model spice matrices. The authors confirmed that DNA-based allergen quantification was strongly dependent on both the food matrix and the method used for the isolation of DNA. However, it was found that DNA isolation using the commercially available kits was more reliable and sensitive than the matrix-independent MCH method. Holzhauser et al. (2014) investigated a matrix-independent approach to PCR analysis with good results. An internal competitive DNA sequence was added to the sample prior to DNA extraction to normalize the extraction and amplification efficiencies. Chocolate, vanilla ice cream, cookie dough, baked cookie, and coconut muesli matrices were incurred with defined levels of peanut cream. The average percent recovery was 87% across all matrices using competitive quantitative real-time PCR. The authors explained that the coconut muesli matrix would have had a 90% reduced recovery if only traditional external DNA standards were used. However, the internal competitive standard used in the study accounted for differences in food matrix extraction efficiencies, resulting in a higher recovery rate for the coconut muesli matrix. This study highlights the advantages of using an internal standard added prior to DNA extraction. Most internal standards are included
in the master mix and only normalize tube-to-tube variations in amplification efficiency, not variations in DNA extraction efficiency.

**Ice Cream**

1. **Consumption**

   The ice cream industry is growing at a rate of >5% worldwide, with the highest growth in Latin America, Eastern Europe, Africa, and the Middle East (>10% per year, 2006-2010). The growth rates in Western Europe and North America are lower, but these regions make up the largest market sectors, with sales of $24.1 billion and $17.1 billion in 2010, respectively. It is estimated that 90% of Americans consume ice cream. Accordingly, in 2010, the U.S. was the country with the most ice cream sales ($15.6 billion) and highest ice cream production (4.4 billion liters). The average American consumes 12.3 L of ice cream annually. Take-home products make up the largest market share in North America, followed by impulse products and artisanal/parlor products. Although artisanal/parlor products make up the smallest market share, global consumption of these products has notably increased from 2006-2010 (Goff and Hartel, 2013).

2. **Food Matrix**

   Ice cream is a complex food matrix that can be classified as a frozen food foam, an oil in water emulsion, and a colloid (Goff, 1997; Goff and Hartel, 2013; Bajad et al., 2016). It is composed of eight major ingredients: fat (dairy or nondairy), milk solids non-fat (MSNF), sweeteners, stabilizers, emulsifiers, water, flavors, and air (once whipped
Fat is integral for ice cream structure; it contributes to air stabilization, flavor, texture, and melting properties (Bajad et al., 2016). Emulsified fat droplets in ice cream mix range from 0.5 to 3 µm in size. After freezing, partial coalescence of fat globules results in cluster formation. Clusters range in size from 5 to >100 µm (Goff and Hartel, 2013). FDA standards require that “ice cream” must contain at least 10% milkfat (FDA, 2016a), but some formulations contain up to 18% milkfat (Goff and Hartel, 2013). Percent milkfat is one of the determining factors in ice cream categorization (Table 1.11).

Table 1.11: Average values of ice cream components by category

<table>
<thead>
<tr>
<th>Component</th>
<th>Economy</th>
<th>Standard</th>
<th>Premium</th>
<th>Superpremium</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fat</td>
<td>Legal minimum, usually 8-10%</td>
<td>10-12%</td>
<td>12-15%</td>
<td>15-18%</td>
</tr>
<tr>
<td>Total solids</td>
<td>Legal minimum, usually 35-36%</td>
<td>36-38%</td>
<td>38-40%</td>
<td>&gt;40%</td>
</tr>
<tr>
<td>Overrun</td>
<td>Legal maximum</td>
<td>100-120%</td>
<td>60-90%</td>
<td>25-50%</td>
</tr>
<tr>
<td>Cost</td>
<td>Low</td>
<td>Average</td>
<td>Higher than average</td>
<td>High</td>
</tr>
</tbody>
</table>

Table originally from Goff and Hartel (2013).

MSNF are the solids found in skim milk, including lactose, proteins, minerals (ash), water-soluble vitamins, enzymes, and some other minor components (Goff and Hartel, 2013). There are two main groups of milk proteins: casein and whey. Casein proteins form aggregates called casein micelles, which contain salts such as calcium and phosphorous. Proteins contribute to ice cream structure by stabilizing the fat emulsion.
and supporting air incorporation during processing (Bajad et al., 2016). Ice cream can be made using a variety of sweeteners (sucrose, dextrose, corn syrup, etc.) and stabilizers (gums such as guar, locust bean, and xanthan). Sugars and stabilizers are dissolved in the unfrozen serum phase of ice cream and become more and more concentrated as water freezes out of solution. Stabilizers promote a smooth texture in ice cream by inhibiting the formation and growth of ice crystals during freeze-thaw cycles (Bajad et al., 2016). Emulsifiers promote smoother, creamier, and more melt-resistant ice cream. The two main types of emulsifiers used in ice cream production are mono- and di-glycerides and sorbitan esters (Goff, 1997). Water is an important ingredient in ice cream because it is necessary for ice crystal formation. Ice cream mix usually consists of 60-65% water, mainly from milk or potable water. Ice crystals in ice cream vary in size from a few microns to over 100 µm, with an average size of around 35 to 45 µm. The number and size of ice crystals affect smoothness, scoopability, hardness, and meltdown rate of the finished product. There are a wide variety of particulate inclusions added to ice cream for flavoring purposes: fruits, nuts, bakery pieces, candy pieces, and variegates (stripes, ribbons, swirls). Non-particulate flavoring ingredients and colors can also be added depending on the flavor and formulation. Finally, air is a vital ingredient in ice cream for texture and volume. The amount of air incorporated into the ice cream mix during processing is called overrun, and can range from 25% to 150%, but a gallon of ice cream must weigh at least 2 kg (4.5 pounds). The average air cell size is between 20 and 25 µm (Goff and Hartel, 2013). The most important changes that occur from the transition of ice cream mix to frozen ice cream are: destabilization of the fat emulsion, ice crystal
formation, and air incorporation. Though much research has been conducted on the physio-chemical properties of ice cream, the complexities of ice cream structure and function continue to be an area of research interest (Goff, 2002; Bajad et al., 2016).

**Conclusions**

Clear and correct allergen labeling is vital to protect the health of food-allergic consumers. Unfortunately, current U.S. regulations do not require labeling of allergen cross-contact for non-prepackaged food products, even though it is rampant in the foodservice industry. Ice cream has been identified as a high-risk food and ice cream scoop shops have been identified as a high-risk environment for consumers with food allergy (Furlong et al., 2001; Brough et al., 2015). Consequently, this study sought to determine if ice cream dipper wells are a potential source of allergen cross-contact in ice cream scoop shops. The purpose of this study was to determine if PAL should be used in ice cream scoop shops from a research-based approach. As a preliminary study, the matrix effect of ice cream on real-time PCR was studied. Then a continuous flow dipper well was evaluated for allergen control by measuring peanut levels in the water over time following treatment with peanut butter ice cream. Peanut levels were quantified using a commercial real-time PCR kit. A survey was also conducted to gather information about practices and procedures regarding the use of dipper wells in ice cream scoop shops.

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http://www.fda.gov/Food/GuidanceRegulation/RetailFoodProtection/FoodCode/ucm374275.htm


Stephan, O., N. Weisz, S. Vieths, T. Weiser, B. Rabe and W. Vatterott. 2004. Protein quantification, sandwich ELISA, and real-time PCR used to monitor industrial cleaning procedures for contamination with peanut and celery allergens. J. AOAC Int. 87:1448-1457.


CHAPTER TWO

EVALUATION OF THE MATRIX EFFECT OF ICE CREAM ON PEANUT DETECTION BY REAL-TIME PCR

Abstract

PCR inhibitors are a common problem in allergen analysis. Ice cream has been identified as a high-risk food for consumers with food allergy, but ice cream contains many known PCR inhibitors. The purpose of this research was to evaluate the matrix effect of ice cream on peanut detection using a commercial real-time PCR kit. Three matrices were evaluated: frozen ice cream, liquid ice cream mix, and water. All matrices had low recovery when compared to the amount of incurred peanut. The recovery rates were 23.9%, 17.7%, and 6.2% for frozen ice cream, liquid ice cream mix, and water matrices, respectively. The recovery rate for plain peanut butter was 5.6%. Based on the results, we conclude that real-time PCR detection of peanut was significantly inhibited in all matrices. Factors that could have contributed to the inhibition include: PCR inhibitors in both the peanut butter and the ice cream, poor extraction efficiency, dilution of the DNA extract, and reference material composition. We recommend using an extraction method specifically for fatty food matrices for future peanut butter analyses, and either a PCR system that has been calibrated with ice cream matrix or a matrix-independent PCR system for future ice cream analyses.
Introduction

Ice cream is a tremendously popular food in the U.S. It is estimated that 90% of Americans consume ice cream, with the average American consuming 12.3 L of ice cream annually. In 2010, the U.S. was the country with the most ice cream sales at $15.6 billion and the highest ice cream production at 4.4 billion liters (Goff and Hartel, 2013). Unfortunately, ice cream has been identified as a high-risk food for consumers with food allergy (Furlong et al., 2001). Food allergies are a major worldwide health problem, and several studies have shown that the prevalence is increasing (Jackson et al., 2013; Husain and Schwartz, 2013; Carrard et al., 2015). It is estimated that food allergies affect 15 million Americans, or 8% of children and 4% of adults (Food Allergy Research & Education, undated). At this time, there is no cure for food allergy, so complete avoidance of the culprit food is the only management technique. Consequently, there is a need for sensitive and accurate assays for detecting allergens in high-risk foods, such as ice cream.

Many allergen detection methods have been developed in recent years, but only ELISA- and PCR-based methods are currently convenient for routine screening and quantification purposes in the catering and food industries (Kirsch et al., 2009). ELISA and PCR are both classified as indirect methods of detection, as ELISA measures antibody-antigen complexes and PCR measures allergen coding genes, but they offer several advantages over direct methods, such as cost, ease of use, and availability of commercial kits. Direct methods include mass spectrometric methods, which can identify and quantify allergens at the protein level, independent of their three-dimensional
structure (Kirsch et al. 2009). The main drawbacks of ELISA are cross-reactivity between similar species, protein denaturation and conformational changes due to processing methods, geographical and seasonal variation in protein content, and the inhibitory effect of food matrices. In comparison to proteins, DNA is more specific, more stable to processing methods, exhibits less seasonal and geographical variation, and has reduced matrix effect due to extraction efficiency, making PCR a good alternative to ELISA (Poms et al., 2004a; Monaci and Visconti, 2010; Prado et al., 2016). The main drawback of PCR is that DNA detection does not necessarily indicate the presence of allergenic proteins. Still, PCR can be used as a useful screening method to determine if further allergen analysis is needed. Additionally, several studies have shown good correlation between ELISA- and PCR-based detection methods (Holzhauser et al., 2002; Stephan and Vieths, 2004; Scaravelli et al., 2009).

Although PCR-based methods are becoming an increasingly popular option for specific and sensitive detection of trace food allergens, these methods are affected by the PCR system, the DNA isolation method, and the food matrix (Kenk et al., 2012). The effect of ice cream matrix on PCR detection is of particular interest because ice cream contains many known PCR inhibitors, such as fats, proteins, polysaccharides, minerals, and enzymes (Poms et al., 2004b; Schrader et al., 2012). Several studies have investigated the matrix effect of complex food matrices on allergen detection by PCR (Kenk et al., 2012; Siegel et al., 2013; Martín-Fernández et al., 2016). The findings suggest that the food matrix can have a significant effect on quantitative detection by PCR. To date, ice cream has only been evaluated for allergen detection in a matrix-
independent real-time PCR approach (Holzhauser et al., 2014). Therefore, the inhibitory effect of ice cream matrix on quantitative PCR detection needs to be studied. This is a preliminary study evaluating the matrix effect of peanut butter ice cream on peanut allergen detection using a commercially available real-time PCR kit.

Materials and Methods

1. Samples

Peanut butter made from 100% peanuts was purchased from a local grocery store (Crazy Richard’s Peanut Butter Co., Dublin, OH). Peanut butter was stirred until homogenous, about 2 min. Plain liquid ice cream mix was obtained from a local dairy farmer (Hunter Farms, High Point, NC). The approximate composition of the mix was 12.5% fat, 11.5% MSNF, 15% sugar, 0.3% stabilizer, and the remainder water. The mix was verified to be peanut-free using the commercial real-time PCR kit.

Three food matrices (frozen ice cream, liquid ice cream mix, and water) were prepared with 10% peanut butter by weight. For the frozen ice cream matrix, a sample of 1,000 g of liquid ice cream mix was needed to meet the minimum volume requirements of the ice cream freezer. To prepare the 1,000 g sample, four separate stomacher bags were prepared with 25 g of peanut butter and 225 g of liquid ice cream mix each. Bags were stomached for 2.5 min at 260 rpm. For the liquid ice cream mix and water matrices, a sample volume of 100 grams was used. Both matrices were prepared by adding 10 g of peanut butter to 90 g of matrix (either liquid ice cream mix or double distilled water) in a stomacher bag. Bags were stomached for 2.5 min at 260 rpm. Ten replicates of each
matrix were prepared. Following matrix preparation, samples were stored at 4 °C for 24 h.

Frozen ice cream matrix was prepared using an Emery Thompson CB-350 6-quart batch ice cream freezer (Emery Thompson, Brooksville, FL). The ice cream freezer was sanitized with a liquid sodium hypochlorite solution prior to use. For each batch of ice cream, four sample bags were poured into the ice cream freezer for a total of 1,000 g of mix. Ice cream was prepared by running the compressor for 1.5 min, or until ice cream was fluffy in texture, followed by 0.5 min with the compressor turned off. The gate was opened and ice cream was allowed to flow out into a quart-size cardboard container. Ice cream was stored at -18 °C while subsequent batches were prepared. Once all ten replicates of frozen ice cream matrix were prepared, the samples were moved to a -40 °C freezer for hardening. Liquid ice cream mix matrix and water matrix samples were also transferred to the -40 °C freezer at this time. After about 2 days of hardening, all samples were moved to a -29 °C freezer for storage. Samples were held at -29 °C for a minimum of 1 week before further analysis.

2. **Real-Time PCR Kit Specifications**

The foodproof Allergen Detection and Quantification Kit system (BIOTECON Diagnostics, Potsdam, Germany) was used for DNA extraction, quantification, and analysis. The kit was advertised as suitable for spices, confectionary, meat, and other food matrices. In reference to Allergen RM 800 reference material, the limit of detection (LOD) was 0.1 mg/kg, the limit of quantification (LOQ) was 0.8 mg/kg, and the range of quantification was 0.8-800 mg/kg.
Foodproof Sample Preparation Kit III. The foodproof Sample Preparation Kit III was used to isolate DNA for allergen detection and quantification analysis. The kit included: extraction buffer, binding buffer, wash buffer, elution buffer, proteinase K, filter tubes, and collection tubes. The composition of the reagents was proprietary.

Allergen RM 800. Allergen RM 800 reference material was designed for the quantitative analysis of celery, soy, gluten, peanut, hazelnut, and walnut allergens in food samples. The reference material contained these species in a proven homogenous concentration of 800 mg/kg spiked in a rice flour matrix.

Foodproof Peanut Detection Kit. The foodproof Peanut Detection Kit provided PCR primers, hydrolysis probes (5’ nuclease probes), and convenient premixed reagents for the species-specific amplification and detection of peanut DNA (Arachis hypogaea). The kit included: master mix (with internal control), control template (positive control), and PCR-grade water (negative control). Absolute quantification was possible when the kit was used in combination with the Allergen RM 800 reference material. The exact primer and target gene sequences were proprietary, but the manufacturer disclosed that the target gene was a multi-copy gene specific to peanut.

3. DNA Extraction

After storage, samples were moved to a 4 °C refrigerator for 48 h for thawing. Prior to analysis, frozen ice cream matrix samples were stirred with a sterile spoon for about 10 s. Liquid ice cream mix and water matrices were massaged in the stomacher bag for about 10 s. Three DNA samples from different areas were extracted from each matrix replicate. Plain peanut butter was extracted and plated in duplicate. The DNA extraction
kit was used to prepare samples according to the manufacturer’s instructions. Briefly, 1.5 mL of extraction buffer was added to 200 mg homogenized sample in a 2 mL microcentrifuge tube. The cells were lysed by incubating the contents for 30 min at 80 °C. The contents were centrifuged for 10 min at 12,000 x g. Then 400 µL of supernatant was transferred to a new microcentrifuge tube containing 600 µL binding buffer and mixed by pipetting up and down. To destroy endogenous nucleases and other proteins, 80 µL of proteinase K working solution was added. The contents were mixed by pipetting up and down and then incubated for 10 min at 72 °C. Two hundred microliters of isopropanol was added and mixed by pipetting up and down. Six hundred fifty microliters of mixture was pipetted into the upper reservoir of a combined filter tube-collection tube assembly and centrifuged for 1 min at 5,000 x g. Flow-through was discarded and the remaining mixture was added and centrifuged for 1 min at 5,000 x g. Four hundred fifty microliters of wash buffer working solution was added and contents were centrifuged for 1 min at 5,000 x g. Wash buffer procedure was repeated once. The filter tube was transferred to a clean 2 mL reaction tube. One hundred microliters of elution buffer (warmed to 70 °C) was added to the glass fiber fleece and incubated for 5 min at room temperature. The contents were centrifuged for 1 min at 5,000 x g. Extracted DNA was used immediately, stored at 4 °C for 1-2 days, or stored at –18 °C for future analysis.

According to the manufacturer’s instructions, the standard curve was prepared by extracting 200 mg of Allergen RM 800 reference material following the extraction procedure described above. DNA extract was ten-fold serially diluted to obtain
concentrations of 800 (undiluted), 80, 8, and 0.8 mg/kg. Extracted DNA was used immediately, stored at 4 °C for 1-2 days, or stored at –18 °C for future analysis.

4. Real-Time PCR

Preliminary data showed that extracted DNA from the liquid ice cream mix and water matrices had to be diluted 1:100 in order to obtain results within the quantification range of the kit (data not shown). Therefore, DNA extract samples were mixed by pipetting up and down and ten-fold serially diluted twice using PCR-grade water (Table 2.1). The final dilution was used for real-time PCR analysis. The PCR reaction mixture contained 20 µL of master mix and either 5 µL of diluted DNA extract, 5 µL of standard, or 5 µL of positive or negative control. After pipetting the reaction mixture into each well, the wells were capped and centrifuged briefly in a swing bucket centrifuge. The samples were analyzed using a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad, Hercules, CA). The program setup was as follows: 1 two-step pre-incubation cycle of 37 °C for 4 min and 95 °C for 10 min, followed by 50 two-step amplification cycles of 95 °C for 5 s and 60 °C for 60 s. Fluorescence was detected in the FAM (peanut) and HEX (internal control) channels during step 2 of amplification. Cycle thresholds (Ct) were automatically determined using Bio-Rad CFX Manager software. Amplification efficiency was calculated with the following equation: 

$$\text{Efficiency} = 10^{-\frac{1}{\text{Slope}}} - 1.$$ 

Milligram per kilogram values were calculated from Ct values using an allergen calculation template provided by the manufacturer. The following equation was used:

$$\frac{mg}{kg} = 10^{\left(\frac{(C_t - y\ \text{intercept})}{\text{slope}}\right)}$$
Table 2.1: Matrix preparation scheme

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Peanut content</th>
</tr>
</thead>
<tbody>
<tr>
<td>Step 1 1 part peanut butter + 9 parts matrix</td>
<td>10% 100,000 mg/kg</td>
</tr>
<tr>
<td>↓ Extraction</td>
<td>10% 100,000 mg/kg</td>
</tr>
<tr>
<td>Step 3 1 part extraction + 9 parts water</td>
<td>1% 10,000 mg/kg</td>
</tr>
<tr>
<td>↓ Step 4 1 part dilution from previous step + 9 parts water</td>
<td>0.1% 1,000 mg/kg</td>
</tr>
</tbody>
</table>

5. Sample Recovery

The recovery rate was calculated by dividing the average detection (in mg/kg) by the expected value (in mg/kg), and then multiplying the quotient by 100.

6. Statistical Analysis

A completely randomized design with subsampling was used for the study. There were three treatments for the study (frozen ice cream, liquid ice cream mix, and water). Ten experimental units were used for each treatment and three measurements were taken for each experimental unit. The “Proc Mixed” procedure from Statistical Analysis Software was used to perform an ANOVA test. Pairwise comparisons were conducted using Fisher’s Protected LSD Method. A significance level of 0.05 was used for all hypothesis tests.
Results

1. Amplification efficiency and R\textsuperscript{2} coefficient

Matrix samples were analyzed on two separate days, and a standard curve was run on each day. The slopes for the standard curve were -3.4117 and -3.2974, y-intercepts were 35.605 and 35.497, amplification efficiencies were 96% and 101%, and R\textsuperscript{2} values were 0.9981 and 0.9997 for days 1 and 2, respectively.

2. Sample Recovery

The frozen ice cream matrix had the highest recovery, followed by the liquid ice cream mix matrix and the water matrix (Table 2.2, Figure 2.1). An average of 56,136 mg/kg peanut (5.6% recovery) was detected in the plain peanut butter samples.

Table 2.2: Peanut recovery in frozen ice cream, liquid ice cream mix, and water matrices

<table>
<thead>
<tr>
<th>Matrix</th>
<th>Frozen ice cream</th>
<th>Liquid ice cream mix</th>
<th>Water</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average peanut detected (mg/kg)</td>
<td>239.3 ± 14.3\textsuperscript{a}</td>
<td>176.9 ± 15.4\textsuperscript{b}</td>
<td>62.2 ± 8.2\textsuperscript{c}</td>
</tr>
<tr>
<td>Recovery rate\textsuperscript{1}</td>
<td>23.9%</td>
<td>17.7%</td>
<td>6.2%</td>
</tr>
</tbody>
</table>

\textsuperscript{1}Recovery rate was based on 1,000 mg/kg incurred peanut
\textsuperscript{abc}Within the rows, averages that do not share a letter are significantly different (p > 0.05)
Figure 2.1: Box and whisker plot comparison of peanut detection values between matrices, where “frozen” is frozen ice cream matrix, “liquid” is liquid ice cream mix matrix, and “water” is water matrix.

Discussion

Peanuts and peanut butter, in the form of nuts, candy pieces, and variegates, are common additions to ice cream. According to Goff and Hartel (2013), crushed peanuts or peanut butter are usually added in amounts of 5-8% by weight, candy or confection pieces are 5-8% by weight, and variegates are 7-10% by weight. Accordingly, peanut-flavored ice creams can contain high amounts of peanut allergen. Previous studies using allergen-spiking materials rich in fat, such as nuts, defatted the materials prior to use (Kenk et al., 2012; Siegel et al., 2013; Linacero et al., 2016). However, most ice cream flavors containing peanut and tree nut allergens contain the full-fat product. Consequently, we used peanut butter made from 100% peanuts as the spiking material.
It was not surprising that the plain peanut butter samples had a low recovery rate, because peanut has a high concentration of proteins and fats, as well as small amounts of tannins and other phenolic compounds (Venkatachalam and Sathe, 2006), all of which are known PCR inhibitors (Poms et al., 2004b; Kontanis and Reed, 2006). The peanut butter used in this study was composed of 50% fat and 25% protein by weight. Interestingly, Hird et al. (2003) found that a DNA extraction kit designed for meat tissue was more applicable to peanut samples than kits designed for plant tissue because peanut is abnormally high in fat for a plant. Special extraction procedures are necessary for fatty matrices in order to obtain high-quality DNA. The DNA extraction kit utilized in this study was designed for the isolation and purification of DNA from material or foodstuffs of plant or animal origin, but several authors have found that commercial DNA extraction kits are not suitable for all food samples. Iniesto et al (2013) obtained high spectrophotometric values when evaluating commercial kits for extraction of hazelnut DNA, indicating co-extraction of inhibitors. Scavavelli et al. (2008) investigated fifteen extraction methods, including five commercial kits, for the isolation of peanut DNA from raw and roasted peanuts. The most suitable extraction method was determined to be a guanidine hydrochloride-based extraction buffer combined with a commercial DNA purification system, which performed better than the all-inclusive commercial DNA extraction kits. These results highlight the need to determine the best extraction method based on the food sample of interest. Commercial DNA extraction kits do not produce the best quality DNA for all food matrices, particularly for fatty matrices. However, these
kits are convenient and may be suitable for purposes that do not require precise quantification.

All three matrices (frozen ice cream, liquid ice cream mix, and water) had higher recovery rates than the plain peanut butter samples. These results makes sense if fat was the main cause of the PCR inhibition in the plain peanut butter samples. The ice cream matrices also contained fat, but only 12.5% by weight compared to 50% fat by weight in the peanut butter, and the water matrix did not contain any fat. The overall decrease in fat content of the matrix samples could have yielded higher quality DNA extract compared to the plain peanut butter samples. Procedures for optimizing DNA extraction are described by Scaravelli et al. (2008). Further studies could apply these methods to peanut butter samples to analyze the effect of fat inhibition and to increase extraction efficiency.

However, the frozen ice cream and liquid ice cream mix matrices still showed relatively low recovery rates. Ice cream contains many known PCR inhibitors, such as fats, proteins, polysaccharides, minerals, and enzymes which could have reduced the recovery. The mechanisms of action, as well as removal techniques, for many PCR inhibitors are discussed in depth by Schrader et al. (2012). Calcium is cited as a polymerase inhibitor, but it is not believed that calcium had a significant effect on PCR inhibition in this study because the internal control was detected in all samples, indicating good amplification efficiency. It is believed that poor DNA extraction efficiency was the main cause of the low recovery rates in the peanut butter and ice cream matrices. We speculate that fat was a major factor in DNA extraction efficiency, because recovery increased as percent fat decreased, but more research is needed to validate this
hypothesis. While many PCR inhibitors have been identified, the mechanisms of actions of these inhibitors is still unknown. Once the mechanisms of action are identified, methods to combat these inhibitors can be developed.

The frozen ice cream had a higher recovery rate than the liquid ice cream mix, despite a nearly identical composition between the two matrices. A possible explanation for this result is overrun, or the amount of air incorporated into ice cream during processing. Overrun creates air cells in the ice cream, increasing the surface area, which might have allowed the extraction buffer to more effectively extract DNA from the frozen ice cream sample. Another possible explanation for this result is the fat destabilization that occurs during frozen ice cream production. During homogenation of liquid ice cream mix, fat is broken down into numerous small droplets. However, during aeration and freezing of liquid ice cream mix to make frozen ice cream, fat destabilization causes the fat droplets to cluster and clump, or partially coalesce. The fat in frozen ice cream may inhibit PCR to a lesser degree than the fat in liquid ice cream mix because there are fewer, albeit larger, fat globules in frozen ice cream. Numerous small fat droplets in liquid ice cream mix are more evenly distributed throughout the matrix. Consequently, these fat droplets might more uniformly coat the DNA and decrease DNA extraction efficiency. The effect of fat coalescence on PCR inhibition is a matter that needs to be studied further. It is well known that fat destabilization is enhanced by the addition of emulsifiers to liquid ice cream mix (Goff, 1997). It would be interesting to investigate if emulsifiers could be added to ice cream samples prior to DNA
extraction to increase partial coalescence, and therefore potentially increase extraction efficiency as well.

The water matrix had a much lower recovery rate than both ice cream matrices, even though the matrix itself did not contain any PCR inhibitors. The most likely reason for the reduced recovery in the water matrix is the difference in polarity between the peanut butter (nonpolar) and the water (polar). The peanut butter and water matrix did not mix well, and visible peanut butter globules were visible after storage. It is believed that the globules were too large to fit up the tip of the pipette, resulting in samples containing very low amounts of peanut. Future studies could add an emulsifier to the water matrix or re-stomach the sample after freezing to break up the peanut butter globules and improve the recovery rate. Notably, the peanut butter was well-dispersed in the frozen ice cream and liquid ice cream mix matrices because ice cream contains both polar and nonpolar components.

In addition to PCR inhibitors, the PCR system has a significant effect on results. The target gene for a PCR assay has to be carefully selected to allow for adequate specificity and sensitivity. Typically, shorter genes with high heat stability are selected. Although the exact target gene sequence was not disclosed by the manufacturer, the fact that the target region was a multi-copy gene probably increased the sensitivity of the assay. López-Calleja et al. (2013) compared a real-time PCR system based on the selective amplification of two different gene sequences: a gene coding for an allergenic protein or a multi-copy gene coding for a species-specific region in the genome. The
results showed that the PCR system based on the multi-copy gene was more sensitive, with a LOD of 0.1 mg/kg as opposed to 10 mg/kg for the other PCR system.

Calibration material is also vital for accurate quantification. Ideally, calibration material will be composed of a similar food matrix as the food sample (Siegel et al., 2013). The Allergen RM 800 reference material was composed of defatted peanut flour in a rice flour matrix, which is dissimilar to both the spiking material and the matrix studied and may have affected the results. Defatted peanut flour is a common calibration and spiking material, but may not be suitable for analysis of samples containing peanut butter if adequate DNA extraction and purification procedures are not used. To date, the only PCR assay to use peanut butter as a spiking material was one designed by Holzhauser et al (2014) in which the peanut butter was also used as the calibration standard. This method had good recovery, with an average recovery rate of 87% across five different matrices. These results demonstrate the importance of using a calibration material similar to the spiking material. A major disadvantage of using calibration standards from a kit is that the calibration material might not be the same as the food sample being tested, which can result in significant differences in quantitative results.

Scaravelli et al. (2009) investigated the effect of heat treatment on peanut detection by PCR kits. There were dramatic differences in recovery between raw and roasted peanut samples, with increased thermal treatment resulting in decreased DNA extraction yield. Similar results were found by Iniesto et al. (2013) for hazelnut samples. Roasted hazelnut samples had 27.7% recovery compared to raw samples and autoclaved samples at harsh conditions had 13.7% recovery compared to raw samples. If the kit
calibration material was made from raw peanut material, it could explain the low recovery rates, as the peanut butter used in this study was made from roasted peanuts. Scaravelli et al. (2009) also found that peanut variety can result in slight differences in DNA detection, but it is not believed that peanut variety would be a major factor in the recovery rate.

There are several limitations of this study. The commercial real-time PCR kit was designed for trace allergen detection. All samples in this study were highly concentrated, and therefore had to be ten-fold serially diluted to obtain results within the quantification range of the kit. Several authors note that dilution of the sample extract can affect the precision of the method (Scaravelli et al., 2009; Kenk et al., 2012; Schrader et al., 2012). Dilution of the DNA extracts may also have contributed to the low recovery rates. Therefore, the quantitative results and recovery rates from this experiment should not be used in absolute terms. It is recommended that further studies using this PCR kit to analyze highly concentrated samples apply a procedure similar to the one described by Holzhauser et al. (2002) for chocolate matrix. It is believed that diluting spiked matrix with blank matrix for analysis rather than diluting DNA extract would result in better recovery rates. Additionally, to better analyze the matrix effect of ice cream, further studies need to control for factors such as the PCR system and the DNA isolation method, which can both have an effect on PCR detection and quantification. The PCR system should be designed or selected with the sample matrix in mind (including the composition of the calibration material) and a DNA isolation method for fatty food matrices should be utilized.
1. Conclusions

Ice cream is a high-risk food for consumers with food allergy, so it is imperative that sensitive and specific detection methods for allergens in ice cream be developed. It is clear from the results of this study that peanut detection by real-time PCR was inhibited by peanut butter ice cream matrix. It is believed that PCR inhibitors, such as fats, proteins, and polyphenolic compounds were the main cause of the inhibition. Once the mechanisms of action of PCR inhibitors are better understood, their effect on PCR results can be minimized. Better DNA extraction and purification methods, specific to the matrix being analyzed, could also minimize PCR inhibitors. The effect of ice cream structure (i.e., percent overrun, degree of fat destabilization, and addition of emulsifiers) on PCR inhibition should be studied to maximize the DNA extraction efficiency in frozen ice cream samples. To more precisely analyze the matrix effect of ice cream, spiking materials that are similar to the calibration material should be used. Development of a PCR assay calibrated with ice cream matrix would be ideal because it would eliminate the matrix effect. Unfortunately, PCR assays for individual food matrices are not always feasible, and therefore a matrix-independent approach might be useful for future ice cream sample analysis.

Acknowledgements

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References


CHAPTER THREE

EVALUATION OF ICE CREAM DIPPER WELL WATER AS A POTENTIAL SOURCE OF PEANUT ALLERGEN CROSS-CONTACT

Abstract

Food allergies are a worldwide health issue, and their prevalence is on the rise. Allergen cross-contact in foodservice establishments is a prominent cause of food-allergic reactions outside the home. Ice cream shops have been identified as a high-risk environment for consumers with food allergy. Accordingly, we evaluated the allergen cross-contact potential of ice cream scoop shop dipper wells using a real-time PCR kit for peanut detection. Following the addition of a full serving of peanut butter ice cream to the dipper well, peanut was detected in dipper well water at levels unlikely to cause an allergic reaction for all rinse times (5, 10, 30, 180, 600 s). Similarly, a continuous use scenario resulted in peanut detection at levels insufficient to cause an allergic reaction. Two cleaning treatments of a dipper well basin were evaluated. Peanut residue was detected, though not quantifiable, following both treatments. A survey of ice cream scoop shop owners (n = 7) found that all respondents believed that dipper wells were a source of allergen cross-contact. Most shops had a standard procedure for serving customers with food allergy, including using a sanitized ice cream scoop (not from the dipper well). However, only two shops had allergen advisory signs posted. Based on the results of this hazard analysis, we conclude that dipper wells are most likely not a significant risk for food-allergic consumers. Nonetheless, we still recommend that ice cream scoop shops
post allergen advisory signs and avoid using scoops from the dipper well to serve food-allergic customers, as a precaution for the worst case scenario.

**Introduction**

Food allergies are considered the fourth most important public health problem by the World Health Organization (Kirsch et al., 2009). They affect a significant proportion of the population, up to 10% of young children and 2-3% of adults in industrialized countries, and several studies have shown that the prevalence is on the rise (Jackson et al., 2013; Husain and Schwartz, 2013; Carrard et al., 2015). It is estimated that food allergy affects 15 million Americans, including 1 in 13 children (Food Allergy Research & Education, undated). An estimated 30,000 Americans require emergency room treatment each year and 150 Americans die each year due to allergic reactions to food (FDA, 2004).

Several studies have identified restaurants and catering establishments as high-risk environments for consumers with food allergy (Wanich et al., 2008; Versluis et al., 2015; Brough et al., 2015). Accordingly, some countries have passed laws regarding allergen labeling of non-prepackaged food items, such as those sold in foodservice establishments. The Food Standards Code of Australia and New Zealand requires that if food is not in a package or if it is not required to have a label, allergen information must be displayed by the food or must be provided to the customer if requested (Food Standards Australia New Zealand, 2016). The European Union passed a similar law: the EU Food Information for Consumers Regulation (EU FIC). Non-prepackaged foods made with intentionally added allergen-containing ingredients will now require allergen
labeling. Allergen information can be provided in a variety of ways, for example, supplied on the menu, on chalk boards, or provided verbally (with a notice informing customers that allergen information can be obtained that way) (Department for Environment Food & Rural Affairs, 2012). Allergen labeling of non-prepackaged food is not currently required in the U.S.

Allergen cross-contact, or unintentional incorporation of an allergen into a food, is another serious issue for consumers with food allergy. Several countries have introduced measures to address allergen cross-contact in the food industry. Switzerland requires allergens to be labeled if their concentration exceeds 1000 mg/kg (100 mg/kg gluten for cereals), even if they are present through unintentional allergen cross-contact (Stephan et al., 2004; Allen et al., 2014b). Japan requires allergens to be labeled if they are present at levels of 10 mg/kg or above (Akiyama et al., 2011). In the U.S., the Food Safety and Modernization Act of 2011 (FSMA) requires that covered establishments address allergen cross-contact in their food safety plan. Allergens are considered a chemical hazard by the FDA and preventive controls must be implemented to significantly minimize or prevent the occurrence of allergens. Prepackaged foods with unidentified allergens will now be considered misbranded, however, a threshold dose has not been established in the U.S. (FDA, 2011).

Dipper wells are a potential source of allergen cross-contact in foodservice establishments. Dipper wells are small, continuously running sinks used in some restaurants, coffee houses, and ice cream shops to store in-use utensils. A traditional dipper well has a single spigot, controlled by a valve, that empties into a receiving well.
When the water reaches a certain level, it begins to overflow and the well is simultaneously filled and emptied as the spigot runs. It is common practice for retailers to leave their dipper well(s) running continuously during operating hours (EPA, 2012). Dipper wells have been approved by the FDA for the storage of in-use utensils. However, Section 3-304.12 D the 2013 Food Code is designed around pathogen control, not allergen control (FDA, 2013). There are several reasons why dipper wells might pose a significant allergen cross-contact risk: (1) ice cream has been deemed a high-risk food (Brough et al., 2015) and ice cream shops have been identified as a high-risk environment for food-allergic consumers (Furlong et al., 2001), (2) improper cleaning of shared equipment is a common cause of allergen cross-contact (Yunginger et al., 1983; Jones et al., 1992; Laoprasert et al., 1998), (3) water has been shown to be a viable source of allergen cross-contact (Stephan et al., 2004; Kerkaert et al., 2012), (4) unlike microorganisms, allergens do not need time to multiply nor do they need nutrients to survive, and (5) very low doses of allergens can cause immediate and potentially fatal allergic reactions in sensitive individuals. Furthermore, Furlong et al. (2001) reported an allergic reaction as a result of serving a nut-allergic individual with an ice cream scoop that was previously used for nut ice cream. There was no information provided as to whether the ice cream scoop was rinsed in a dipper well between uses, but this anecdote highlights the need to investigate this matter further. In light of these reasons, it is believed that ice cream dipper wells should be evaluated for their ability to control allergens.
This is a pilot study to investigate the risk of allergen cross-contact in ice cream scoop shops via dipper well usage. A traditional dipper well was evaluated for its ability to remove peanut residue from the dipper well water over time as well as during continuous use. Additionally, two cleaning procedures were tested for their ability to remove traces of peanut from the dipper well basin. Peanut levels were quantitatively evaluated using a commercial real-time PCR kit for peanut detection. A survey of practices and procedures regarding dipper well usage in ice cream scoop shops was also conducted.

Materials and Methods

1. Ice Cream Preparation

Two batches of peanut butter ice cream were prepared with 10% peanut butter by weight. Briefly, 25 g of peanut butter made from 100% peanuts (Crazy Richard’s Peanut Butter Co., Dublin, OH) was added to 225 g of liquid ice cream mix (12.5% fat) (Hunter Farms, High Point, NC) in a stomacher bag and stomached for 2.5 min at 260 rpm. The procedure was repeated for a total of eight bags. Bags were stored at 4 °C for 24 h. An Emery Thompson CB-350 6-quart batch freezer (Emery Thompson, Brooksville, FL) was used to prepare the ice cream. The machine was sanitized with a liquid sodium hypochlorite solution prior to use. For each batch of ice cream, four sample bags were poured into the machine. Ice cream was prepared by running the compressor for 1.5 min, or until ice cream was fluffy in texture, followed by 0.5 min with the compressor turned off. The gate was opened and ice cream was allowed to flow out into a quart-size ice
cream carton. Ice cream was stored at -18 °C while subsequent batches were prepared. The ice cream was moved to a -40 °C freezer for 2 days for hardening. Then the ice cream was moved to a -29 °C freezer for storage.

2. *Dipper Well Set-Up*

A Fisher brand dipper well (Fisher Manufacturing Company, Tulare, CA) was used for this study (approximately 41 cm x 11.5 cm x 8 cm) (Figure 3.1). A flexible hose was attached to the dipper well spigot using a hose clamp. The hose was used to allow the flow rate to remain constant during pauses to collect samples. A flow rate of 0.3 gallons per min was used for all experiments, based on EPA guidelines for water conservation (EPA, 2012). The mouth of the hose was held next to the spigot to accurately represent dipper well filling and emptying behavior. The dipper well and ice cream scoop were washed using a non-chlorinated alkaline cleaner (Ecolab, Saint Paul, MN) with manual scrubbing prior to beginning each experiment. The temperature, pH, and hardness of the water were recorded to ensure measurements were within normal parameters. The hardness was measured using a Hardness Drop Count Test Kit (AquaPhoenix Scientific, Hanover, PA).
3. **Treatment of Dipper Well**

For Treatment 1, the dipper well was filled about halfway with water. Then 66 g of melted peanut butter ice cream was poured into the dipper well and stirred until homogenous. The dipper well was filled the rest of the way with water. Once the water started overflowing, a timer was started. Water samples were collected at the 5, 10, 30, 180, and 600 s mark. Prior to taking each sample, the hose was transferred to a bucket and the water in the basin was stirred until visually homogenous.

For Treatment 2, an identical procedure was used except that the dipper well was filled all the way with water before pouring in the melted ice cream. Both scenarios were repeated in quadruplicate. The dipper well basin was cleaned between replicates (cleaning procedures described below). If residual levels of peanut were detected, the amount was subtracted out from the following replicate.

A continuous use scenario was tested by serving multiple scoops of ice cream in succession. The dipper well was filled all the way with water and water was left running for the duration of the experiment. Five scoops of peanut butter ice cream were scooped.
within 60 s, rinsing the utensil in the dipper well for 5 s with swirling between scoops. After the final scoop, the hose was removed from the dipper well. The basin was stirred until visually homogenous and a water sample was collected. The hose was returned to the dipper well and the procedure was repeated for 5 more scoops in 60 s, totaling 10 scoops in 120 s. The hose was removed from the dipper well, the basin was stirred until homogenous, and a water sample was collected. The experiment was repeated in duplicate.

4. Basin Cleaning Techniques

Two different basin cleaning techniques were evaluated. For the first cleaning technique (rinse only), the hose was used to rinse all dipper well surfaces until visually clean. For the second cleaning technique (rinse, alkaline detergent, rinse), the hose was used to rinse all dipper well surfaces until visually clean. Then, a non-chlorinated alkaline cleaning solution and a cloth were used to scrub all dipper well surfaces, followed by a post-rinse. For both techniques, the drain stopper was removed during cleaning and cleaned in a similar fashion as the dipper well basin. Cleaning techniques were employed between dipper well treatments (described above), for four replicates each. In order to evaluate the efficacy of the cleaning techniques, a water sample was collected after filling the basin with water but before adding ice cream.

5. Real-Time PCR Kit Specifications

The foodproof Allergen Detection and Quantification Kit system (BIOTECON Diagnostics, Potsdam, Germany) was used for DNA extraction and quantification. Based
on the Allergen RM 800 reference material, the limit of detection (LOD) was 0.1 mg/kg, the limit of quantification (LOQ) was 0.8 mg/kg, and the range of quantification was 0.8-800 mg/kg.

**Foodproof Sample Preparation Kit III.** The foodproof Sample Preparation Kit III was used to extract and purify DNA for further analysis. The kit included: extraction buffer, binding buffer, wash buffer, elution buffer, proteinase K, filter tubes, and collection tubes. The composition of the reagents was proprietary.

**Allergen RM 800.** The Allergen RM 800 reference material was composed of celery, soy, gluten, peanut, hazelnut, and walnut at 800 mg/kg each in a rice flour matrix.

**Foodproof Peanut Detection Kit.** The foodproof Peanut Detection Kit was used to quantify peanut (*Arachis hypogaea*) DNA. The kit included: master mix (with internal control), control template (positive control), and PCR-grade water (negative control). Absolute quantification was possible when the kit was used in combination with the Allergen RM 800 reference material. The exact primer and target gene sequences were proprietary, but the manufacturer disclosed that the target gene was a multi-copy gene specific to peanut.

6. DNA Extraction

A commercial real-time PCR kit for peanut detection (BIOTECON Diagnostics, Potsdam, Germany) was used according to the manufacturer’s instructions. Briefly, 200 mg of sample was extracted using 1.5 mL of extraction buffer and incubated for 30 min at 80 °C followed by centrifugation for 10 min at 12,000 x g. Supernatant was transferred to a new tube and mixed with 600 µL binding buffer and 80 µL proteinase k solution
followed by incubation for 10 min at 72 °C. Next 200 μL of isopropanol was added. Contents were transferred to a filter tube-collection tube assembly and centrifuged for 1 min at 5,000 x g. Wash buffer was added twice, centrifuging for 1 min at 5,000 x g after each addition. Finally, 100 μL of pre-warmed elution buffer was added to the filter tube and contents were centrifuged for 1 min at 5,000 x g. Extracted DNA was collected in the collection tube and used immediately, stored at 4 °C for 1-2 days, or stored at –18 °C for future analysis.

To prepare the standard curve, 200 mg of Allergen RM 800 reference material was extracted according to the procedure above. Undiluted DNA extract had a concentration of 800 mg/kg peanut. Extract was ten-fold serially diluted to obtain solutions of 80, 8, and 0.8 mg/kg. All four calibration solutions were plated in duplicate to obtain a standard curve.

7. Real-Time PCR Analysis

The PCR reaction volume was 25 μL: 20 μL master mix and 5 μL of sample (DNA extract, control, or calibration standard). The time-temperature protocol for PCR was based on the kit manufacturer’s recommendations. There was one pre-incubation cycle that consisted of 4 min at 37 °C and 10 min at 95 °C. There were 50 cycles of amplification; each consisted of 5 s at 95 °C and 60 s at 60 °C. PCR thermocycling was carried out using a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad, Hercules, CA). Fluorescence was detected in the FAM (peanut) and HEX (internal control) channels during step 2 of amplification. Cycle thresholds (Ct) were automatically determined using Bio-Rad CFX Manager software. Milligrams per kilogram values were
calculated by plotting the C\textsubscript{t} values against the standard curve on a calculation template supplied by the kit manufacturer. Amplification efficiency was calculated with the following equation: Efficiency = 10\(^{(-1/Slope)}\)-1. Milligram per kilogram values were calculated from C\textsubscript{t} values using an allergen calculation template provided by the manufacturer. The following equation was used:

\[
\frac{mg}{kg} = 10^{\frac{(C_t-y \text{ intercept})}{slope}}
\]

8. **Ice Cream Shop Survey**

Members of the National Ice Cream Retailers Association (NICRA) were contacted by email to voluntarily participate in a survey regarding dipper well practices and procedures in ice cream scoop shops. To increase participation, members who did not respond via email were contacted by phone. Surveys were distributed and returned via email. Most questions were multiple choice, but a few were free response (Appendix D). All materials were approved by the Institutional Review Board. After the survey responses were collected, a follow-up question regarding the dipper well water source (e.g., municipal or well water) was sent via email.

**Results**

1. **PCR Efficiency**

The slope of the standard curve was -3.419, the y-intercept was 35.457, the efficiency was 96\%, and the R\textsuperscript{2} value was 0.998.
2. *Peanut Detection in Ice Cream Dipper Well Water at Varying Rinse Times*

Following the dipper well treatments, peanut was detected in all water samples, with the lowest levels detected at 600 s. For Treatment 1, there was a noticeable upward trend in peanut detection from the 5 s sample to the 10 and 30 s samples. On average, the 30 s sample had the highest level of peanut. Peanut detection decreased considerably after the 30 s sample (Figure 3.2). For Treatment 2, there was a consistent overall decrease in peanut detection over time. On average, the 5 s sample had the highest level of peanut (Figure 3.3).

![Graph of Treatment 1](image)

**Figure 3.2.** Graph of Treatment 1. The reduction in peanut detection in dipper well water over time when a serving of peanut butter ice cream was added to a half-full basin.
Figure 3.3. Graph of Treatment 2. The reduction in peanut detection in dipper well water over time when a serving of peanut butter ice cream was added to a full basin.

3. **Control of Allergens during Continuous Use of Dipper Well**

Peanut detection increased from 5 to 10 scoops for both replicates. Replicate 1 increased from 17 to 20 mg/kg peanut. Replicate 2 increased from 9 to 16 mg/kg. On average, peanut detection was 13 and 18 mg/kg for 5 and 10 scoops, respectively.

4. **Comparison of Cleaning Techniques**

Peanut was detected after both cleaning techniques. Rinse only removed all traces of peanut 25% of the time, compared to rinse, alkaline detergent, rinse, which removed all traces of peanut 50% of the time (Table 3.1). All residuals were below the limit of quantification of the kit (0.8 mg/kg peanut).
Table 3.1: Comparison of cleaning procedures

<table>
<thead>
<tr>
<th>Cleaning Method</th>
<th>Replicate</th>
<th>Peanut detection</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Rinse only</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Rinse, alkaline detergent, rinse</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

Where “+” represents peanut was detected and “-” represents peanut was not detected

5. Water Quality Measurements

Water measurements were taken on two separate days. On average, the temperature was 22.7 °C, the pH was 7.44, and the hardness was 55 mg/kg CaCO₃. The water quality measures fell within normal parameters (United Utilities, Undated).

6. Current Dipper Well Practices and Procedures in Ice Cream Scoop Shops

Nineteen NICRA members were contacted, and seven survey responses were obtained (37% response rate). The survey responses were collected from six different states, representing the Midwest, Southeast, and Northeast regions of the U.S. Dipper well brands used by shops included Dipwell, Fisher, and Winco. One shop utilized a dipper well and a Taylor spray shower. The results showed that 100% of establishments continuously ran their dipper well from open of business until close of business. Only six out of the seven original respondents answered the follow up question, but of those six, all had dipper wells connected to municipal water. In regards to flow rate, 57% ran their dipper well at half force, 29% at minimum force, and 14% at a variable rate. All establishments had a Standard Operating Procedure (SOP) for cleaning their dipper well. Forty-three percent of establishments followed this SOP all of the time, 29% followed it
most of the time, and 14% followed it sporadically. The majority of establishments also had an SOP for serving customers with a food allergy (Table 3.2). Of those establishments, the procedure for serving customers with a food allergy varied from business to business, but 83% of respondents mentioned that a clean or sanitized ice cream scoop (not from the dipper well) was used and 50% mentioned that a fresh tub of ice cream (not from the dipping cabinet) was used.

Table 3.2: Dipper well survey responses

<table>
<thead>
<tr>
<th>Survey Question</th>
<th>Yes</th>
<th>No</th>
<th>Blank</th>
</tr>
</thead>
<tbody>
<tr>
<td>Do you have an SOP(^1) for serving customers with food allergy?</td>
<td>86%</td>
<td>14%</td>
<td></td>
</tr>
<tr>
<td>Do employees have convenient access to this SOP during operating hours?</td>
<td>86%</td>
<td>14%</td>
<td></td>
</tr>
<tr>
<td>Are employees trained on this SOP at the beginning of their employment?</td>
<td>86%</td>
<td>14%</td>
<td></td>
</tr>
<tr>
<td>Do you have any advisory allergen statements posted in your store?</td>
<td>29%</td>
<td>71%</td>
<td></td>
</tr>
<tr>
<td>Do you or your employees verbally inform customers that have a food allergy that your products are not guaranteed to be allergen-free?</td>
<td>100%</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^1\)Standard Operating Procedure

The proportion of ice cream scoop shop owners (n = 7) who said they had the following controls in place to prevent allergen cross-contact in their shops.

Seventy-one percent of establishments had peanut-containing flavors all of the time, 14% had them sporadically, and 14% had them rarely or never. When asked how much they
agreed with the statement, “Dipper wells are a potential source of allergen cross-contact in ice cream scoop shops,” 43% strongly agreed and 57% agreed.

**Discussion**

This was a pilot study to investigate the risk of allergen cross-contact in ice cream scoop shops via dipper well water. We evaluated the ability of a continuous flow dipper well to remove peanut residue by adding a full serving of peanut butter ice cream to the dipper well and analyzing the peanut levels over time by real-time PCR. We used a full scoop of ice cream to represent a worst case scenario. Ice cream was formulated to have 10% peanut butter by weight based on recipe recommendations by Goff and Hartel (2013). We decided to use a PCR-based method over an ELISA-based method because DNA-based detection is more specific, less variable, more heat stable, and has reduced matrix effect compared to protein-based detection (Poms et al., 2004a; Monaci and Visconti, 2010; Prado et al., 2016). Although DNA-based methods do not directly quantify allergens, but rather the presence of the allergen-containing species, they are useful for screening purposes when absolute quantification is not necessary. The commercial real-time PCR kit used in this study contained an internal standard in the master mix to control for differences in amplification efficiency, but DNA extraction efficiency was not accounted for. Several studies have found that the food matrix can affect quantitative PCR analysis (Kenk et al., 2012; Siegel et al., 2013; Martín-Fernández et al., 2016). Our preliminary research showed that the peanut butter ice cream matrix had around a 25% recovery rate (defined as average detected peanut divided by incurred peanut, both in milligrams per kilogram, multiplied by 100) with this real-time PCR kit.
Based on these results, a corrective multiplication factor of 4 could be applied to the quantitative data in this study. However, there were several factors in the preliminary study that could have decreased the recovery rate, such as a high concentration of PCR inhibitors in the sample and dilution of the DNA extract in order to obtain quantitative results. We do not believe that PCR inhibitors significantly influenced the results of the current study because the samples were largely diluted by water, therefore also diluting the concentration of inhibitors. Furthermore, the samples in this study did not need to be diluted in order to obtain quantifiable results, eliminating that source of error. Of note, the kit reference material was composed of defatted peanut in rice flour matrix, whereas the samples were composed of full-fat peanut butter in ice cream matrix. The difference in composition between reference material and samples could have affected the accuracy of the peanut quantification. Overall, the PCR analysis had good efficiency and was adequate for an initial analysis of allergens in dipper well water, but further research should be conducted to verify if a corrective multiplication factor needs to be applied to the quantitative results.

Rinse times were selected based on previous dipper well studies (Gibson and Almeida, 2015; Almeida and Gibson, 2016). Two different treatments were used to add the peanut butter ice cream to the dipper well. In Treatment 1, ice cream was added to the basin when it was filled halfway with water in order to prevent overflow (and therefore, loss of peanut). Treatment 1 showed an unexpected increase in peanut detection at the 10 and 30 s mark. This anomaly can be explained by the density of the two components. Ice cream is less dense than water, so it should float on top of the water and get washed down
the dipper well drain. However, water was added on top of the ice cream for Treatment 1. Therefore, it may have taken 10 or 30 s for the ice cream to float to the top, at which point the water sample was collected. The level of peanut detected drastically decreased after the 30 s mark, supporting this hypothesis. In Treatment 2, ice cream was added into a full dipper well. This treatment showed a consistent decrease in peanut detection over time, as expected. Because the ice cream was added on top of the water, it was washed down the drain quickly, explaining the lower average detection levels for Treatment 2. In a real-life dipper well usage scenario, some ice cream would be expected to come off the ice cream scoop immediately when the utensil hit the surface of the water and some ice cream would be expected to adhere to the utensil after it was submersed. Therefore, we believe that actual allergen removal behavior would be a hybrid of Treatments 1 and 2. Both treatments followed an exponential decay pattern, with $R^2$ values of 0.9894 and 0.9998, respectively. Quantitative allergen detection is only significant if thresholds, or limits below which only the most sensitive allergic subjects might react, are defined. Several countries have defined allergen thresholds, but the VITAL scheme, created by the Allergen Bureau of Australia and New Zealand, appears to be the most robust and reliable (Allen et al., 2014a; Allen et al., 2014b). If allergens are present at or above the defined threshold, they must be labeled, regardless of whether they were intentionally added or present through accidental cross-contact. The VITAL threshold dose for peanut is 0.2 mg of peanut protein, which equates to about 0.8 mg of whole peanut (Taylor et al., 2002; Lexmaulová et al., 2013). Assuming a volume of 1 mL of dipper well water, the clinically relevant dose of peanut is 800 mg/kg, which is well above the highest levels of
peanut detected in this study. If the volume of dipper well water was changed to 2 mL, the clinically relevant dose of peanut would be 400 mg/kg. Several of the water samples from Treatments 1 and 2 were above or close to 400 mg/kg. However, Treatments 1 and 2 represent a worst case scenario. It is unlikely that an entire scoop of ice cream would be added to a dipper well. Furthermore, it is unlikely, though definitely possible, that 2 mL of dipper well water would end up in a scoop of ice cream. The continuous use scenario is much more realistic and representative of actual dipper well use in ice cream scoop shops. Peanut levels from this experiment were well below the clinically relevant doses referenced above. Even if a corrective multiplication factor of 4 was applied to the continuous use scenario results (to account for the potential reduced recovery rate), peanut levels would still be well below the clinically relevant dose. Based on the results of these experiments, we conclude that it is highly unlikely that cross-contact via dipper well water would cause an allergic reaction in a sensitive individual, although it is possible.

Interestingly, the cleaning techniques we tested only removed residual peanut traces 25% (rinse only) and 50% (rinse, alkaline detergent, rinse) of the time. Peanut traces were below the level of quantification of the PCR kit, but these results indicate that more robust cleaning procedures might needed to remove high concentrations of allergens from dipper well basins. Water measurements fell within normal parameters, so water quality was not believed to be a factor in cleaning efficacy (United Utilities, undated). In light of these results, we recommend a rinse, alkaline detergent, rinse, and sanitize cleaning procedure to clean dipper wells. Velocity, contact time, and temperature
all effect cleaning efficacy, so increased water velocity and temperature are recommended. We recommend following the manufacturer’s directions for contact time, as contact time requirements depend on the specific detergent and sanitizer used. Special care should be taken to scrub uneven areas, such as the area around the drain, in order to remove all traces of allergens.

Survey results showed that only 29% of respondents had advisory allergen signs posted in their store. Though our sample size was small, a study of peanut allergen cross-contact at takeaway establishments in the U.K. found even lower rates of allergy warning signs (Leitch et al., 2005). Warning signs were not common in the ice cream shops surveyed, but standard procedures for serving a customer with food allergy were well-established. Notably, using a clean, sanitized scoop (not from the dipper well) was common practice. Several studies have shown that allergen cross-contact is a poorly understood concept by foodservice workers (Abbot et al., 2007; Radke et al., 2016; Dupuis et al., 2016), but the results of our survey indicate that the cross-contact potential of dipper wells is a well understood concept by ice cream scoop shop owners. That being said, other ice cream scoop shop staff members may not fully understand allergen cross-contact, which is why cross-contact prevention procedures and research-based precautionary allergen labeling is of utmost importance to protect sensitive consumers. Ideally, ice cream scoops shops should post allergen advisory signs, and when informed of an allergy, they should use a sanitized ice cream scoop and a fresh container of ice cream to serve the customer.
Our survey results showed that all establishments ran their dipper well from open of business until close of business, with the majority of them running their dipper well at half force. Recent research has focused on developing a reduced water alternative to continuous flow dipper wells (Gibson and Almeida, 2015; Almeida and Gibson, 2016). Unfortunately, the measures taken to reduce water usage while controlling for microbial growth (i.e., UV light and ozone treatment) would not be effective against allergens, as allergens are proteins and not living organisms. When evaluating the efficacy of reduced water dipper well systems, it is important to concurrently consider microbial and allergen removal.

There are several limitations of this study. As stated previously, real-time PCR is an indirect method for allergen analysis, but absolute quantification was not necessary for this study, as it was a pilot study to determine if further analysis was required. Follow-up studies could verify these findings with an ELISA-based method, or even a mass spectrometry method for direct allergen analysis. The survey response rate was relatively low, so the data might not be representative of all ice cream scoop shops. Future studies could investigate dipper well practices and procedures in retail foodservice establishments in more depth. Several types of foodservice establishments use dipper wells to store in-use utensils, but these results are only applicable to ice cream scoop shops. More studies would be necessary to determine if dipper well model, flow rate, food matrix, and allergen type significantly affect results.
1. Conclusions

Treatment of a dipper well with high loads of peanut allergen showed that allergen removal behavior follows an exponential decay pattern. Quantitative analyses showed that peanut was present in ice cream dipper well water for all rinse times tested, during a continuous use scenario, and following cleaning treatments. However, based on a thorough hazard analysis, we do not believe that ice cream dipper wells pose a significant risk to food-allergic customers. We still recommend that ice cream scoop shops post allergen warning signs to notify customers of potential cross-contact. In addition, we recommended that ice cream scoop shops use clean, sanitized scoops (not from the dipper well) to serve customers with a food allergy as an extreme precaution.

Acknowledgements

We would like to thank members of the National Ice Cream Retailers Association (NICRA) for participating in our survey.

References


Stephan, O., N. Weisz, S. Vieths, T. Weiser, B. Rabe and W. Vatterott. 2004. Protein quantification, sandwich ELISA, and real-time PCR used to monitor industrial cleaning procedures for contamination with peanut and celery allergens. J. AOAC Int. 87:1448-1457.


APPENDIX A

foodproof Sample Preparation Kit III

A. Kit Contents
All solutions are clear and should not be used when precipitates have formed. If precipitates have formed, simply warm
the solutions at 15 - 25 °C or in a 37 °C water bath until the precipitates have dissolved.

<table>
<thead>
<tr>
<th>Vial / Cap Color</th>
<th>Label</th>
<th>Contents / Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 red</td>
<td>foodproof Sample Preparation Kit III Extraction Buffer</td>
<td>2 x 45 ml for extraction of DNA</td>
</tr>
<tr>
<td>2 green</td>
<td>foodproof Sample Preparation Kit III Binding Buffer</td>
<td>25 ml for binding of DNA to glass fibre fleece</td>
</tr>
<tr>
<td>3 blue</td>
<td>foodproof Sample Preparation Kit III Wash Buffer</td>
<td>10 ml, add 40 ml absolute ethanol for removing impurities</td>
</tr>
<tr>
<td>4 colorless</td>
<td>foodproof Sample Preparation Kit III Elution Buffer</td>
<td>44 ml for elution of DNA</td>
</tr>
<tr>
<td>5 purple</td>
<td>foodproof Sample Preparation Kit III Proteinase K</td>
<td>100 mg lyophilized, dissolve in 5 ml ddH₂O, aliquot and store at -15 to -25 °C, for protein digestion and inactivation of endogenous nucleases</td>
</tr>
<tr>
<td>6</td>
<td>Filter Tubes</td>
<td>Bag with 50 polypropylene tubes with two layers of glass fibre fleece, for use of up to 700 μl sample volume</td>
</tr>
<tr>
<td>7</td>
<td>Collection Tubes</td>
<td>3 x Bag with 50 polypropylene tubes (2 ml)</td>
</tr>
</tbody>
</table>

B. Storage and Stability
The foodproof Sample Preparation Kit III components must be stored at 15 - 25 °C. Kit components are guaranteed to be stable until the expiration date printed on the label.

Note: Improper storage at 2 - 8 °C (refrigerator) or -15 to -25 °C (freezer) will adversely impact DNA purification when precipitates form in the solutions.

After dissolution of Proteinase K the solution should be aliquoted and stored at -15 to -25 °C. The solution is stable at -15 to -25 °C for 12 months.

C. Basic Steps

<table>
<thead>
<tr>
<th>Stage</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>DNA is extracted by incubation with the Extraction Buffer.</td>
</tr>
<tr>
<td>2</td>
<td>Proteinase K digestion of endogenous nucleases and other impurities.</td>
</tr>
<tr>
<td>3</td>
<td>DNA is bound to the glass fibers pre-packed in the filter tube.</td>
</tr>
<tr>
<td>4</td>
<td>Washing of bound DNA, and purification from salts, proteins, and other cellular impurities.</td>
</tr>
<tr>
<td>5</td>
<td>Purified DNA is recovered using the Elution Buffer.</td>
</tr>
</tbody>
</table>
### D. Procedure

The following protocol describes the isolation of DNA from 200 mg homogenized food sample.

<table>
<thead>
<tr>
<th>Step</th>
<th>Action</th>
<th>Volume</th>
<th>Time/Temp.</th>
</tr>
</thead>
</table>
| 1    | • Add Extraction Buffer (bottle 1, red cap) to 200 mg homogenized sample (in 2 ml microcentrifuge tubes).  
      • Vortex for 30 s.  
      • Incubate.  
      Note: Mix 2-3 times during the incubation by inverting the tube. If the matrix absorbs the Extraction Buffer, add additional buffer. | 1.5 ml | 30 s  
80 °C for 30 min |
| 2    | Centrifuge. | | 10 min at 12,000 x g |
| 3    | Add Binding Buffer (bottle 2, green cap) to a new 2 ml microcentrifuge tube. | 400 µl | |
| 4    | • Transfer supernatant to the new 2 ml microcentrifuge tube with Binding Buffer, then mix gently but thoroughly by pipetting up and down.  
      • Add Proteinase K working solution (100 mg/5 ml ddH₂O)  
      • Mix gently but thoroughly by pipetting up and down.  
      • Incubate. | 600 µl  
80 µl | 72 °C for 10 min |
| 5    | • Add isopropanol.  
      • Mix well by pipetting up and down. | 200 µl | |
| 6    | Pipet the mixture into the upper reservoir of a combined Filter Tube-Colletion Tube assembly. | 650 µl | |
| 7    | Centrifuge in a microcentrifuge. | | 1 min at 5,000 x g |
| 8    | • Discard flow-through and Collection Tube, place Filter Tube in new Collection Tube.  
      • Apply the remaining mixture to the same Filter Tube and centrifuge.  
      Note: If DNA must be pooled, steps 6-7 can be repeated with additional sample preparations. | | 1 min at 5,000 x g |
| 9    | • Discard flow-through and Collection Tube, place Filter Tube in new Collection Tube.  
      • Add Wash Buffer working solution (bottle 3, blue cap) to the upper reservoir.  
      • Centrifuge. | 450 µl | 1 min at 5,000 x g |
| 10   | • Discard the flow-through and reuse the Collection Tube.  
      • Add Wash Buffer working solution (bottle 3, blue cap) to the upper reservoir.  
      • Centrifuge. | 450 µl | 1 min at 5,000 x g |
| 11   | • Discard the flow-through and reuse the Collection Tube.  
      • Centrifuge to remove residual Wash Buffer. | | 10 s at max speed (13,000 x g) |
| 12   | • Insert Filter Tube in a clean 1.5 ml reaction tube.  
      • Add pre-warmed (70 °C) Elution Buffer (bottle 4, colorless cap) onto the glass fibre fleece.  
      • Incubate. | 100 µl | 15 - 25 °C for 5 min |
| 13   | Centrifuge. | | 1 min at 5,000 g |
| 14   | The microcentrifuge tube now contains the eluted DNA. | | |
APPENDIX B

Allergen RM 800

A. Contents

<table>
<thead>
<tr>
<th>Product</th>
<th>Storage</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 bottle with 2 g Allergen RM 800</td>
<td>-15 to -25 °C</td>
</tr>
<tr>
<td>5 x 1 ml Dilution Buffer</td>
<td>4 °C</td>
</tr>
</tbody>
</table>

B. Test Principle

The DNA of Allergen RM 600 should be prepared in combination with the foodproof Sample Preparation Kit III and the foodproof Magnetic Preparation Kit III. For that, 200 mg is set up as the initial sample size for the DNA extraction purposes. The purified DNA is used for the preparation of a decadal calibration curve, ranging from 800 mg/kg (undiluted) up to 0.0 mg/kg, while 0.0 mg/kg is equal to the limit of quantification. The calibration curve should be used together with foodproof Celery Detection Kit, foodproof Soya Detection Kit, foodproof Hazelnut Detection Kit, and foodproof Peanut Detection Kit for reliable quantitative results.

C. Procedure A – Distribution of wells for a quantification using included (in run) calibration curve:

- Calibration curve: 4 dilution steps with 2 replicates each of the Allergen RM 600,
- A variable number of sample preparations to be analyzed for allergenic contents,
- At least one negative control reaction for the respective allergen-PCR.

A typical experiment consists of 9 wells needed for calibration curve and negative control, plus 2 × n wells (n = number of food samples). Since a multiwell plate has 96 wells, up to 43 food samples can be analyzed during one PCR run if the Allergen RM 600 calibration curve is analyzed in the same run.

Dilution of Allergen RM 800 DNA

Quantification of the Allergen content via procedure A requires the stepwise dilution of the Allergen RM 800 purified DNA in the Dilution Buffer as shown below:

<table>
<thead>
<tr>
<th>Dilution step</th>
<th>Dilution</th>
<th>Concentrations to be entered as standards for the Allergen PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>undiluted</td>
<td>800</td>
</tr>
<tr>
<td>2</td>
<td>1:10</td>
<td>60</td>
</tr>
<tr>
<td>3</td>
<td>1:100</td>
<td>8</td>
</tr>
<tr>
<td>4</td>
<td>1:1,000</td>
<td>0.8</td>
</tr>
</tbody>
</table>
D. Procedure B – Distribution of wells for a quantification using external (imported) standard curves:

- One positive reaction with the purified DNA of Allergen RM 800.
- A variable number of sample preparations to be analyzed for allergenic contents.
- At least one negative control reaction for the respective allergen-PCR.

A typical experiment consists of 2 wells needed for controls, plus 2 x n wells (n = number of food samples). Since a multiwell plate has 96 wells, 47 food samples can be analyzed during one PCR run.

**Note:** Procedure B is only applicable if the used real-time PCR instrument provides the opportunity to import an external standard curve generated in a previous run.

E. Application in PCR Mixes

For reliable quantitative results, it is recommended to apply the Allergen RM 800 in combination with the:
- **foodproof** Celery Detection Kit
- **foodproof** Soya Detection Kit
- **foodproof** Hazelnut Detection Kit
- **foodproof** Peanut Detection Kit

Proceed as described below to prepare a 25 µl standard reaction. Always wear gloves when handling the PCR-vessels and do not touch the upper surface of the PCR plate:

1. Thaw the solutions and, for maximal recovery of contents, briefly spin vials in a microcentrifuge before opening.
2. Mix carefully but thoroughly by pipetting up and down. Do not vortex.
   - **Procedure A:** For the included (in-run) calibration curve, add 5 µl of each dilution and replicate of Allergen RM 800 DNA to the wells containing 20 µl PCR-Master Mix.
   - **Procedure B:** For the use of the external calibration curve, add 5 µl of the purified of Allergen RM 800 DNA.
   - For the samples of interest, add up to 5 µl sample DNA (if less than 5 µl add H₂O to 5 µl) to a well.
   - For the negative control, add 5 µl PCR-grade H₂O.
3. Seal the plate accurately with an optical sealing foil.
4. Place the plate in a swing bucket centrifuge and centrifuge at 1,500 x g for 30 s.
5. Cycle the samples as described in respective QRP.
APPENDIX C

foodproof Peanut Detection Kit

A. Real-time PCR Time-Temperature Protocol
The following procedure is optimized for a real-time PCR instrument with FAM (detection of peanut) and HEX (detection of the internal control). Program the PCR instrument before preparing the reaction mixes. The amplification is carried out according to the following temperature-time-program (for details on how to program the experimental protocol, see the operation manual of your real-time PCR cyclo):

Program the PCR instrument before preparing the reaction mixes

<table>
<thead>
<tr>
<th>Program for real-time PCR instrument</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-incubation</td>
</tr>
</tbody>
</table>

Step 1: 37 °C for 4 minutes
Step 2: 95 °C for 10 minutes

Amplification: 50 cycles
Step 1: 95 °C for 5 seconds
Step 2: 60 °C for 60 seconds

Fluorescence detection in step 2

Note: The instrument compatibility was tested for LightCycler® 480 Systems I and II, Mx3000p, ABI 7300 FAST, PikoReal, and Bio-Rad iCycler. For some real-time PCR instruments (e.g. ABI7500) the type of the probe quencher as well as the usage of a passive reference dye has to be determined. The foodproof Peanut Detection Kit contains probes with a nonfluorescent quencher and no passive reference dye. For users of the Agilent Mx3000p instrument: Click “Instrument Filter Set Gain Settings” to open the Filter Set Gain Settings dialog box in which the gains settings may be viewed and modified. For FAM and HEX the Filter Set Gain Setting must be modified to “4”.

B. Preparation of the PCR Mixes
Proceed as described below to prepare a 25 µl standard reaction. Always wear gloves when handling the PCR-vessels and do not touch the upper surface of the PCR plate.

1. Thaw the solutions and, for maximal recovery of contents, briefly spin vials in a microcentrifuge before opening.
2. Mix carefully but thoroughly by pipetting up and down. Do not vortex.
   - Pipet 20 µl of the Peanut Master Mix (via 1, yellow cap) into each well
   - For the samples of interest, add up to 5 µl sample DNA (if less than 5 µl add H2O to 5 µl) to a well.
   - For the positive control, add 5 µl Peanut Control Template (via 2, purple cap) to a well
   - For the negative control, add 5 µl H2O PCR-grade (via 3, colorless cap)
3. Seal the plate accurately with an optical sealing foil
4. Place the plate in a swing bucket centrifuge and centrifuge at 1,500 x g for 30 s
5. Cycle the samples as described above.

Note: For quantification purposes please refer to our reference material Allergen RM 000 at www.bc-diagnostics.com
**Product Characteristics**

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Specificity</strong></td>
<td>The primers and hydrolysis probes (5’ nuclease probes) provided in the Master Mix, (vial 1, yellow cap) are sequence-specific for peanut and the Internal Control, respectively. Specificity of the assay was proven by 81 plant and animal species, as well as 13 commercial food products.</td>
</tr>
<tr>
<td><strong>Sensitivity</strong></td>
<td>The limit of detection was determined to be 0.2 peanut genome equivalent and 1 ppm in peanut spiked rice flour matrix. The limit of quantification was determined to be 0.8 ppm based on the threshold set by the standard curve.</td>
</tr>
<tr>
<td><strong>Precision</strong></td>
<td>The Repeatability Relative Standard Deviation (RSDr) of high and low concentrations of peanut in food samples was measured to be below 13.7 % for Allergen RM 600 at 800 ppm, 60 % (0.3ppm ±0.2) for 1 ppm in ice cream, and 53.8 % (0.2ppm ±0.1) for 1 ppm in chocolate.</td>
</tr>
<tr>
<td><strong>Robustness</strong></td>
<td>Reproducibility of Cp-values was successfully tested with different real-time PCR-instruments, including Roche LightCycle™ 430 II, Agilent Mx3005p, Applied Biosystems® 7500 FAST, Thermo Scientific PikoReal, and Bio-Rad iQ™5 Cycler.</td>
</tr>
</tbody>
</table>

**Note:** More detailed information is listed in the Validation Data Report of the foodproof Peanut Detection Kit. Please contact our Technical Support (bdc@bh-diagnostics.com).
APPENDIX D

NICRA Dipper Well Survey Questions

Directions:
- Your responses are very valuable to us! Thank you for taking the time to complete this survey.
- Please contact Lindsey Lake at LKEATIN@G.CLEMSON.EDU or 630-806-5188 if you have any questions.
- Your participation in the survey will remain anonymous. Please answer all questions as honestly and completely as possible.
- To complete a multiple choice question, select the answer that you have chosen by highlighting it in yellow as shown below in the example question.
- To complete a non-multiple choice question, please type in your answer.

Example Question:
Example question one:
  a. Yes
  b. No

Inclusion Criteria:
1. Does your ice cream shop currently use a dipper well?
   a. Yes
   b. No
   c. I don't know
2. Are you an owner/operator of an ice cream scoop shop?
   a. Yes
   b. No

If you did not answer ‘yes’ to both questions above, please end the survey and proceed to the return directions that are found at the end of this document.
If you answered ‘yes’ to both questions above, please proceed to the survey questions.
Survey Questions:

3. If convenient, please list the brand and model of your dipper well below.

4. Which of the following statements best describes the operation of your dipper well:
   a. Continuously run from open of business until close of business
   b. Turned on and off according to how busy we are
   c. Turned on and off according to a timed schedule
   d. Other (please describe):
   e. I don’t know

5. What is the typical flow rate of the dipper well faucet when turned on? Select the answer that is closest to the estimated flow rate.
   a. Full force
   b. Three quarters force
   c. Half force
   d. Quarter force
   e. Minimum force
   f. Variable
   g. I don’t know

6. Do you have an SOP (Standard Operating Procedure) for cleaning your dipper well?
   a. Yes
   b. No
   c. I don’t know
   d. Most of the time
   e. Sporadically
   f. Rarely or never
   g. I don’t know

7. Do you have an SOP for serving customers with a food allergy? (If No, proceed to question #12)
   a. Yes
   b. No
   c. I don’t know

8. Do employees have convenient access to this SOP during operating hours?
   a. Yes
   b. No
   c. I don’t know
9. Are employees trained on this SOP at the beginning of their employment?
   a. Yes
   b. No
   c. I don’t know

10. Please write down your SOP for serving customers who have informed you that they have a food allergy.

11. Do you have any advisory allergen statements, such as ‘may contain [allergen]’ statements, posted in your store on signs or boards?
   a. Yes
   b. No
   c. I don’t know

12. If yes, where are these signs located?

13. Do you or your employees verbally inform customers that have a food allergy that your products are not guaranteed to be allergen-free?
   a. Yes
   b. No
   c. I don’t know

14. How often does your shop have at least one ice cream flavor that contains peanut or peanut-containing ingredients (such as peanuts, snickers, peanut butter, peanut butter cups, peanut butter swirls, etc.)?
   a. All of the time
   b. Most of the time
   c. Sporadically
   d. Rarely or never
   e. I don’t know
15. How much do you agree with the following statement: Dipper wells are a potential source of allergen cross-contact in ice cream scoop shops.
   a. Strongly agree
   b. Agree
   c. Neutral
   d. Disagree
   e. Strongly disagree
   f. I do not know what allergen cross-contact is

Return Directions:
- Thank you so much for participating in our survey!
- Please save your responses and send the completed survey to Lindsey Lake at LKEATIN@G.CLEMSON.EDU