

Sandwich Enzyme-Linked Immunosorbent Assay (ELISA) for Detection of Cashew Nut in Foods

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Abstract: The presence of undeclared cashew can pose a health risk to cashew-allergic consumers. The food industry has the responsibility to declare the presence of cashews on packaged foods even when trace residues are or might be present. The objective of this study was to develop a rapid, sensitive, and specific enzyme-linked immunosorbent assay (ELISA) for the detection of cashew residues. Raw and roasted cashews were defatted and used separately to immunize sheep, goats, and rabbits. The cashew ELISA was developed using sheep and rabbit polyclonal anti-roasted cashew sera as capture and detector reagents, respectively, with visualization through an alkaline phosphatase-mediated substrate reaction. The cashew ELISA was shown to have a limit of quantification of 1 ppm (1 μg cashew/g). The ELISA was highly specific except that substantial cross-reactivity was noted with pistachio and a lesser degree of cross-reactivity was noted with hazelnut. The performance of the ELISA was assessed by manufacturing cookies, ice cream, and milk chocolate with added known amounts (0 to 1000 ppm) of cashew. The mean percent recoveries for ice cream, cookies, and milk chocolate were $118\% \pm 2.9\%$, $84.3\% \pm 4.0\%$, and $104\% \pm 3.0\%$, respectively. In a limited retail survey, 4/5 retail samples with cashew declared on ingredient labels tested positive for cashew compared to 5/36 samples of foods with precautionary labels indicating the possible presence of one or more tree nuts and 0/18 samples without cashew declared on the label in any manner. The cashew ELISA can be used to detect undeclared cashew residue in foods and as a potential tool for the food industry to assess the effectiveness of allergen control strategies and to guarantee compliance with food labeling regulatory requirements.

Keywords: allergen control, allergy, cashew, detection, ELISA

Introduction

Cashew (*Anacardium occidentale* L.) is a tree in the flowering plant family of Anacardiaceae, which also includes pistachio, mango, Asian sumac, hog plum, poison ivy, and others (Garcia and others 2000). This family contains 75 genera and about 700 species. The *Anacardium* genus contains 8 species; cashew is by far the most important economically (Aliyu 2005). Originally spread from Brazil by the Portuguese, cashew is now cultivated in all regions with sufficiently warm and humid climates.

Internationally, the cashew nut kernel is a very desirable food. Cashew is consumed as a snack nut, as an ingredient incorporated into a variety of foods such as ice creams, sweets, cakes, and chocolates both at home and industrially, and as a paste to spread on bread or crackers. An estimated 60% of cashew kernels are consumed in the form of snacks, while the remaining 40% are mostly included in confectionery products (Azam-Ali and Judge 2001). The composition of the kernel is protein 21%, carbohydrate 25%, and fat 46% (Nandi 2005); cashew is also a very good source of minerals such as calcium, magnesium, phosphorus, sodium, iron, and others.

The presence of undeclared cashews in foods poses a risk to cashew-allergic consumers. Cashew allergy is primarily an IgE-mediated allergic reaction (Teuber and others 2002). Tree nuts

are collectively considered as among the most common allergenic foods on a worldwide basis (Taylor and Hefle 2001). Approximately 0.6% of the U.S. population is allergic to tree nuts (Sicherer and others 2010), and cashew is the second leading cause (after walnut) of food allergy among patients with tree nut allergy (Fleischer and others 2005). The prevalence of cashew allergy has considerably increased in recent years (Rance and others 2003). Cashew appears to be a very potent allergenic food. The ingestion of foods containing cashews has caused systemic anaphylactic reactions and fatalities in both children and adolescents (Sampson and others 1992; Tariq and others 1996; Moneret-Vautrin and others 1998; Rasanen and others 1998; Quercia and others 1999; Hourihane and others 2001; Ferdman and others 2006; Hasegawa and others 2009). In recent studies, cashew nut allergy was more frequently associated with severe clinical reactions than was peanut allergy, a legume often implicated in severe allergic reactions (Davoren and Peake 2005; Clark and others 2007).

The elimination of cashews from the diet is still the only proven approach for the prevention of allergic reactions in individuals with cashew allergy (Sampson 2004). Cashew-allergic individuals must adhere to strict avoidance diets and are reliant on the accuracy of the ingredient statement on packaged foods. Undeclared cashew residues can occur in foods due to inadequate cleaning of shared processing equipment, inappropriate use of cashew-containing rework, packaging errors, formulation mistakes, and other factors (Taylor and Hefle 2005; Jackson and others 2008). Reliable detection and quantification methods for food allergens are necessary in order to ensure the adequacy of allergy control measures in processing facilities and compliance with food labeling laws and regulations (Taylor and others 2007). Because undeclared cashew

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residues in foods pose a potential hazard to cashew-allergic individuals, a sensitive, specific, and rapid method is needed in the food industry and by regulatory agencies to detect cashew residues (Hefle 1998; Wei and others 2003).

ELISA is usually the method chosen for the quantitative analysis of allergenic residues of various foods because it has excellent sensitivity and selectivity and detects the allergenic protein residues (Taylor and Nordlee 1996; Teuber and others 2002). It offers a significant improvement in versatility and performance advantages over other detection technologies (Yeung 2006). Previously, Wei and others (2003) developed a cashew ELISA. We now report the development of a sensitive and specific sandwich-type ELISA for the detection of cashew residues in foods using polyclonal antibodies raised against a mixture of cashew proteins.

Materials and Methods

Immunogen preparation

Cashews were purchased locally, pooled, and washed several times to remove possible residues of any other tree nuts and other materials, and air-dried. A portion of the ground cashews was roasted at 132 °C for 8 min. The raw and roasted cashews were chopped, frozen, and ground to a small particle size (16-speed Oster, Niles, Ill., U.S.A.). Each portion was separately defatted by 5 repetitive treatments with 1:5 (w/v) ethyl ether. The defatted powdered raw and roasted cashews suspended in a slurry in Complete Freund's Adjuvant (CFA) were used as immunogens. The soluble protein contents of the raw and roasted cashew immunogens were determined by the Lowry method (Lowry and others 1951).

Polyclonal antisera production

Polyclonal antibodies were produced at Covance Research Products (Denver, Pa., U.S.A.) using standard immunization protocols described by Harlow and Lane (1988). The powdered and defatted raw and roasted cashews were used separately as the immunogens, although ultimately only antisera directed against roasted cashew was used for development of the immunoassay and subsequent analysis. Each immunogen was used to immunize 1 sheep, 1 goat, and 3 New Zealand white rabbits. For the initial immunization, each rabbit was injected intradermally (back) with 250 µg of immunogen emulsified with CFA. For the goats and sheep, multiple sites were injected subcutaneously with 1.0 mg of the appropriate immunogen emulsified with CFA. On the 14th d after immunization, the first booster dose was administered subcutaneously to each animal using the appropriate immunogen (125 µg for rabbits, 0.5 mg for goats and sheep) emulsified with Incomplete Freund's Adjuvant (IFA). Every 21 d for the first 98 d, each rabbit was injected subcutaneously with 125 µg immunogen emulsified with IFA. From day 119 to 448, the rabbits were injected every 28 d with 100 µg immunogen emulsified with IFA. The same procedure was followed for the goat and sheep except that 0.5 mg cashew immunogen was used for each booster dose. Every 14 d after the booster injection, blood was collected from each animal to monitor antibody production. Polyclonal antibodies were partially purified from the sera by precipitation with ammonium sulfate using the procedure outlined by Harlow and Lane (1988). The partially purified IgG was briefly dialyzed against deionized water, and then extensively dialyzed against 0.01 M phosphate-buffered saline (PBS) (0.002 M NaH₂PO₄, 0.008 M Na₂HPO₄, 0.85% NaCl, and 0.02% NaN₃, pH 7.4) as described by Hefle and others (2001). The specificity and titer of

the antisera were monitored throughout the immunization period with a noncompetitive ELISA essentially as described by Hefle and others (2001). First, microtiter plates (NUNC-ImmunoTM MaxiSorpTM 96-MicroWellTM plates; Nalgel Nunc International, Rochester, N.Y., U.S.A.) were coated with 1 µg of soluble roasted cashew protein/mL of coating buffer (0.015 M Na₂CO₃, 0.035 M NaHCO₃, 0.02% NaN₃, pH 9.6) and allowed to incubate overnight at 4 °C. Plates were washed throughout the assay in an automatic plate washer (Dynatech Laboratories, Inc., Chantilly, Va., U.S.A.) with 0.01 M PBS containing 0.05% Tween 20 (v/v) (Bio-Rad Laboratories, Inc., Hercules, Calif., U.S.A.). Then, serial dilutions of the antisera prepared in conjugate buffer (0.01 M PBS containing 0.1% BSA [bovine serum albumin] [RIA grade, Sigma-Aldrich Chemical Company, St. Louis, Mo., U.S.A.]) were applied to the plates and allowed to incubate for an hour as described by Hefle and others (2001). The titer values of the antisera were calculated using GraphPad Prism[®] Software (GraphPad Software, Inc., San Diego, Calif., U.S.A.) to determine the dilution of the antisera needed to achieve half-maximal binding (the midpoint) of the linear portion of the dilution curve (Hefle and others 2001). Antibodies from bleeds having the highest titers (>10000) were pooled accordingly and used for the development of the cashew ELISA. The protein content of pooled sera was determined by the Lowry method (Lowry and others 1951).

Gel electrophoresis and IgG immunoblotting

The specificity of the polyclonal rabbit, goat, and sheep antibodies used in the sandwich-type cashew nut ELISA to bind to various proteins was investigated by separating the proteins on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and assessing binding with immunoblotting. Immunoblotting was performed using roasted cashew nut, pistachio, hazelnut, and pectin extracts. The protein samples (10 µg/well) were separated under reducing conditions using the Laemmli (1970) method with minor modifications. Electrophoresis was performed using a Bio-Rad Mini Protean[®] II (Bio-Rad Laboratories) with Ready Gel[®] precast gels (15% Tri-HCl; Bio-Rad Laboratories). Proteins were located on the gels by staining with brilliant blue G-collodial stain and then scanned with the Kodak Gel Logic 440 imaging system (Kodak Scientific Imaging Systems, New Haven, Conn., U.S.A.). Proteins from the remaining unstained SDS-PAGE gels were transferred to the polyvinylidene difluoride (PVDF) membrane (Immoblin-P PVDF membrane, 0.45 µm, Millipore Corporation, Billerica, Mass., U.S.A.) as described by Towbin and others (1979) and Harlow and Lane (1988). The PVDF membranes were then blocked with 0.2% BSA in PBS, pH 7.4. Rabbit and sheep roasted cashew antisera (IgG) were used as the primary antibodies. The primary antibodies were diluted 1:10000 (v/v) and 1:15000 (v/v), respectively, in PBS-T (0.05% Tween 20) containing 0.2% BSA RIA grade (USB Corporation, Cleveland, Ohio, U.S.A.). Then the membrane was incubated with either commercial goat anti-rabbit or rabbit anti-sheep antisera conjugated with horseradish peroxidase (diluted 1:20000 [v/v] in PBS-T) (Immunopure[®], Pierce Biotechnology, Inc., Rockford, Ill., U.S.A.). The membrane was then treated for at least 15 min with DAB (3/3'-diaminobenzidine) substrate solution (Pierce Biotechnology, Inc.) prepared according to the manufacturer's recommendation. After washing with PBS-T, the membranes were air-dried overnight at room temperature. A digital image of the stained, dried PDVF membranes was then captured using a Kodak Gel Logic 440 imaging system and analyzed with the Kodak Gel Logic ID v. 3.6.5 software (Kodak Scientific Imaging Systems).

Standard and sample extractions

Ground, washed, and roasted cashews were used as standards throughout the study. All food samples used for various aspects of this project were purchased from local grocery and specialty stores (Lincoln, Nebr., U.S.A.), except for milk chocolate which was produced in a chocolate industry pilot plant (see below). Samples of a solid nature were ground to homogeneous consistency utilizing a commercial food processor (KPF600 Food Processor, Kitchen Aid®, St. Joseph, Mo., U.S.A.) or an Osterizer® blender (Sunbeam Corporation, Delray Beach, Fla., U.S.A.). Fluid samples including oils were used “as is” without further processing.

The standard containing 1000 ppm roasted cashew, samples, model foods (ice cream, sugar cookies, and milk chocolate) were extracted 1:10 in 0.01 M PBS, pH 7.4 with 1% nonfat dry milk (NFD) (PBS + NFD) for 1 h at 60 °C in a shaking water bath (Julabo SW22, Julabo, Inc., Allentown, Pa., U.S.A.) at 140 rpm. The extracts were subsequently centrifuged for 30 min at $3612 \times g$ (4100 rpm) at 10 °C in a table top centrifuge (Sorvall® Legend™ RT; Kendro Laboratory Products, Newton, Conn., U.S.A.). The clarified supernatants were divided into aliquots and stored at –20 °C until used. The protein content of each sample extract was determined using the Lowry method (Lowry and others 1951); in cases where NFD was present in the extract, the protein in NFD was accounted for.

Cashew ELISAs

The cashew sandwich-type ELISA method was developed according to the procedure described by Hefle and others (2001) with slight modifications. Two sandwich-type ELISAs were developed using goat and sheep anti-roasted cashew antibodies as capture reagents. Microtiter plates were used as solid support. The plates were coated with 100 μ L of sheep or goat anti-roasted cashew antibody at 2.25 μ g/mL in coating buffer (0.015 M Na_2CO_3 , 0.035 M NaHCO_3 , and 0.02% NaN_3 , pH 9.6), and plates were incubated overnight at 4 °C. All subsequent incubations were carried out at 37 °C for 1 h (except incubation for color development with substrate). On the following day, each incubated plate was washed with 0.01 M PBS containing 0.05% Tween 20 and 0.02% NaN_3 4 times using a microplate washer (AM6, Dynatech Laboratories). Plates were washed after each immunological incubation step to remove unbound proteins. All of the wells were then blocked with 350 μ L of 0.1% porcine gelatin in 0.01 M PBS followed by incubation. For generation of the standard curve, 100 μ L of serially diluted roasted cashew standards (0 to 1000 ppm) was added to the plates. The bound antigen was detected by the addition of 100 μ L of 1.5 μ g/mL rabbit anti-roasted cashew antibody diluted in conjugate buffer (PBS-B) (0.01 M PBS that contained 0.1% BSA), followed by incubation with 100 μ L of commercial goat anti-rabbit IgG labeled with alkaline phosphatase (Immunopure®, Pierce Biotechnology; diluted 1:5000 [v/v] in PBS-B). The p-nitrophenyl phosphate substrate (SigmaFast™ Sigma Aldrich Co.) was added to produce color and the absorbance was measured at 405 nm using a microplate reader (ELx808 Ultra-plate Reader, Bio-Tek Instruments, Inc., Winooski, Vt., U.S.A.). Standard curves were generated using GraphPad Prism® Software. For the majority of the described experiments, the sheep capture cashew ELISA was used after demonstration that the sheep and goat capture ELISAs yielded quite similar results (see below). Similarly, antisera were obtained to both raw and roasted cashew but only results with antisera against roasted cashew are shown because very similar results were obtained with both antisera.

Reference food standards

Food components can potentially impede the interaction of antigen and antibody in an ELISA. Because cashew is used widely in various popular products, vanilla ice cream, milk chocolate, and sugar cookies were selected as reference foods to evaluate matrix interference and sensitivity of the assay. Chocolate is a challenging food matrix for the extraction of proteins because of its high content of tannins and other phenolic compounds that have a high binding affinity to proteins. Tannins can bind to proteins during extraction and can also interfere with the ELISA test by binding to the antibody. Ice cream, cookies, and milk chocolate (10 g each) were spiked with 0.01 g cashew powder (resulting in 1000 ppm cashew concentration) and were extracted at 1:10 (w/v) with PBS + NFD. Negative controls (cashew-free vanilla ice cream, sugar cookies, and milk chocolate) were also extracted using the same extraction protocol. The clarified supernatants of the spiked food samples were diluted 1:3 (v/v) using each corresponding negative control extract as the diluent to achieve different levels of cashew proteins (0 to 1000 ppm). The spiked food extracts were then analyzed in the sheep capture cashew ELISA.

Cross-reactivity studies

A total of 99 food and food ingredients (Table 1) were investigated for potential cross-reactivity. The foods included mango (skin, flesh, and seed), pistachio, and sumac (belonging to the Anacardiaceae family). These foods and food ingredients were tested with the sheep capture cashew ELISA. The protein content of each extract was determined using the Lowry method (Lowry and others 1951). All food samples were diluted in 0.01 M PBS, pH 7.4, and then analyzed in the cashew ELISA on both protein content (10, 1, and 0.1 μ g/mL) and concentration (1:10, 1:100, and 1:1000) bases. The cross-reactivity of the food extracts was expressed as apparent cashew equivalents by comparing the absorbance readings obtained for these extracts with the standard curves that were constructed based on ground roasted cashew standards prepared in 0.01 M PBS, pH 7.4 in the sheep capture cashew ELISA.

Preparation of manufactured model foods

Vanilla ice cream. The vanilla ice cream formulation was patterned after the recipe for vanilla ice cream VIII from all-recipe.com with revisions. The basic formulation of the vanilla ice cream consisted of 82.4% half-and-half cream, 16.4% white sugar, 0.6% powdered sugar, and 0.68% vanilla extract to provide 886 g of product. The vanilla ice cream mixture was prepared by combining the half-and-half cream with the sugar in an Erlenmeyer flask until the sugar was dissolved. The mixture was stored in a refrigerator overnight for chilling, while the Speed Freeze canister of the ice cream maker (Deni Scoop Factory Compact Automatic Ice Cream & Frozen Dessert maker, Model 5000, Keystone Manufacturing Company, Buffalo, N.Y., U.S.A.) was stored under freezing conditions overnight before use. On the following day, the chilled mixture was placed in the frozen canister while vanilla was slowly poured into the rotating frozen canister. The ice cream maker was allowed to rotate for 10 min until the consistency of the mixture was a soft custard-like ice cream. Ground roasted cashew nuts, powdered in a coffee grinder (Krup's Fast-Touch Coffee Mill™, Shelton, Conn., U.S.A.) with sugar to get a fine homogeneous mass, at 0, 1, 5, 10, 25, 51, and 102 ppm (μ g of cashew nut/g of vanilla ice cream) were spiked independently for each concentration level into the ice cream. The finished ice cream was stuffed into 50 mL conical tubes (BD Falcon™, Franklin Lakes,

N.J., U.S.A.). Three subsamples from each batch were analyzed individually in 2 independent trials for homogeneity and recovery of cashew using the sheep capture cashew ELISA. Recovery was calculated by comparison to the predicted spike concentration.

Sugar cookie. The sugar cookie formulation consisted of 73% Betty Crocker™ sugar cookie mix, 17% butter, 9% egg, and 0.7% powdered sugar to make a 680 g batch of sugar cookie dough. Batches of sugar cookies were individually prepared with 7 different levels of cashew nut (0, 1, 5, 10, 25, 50, and 100 ppm or μg of cashew nut/g of sugar cookie). First, a calculated 100 ppm powdered cashew-in-sugar cookie mixture was prepared by adding 10 g of calculated cashew-in-sugar mix to 992 g of Betty Crocker™ sugar cookie mix in a Kitchen Aid™ 5 Quart Artisan Mixer, KSM150OPS (Kitchen Aid™). The cashew-spiked cookie mixture was mixed for 30 min. This mixture was used as the stock cashew concentrate for each subsequent desired level. The premixed stock was stored in a refrigerator until use.

Each of the 7 different levels was prepared individually starting from zero ppm to the next lowest cashew level. Appropriate amounts of premixed stock corresponding to each desired level were added to the preweighed sugar cookie mix by mixing for 30 min in the Kitchen Aid™ mixer. Preweighed melted butter and preweighed whisked eggs were added to the mixture and mixed thoroughly using the mixer at medium speed for 15 min. Each spoonful of cookie dough (approximately 30 g) was weighed

before and after baking to monitor the moisture loss. The weighed cookie dough from different levels was baked at 375 °F for 9 min. Three fresh cookies from each batch were taken and tested separately in 2 independent trials for homogeneity and recovery of cashew using the sheep capture cashew ELISA. The remainder of the sugar cookies was stored at -20°C .

Milk chocolate. The milk chocolate was manufactured in a chocolate processing facility. The formulation of the milk chocolate consisted of 56.86% fine sugar, 19.89% cocoa butter, 10.01% natural chocolate liquor, 9.586% skim milk powder, 3.175% butter oil, 0.479% soy lecithin, and 0.009% vanillin to make a batch of 50 kg. Ten levels of roasted cashew (0, 1, 5, 10, 25, 50, 100, 250, 500, and 1000 ppm) in milk chocolate were individually prepared. The 0 and 1000 ppm batches of the milk chocolate were made at a chocolate manufacturer's facility. To prepare the 1000 ppm cashew in milk chocolate batch, a calculated amount of cashew nut-in-sugar mixture was introduced into 50 kg of milk chocolate during the conching step. The remaining levels were prepared by mixing appropriate proportions of the 0 and 1000 ppm cashew in milk chocolate formulations. The 0 and 1000 ppm cashew milk chocolates were melted in a 45 °C incubator, and then mixed thoroughly in appropriate proportions to achieve the different designated levels using a commercial mixer (Legacy™ HL200 Mixer, Hobart Corporation, Troy, Ohio, U.S.A.). The mixture was transferred to a pitcher and mixed again using a big

Table 1—List of foods and food ingredients showing no cross-reactivity in the cashew ELISA.

<i>Anacardiaceae</i>	<i>Flours/starches/meal</i>	<i>Herb and spices</i>
Mango (dried)	Corn flour	Basil
Mango (flesh and skin)	Corn starch	Cinnamon
Mango (seeds)	Oats	Cumin
Sumac	Potato flour	Garlic powder
	Rice flour	Ginger powder
<i>Dairy and dairy ingredients</i>	Tapioca starch	Mustard ground
Butter	Wheat flour	Onion powder
Coconut milk	Wheat gluten	Oregano
Cream cheese	White corn meal	Paprika
Fresh cows milk	Whole wheat flour	Pepper black
Nonfat dry milk	Yellow corn meal	Thyme
Parmesan cheese		
Whey	<i>Functional Ingredients</i>	<i>Legumes and legume products</i>
<i>Fruits</i>	Annatto	Carob bean powder
Apple	Barley malt	Green peas (split)
Banana	Calcium sulfate	Peanut flour (roasted defatted)
Beets (flesh)	Caramel color	Peanuts
Beets (water)	Carrageenan	Snow peas
Cherries	Coconut flakes	Soy beans
Cocoa powder	Gelatin	Soy lecithin
Dates	Guar gum	Soy meal defatted
Green bell pepper	Lemon juice	Soy sauce (acid-hydrolyzed)
Pineapple	Orange juice	Soy sauce (naturally brewed)
Plum (flesh)	Pectin	
Plum (seeds)	Potassium sorbate	<i>Sugars and sweeteners</i>
Raisins	Salt	Corn syrup (heavy)
Strawberry	Spray-dried whole egg	Dextrose
	Sodium alginate	Honey
<i>Oils</i>	Sodium bicarbonate	Molasses
Fully hydrogenated palm oil	Vanilla extract (imitation)	Sugar (beet/pure granulated)
Partially hydrogenated palm oil	Vanilla extract (pure extract)	Sugar (cane /white granulated)
Refined canola oil	Xanthan gum	
Refined soybean oil	Yeast-active dry	<i>Tree nuts</i>
	<i>Seeds</i>	Almond
	Flax seeds	Brazil nut
	Sesame seeds	Chestnut
	Sunflower seeds	Macadamia
		Pecan
		Pine nuts
		Walnut

spatula, then tempered on a tempering stainless steel table with the temperature of milk chocolate brought down to 26 °C and back up to 28 °C. The milk chocolates were then molded in a plastic container and stored at room temperature until use. Three subsamples from each desired level were analyzed for the recovery of cashew in 2 independent trials using the sheep capture cashew ELISA.

Limited retail survey. To validate the performance and applicability of the sandwich-type cashew ELISA, different food samples were analyzed. A total of 59 commercial samples from 6 categories, ice cream, cookies, chocolate, nuts/mixed nuts, breakfast/granola or nutritional bars, and nut butters, were selected because they are common types of food in which cashew is often incorporated as one of the ingredients and thus might have experienced cross-contamination with cashew. All samples were extracted, and each extract was analyzed in triplicate in a single trial using the developed sheep capture cashew ELISA.

Statistical analysis

One-way analysis of variance (ANOVA) (GraphPad Software, Inc.) was used for comparison of the slopes of the standard curves obtained in the goat and sheep cashew ELISA, and also for the differences between the standard curves of reference food standards. Results of the percent recoveries of cashew from each manufactured model foods were expressed as the mean $\pm$ standard error of the mean based on the results obtained from triplicate extractions of each model food that was analyzed in 2 trials on separate days. A *P*-value of < 0.05 was considered significant.

Results and Discussion

Sandwich-type cashew ELISAs were successfully developed for the detection of cashew nut residues by using roasted cashew-specific polyclonal antisera from either sheep or goat as the capture antibodies and from rabbit as the detector antibody. The cashew ELISAs were developed using antisera against roasted cashew to increase the probability of detecting proteins that are modified by heat, although antisera against raw cashew appeared to function equivalently well (data not shown). The antisera against roasted cashew antigens from rabbits, goat, and sheep had titers of 130000 ($10^{5.11}$), 98000 ($10^{4.99}$), and 210000 ($10^{5.32}$), respectively. No significant difference ($P > 0.05$) was observed between the slopes of the standard curves of the sheep and goat capture cashew ELISAs (Figure 1) after optimization (data not shown). Both ELISAs have a limit of detection (LOD) estimated at 0.11 ppm (μg of roasted cashew/g). The limit of quantification (LOQ) for both the goat and sheep capture cashew ELISAs was 0.46 ppm. LOQ is the lowest amount of cashew residues that can be quantified with an acceptable level of accuracy and high degree of confidence and precision (Armbruster and others 1994; Westphal 2006). While statistically similar, the sheep capture cashew ELISA was chosen as the method for further analyses. The ELISA results are expressed as ppm cashew as opposed to cashew protein because clinical threshold doses for cashew-allergic subjects are known on the basis of whole cashew. Figure 2 shows that the vanilla ice cream, sugar cookies, and milk chocolate matrices did not significantly ($P > 0.05$) affect the sensitivity of the cashew ELISA. The background absorbances for the negative controls for sugar cookie, vanilla ice cream, and milk chocolate extracts were still < 0.10 AU showing that the signal-to-noise ratio remains very minimal.

The sheep capture cashew ELISA was quite specific for the detection of cashew residues as demonstrated by the cross-reactivity evaluation. Of 99 common foods and food ingredients, only pista-

chio and hazelnut showed any cross-reactivity in the cashew ELISA (Table 1). Minor cross-reactivity was detected with hazelnut at 1 ppm of cashew equivalents while the cross-reaction encountered with pistachio was greater at 15 ppm of cashew equivalents. As shown in Figure 3, immunoblotting with rabbit and sheep antisera showed strong binding with cashew, weaker binding with pistachio, and very weak binding with hazelnut. The results obtained in the immunoblot verify the finding of Barre and others (2007) that the cross-reactivity of hazelnut with cashew is due to amino acid sequence homologies between the 11S globulins. Both the 11S and 7S globulins of hazelnut showed weak binding to the rabbit polyclonal anti-cashew antibodies, while only the 11S globulin showed weak binding with the sheep polyclonal anti-cashew antibodies. Pistachio showed binding with rabbit antisera, but more extensive binding was observed with sheep antisera. These results also coincide with the assertion of Willison and others (2008) that cross-reactivity is due to the sharing of the homologous seed storage proteins in pistachio and cashew as they both belong to the Anacardiaceae family. Because of this cross-reactivity, it is not advised to analyze a food product that has pistachio as one of the ingredients using the cashew ELISA to detect the possible presence of undeclared cashew residues in the food product. The cross-reactivity with hazelnut is likely insufficient to raise such concerns except in the analysis of pure hazelnuts.

Recently, pectin allergy was suggested to have a possible association with cashew allergy (Ferdman and others 2006) and may be considered clinically in cashew-allergic or pistachio-allergic patients who have unexplained reactions. Pectin did not show any cross-reactivity in the cashew ELISA (Table 1), nor was binding seen to either the sheep or rabbit anti-cashew antibodies in the immunoblot (Figure 3). These results do not seem to support the hypothesis that pectin allergy should be considered in cashew- and pistachio-allergic patients with unexplained allergic reactions as proposed by Ferdman and others (2006). In fact, allergies to ingested pectin are rarely encountered. Also, Ferdman and others (2006) assessed pectin with human sera from patients allergic to cashews and pistachios, while IgG-containing rabbit and sheep sera were used in these experiments. Pectin is rich in carbohydrate polymers so the possibility also exists that the results reported by Ferdman and others (2006) were due to clinically insignificant carbohydrate cross-reactive determinants (Ebo and others 2004).

Model foods (naturally incurred standards) provide a rigorous validation of allergen residue detection methods including extraction of the residues from the food after processing (Taylor and others 2009). This validation also allows the determination of the sensitivity and specificity in the food matrix to assure the applicability of the developed ELISA to that particular matrix (Hefle and Koppelman 2006). The true extraction efficiency of a method and the true recovery from the food matrix can only be ascertained with the use of naturally incurred model foods. However, it is not practical to make model foods for every matrix, so several manufactured model foods should be selected that cover a range of applications (Hefle and Koppelman 2006).

Excellent recovery of cashew residues was obtained from the naturally incurred standard of vanilla ice cream with a mean percent recovery of $118\% \pm 2.94\%$ (Table 2). From a processing perspective, vanilla ice cream is a minimally processed matrix involving pasteurization and mixing.

Somewhat lower recovery of cashew residues was obtained from sugar cookies with a mean percent recovery of $84.34\% \pm 4.03\%$ (Table 3) after compensation for the moisture loss after baking ($10.66\% \pm 0.38\%$). The lower recovery of cashew from the sugar

cookies indicates that the heat treatment in baking either had an effect on the immunological properties of the cashew proteins in the cookie matrix or reduced the extractability of the cashew proteins from the cookie matrix. Neto and others (2001) reported that heat treatment of a cashew nut protein isolate reduced the solubility of the cashew protein.

Proteins can be affected by different food processes and to different degrees (Maleki 2004; Poms and Anklam 2004). Proteins can be broken down or denatured by disruption of their tertiary and secondary structures resulting in the modification of the antibody-binding epitopes (Westphal 2006). Furthermore, some proteins can be rendered insoluble and therefore not extractable due to the exposure of the hydrophobic amino acid residues that are buried in the core of the native protein or due to protein aggregation (Koppelman and others 2002). Although the mean percent recovery was lower than ice cream, the extraction method was still reasonably efficient with this baked product.

For milk chocolate, the mean percent recovery of cashew residues was $104\% \pm 2.99\%$ (Table 4). The high recovery of cashew from the milk chocolate indicates that the heat treatment in the conching at 80°C did not have an effect on the immunological properties of the cashew proteins in the milk chocolate food matrix. Moreover, results showed that the extraction method was efficient with the milk chocolate product containing tannins and phenolic compounds that might be expected to bind proteins. The antibodies in the cashew ELISA were capable of efficiently recognizing the cashew proteins extracted from the milk chocolate.

A limited retail survey was conducted to assess the sensitivity, specificity, and applicability of the cashew ELISA in a wider variety of food matrices (Lipp and others 2005). This study was also performed to verify that the requirements of the U.S. Food Allergen Labeling & Consumer Protection Act (FALCPA) were being implemented with respect to cashew. FALCPA mandates source labeling of ingredients from common allergenic foods including cashew. Of a total of 59 commercial food samples in the

limited retail survey, 5 food samples had a cashew declaration on the label, 36 samples had precautionary labeling for tree nuts, and 18 samples were without any cashew or nut declaration on the food labels. Food samples were considered positive if cashew presence was detected above 1.0 ppm (μg cashew/g of sample product). As shown in Table 5, detectable cashew was found in 4/5 retail samples with cashew declared on their ingredient labels, 5/36 samples with precautionary labels, and 0/18 samples that did not have cashew declared on the label. One of five retail samples with declared cashew failed to test positive for cashew. This product was a nut butter with cashew listed as a minor ingredient. In all likelihood, this nut butter was made on shared equipment with cashew butter leading to a decision to declare cashew as a minor ingredient due to the high risk of cross contact. This particular sample did not contain detectable cashew but

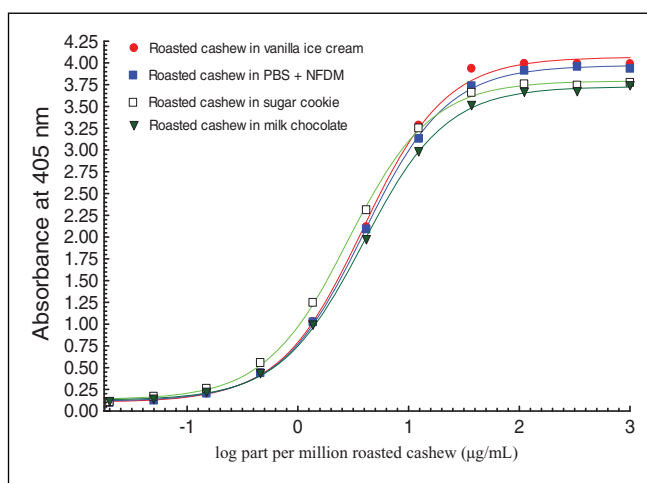


Figure 2—The standard curves of various concentrations of cashew residues in PBS + NFDM (■), vanilla ice cream (●), sugar cookie (□), milk chocolate (▼). Each data point represents 6 readings, with standard error of each point less than < 0.03 absorbance unit.

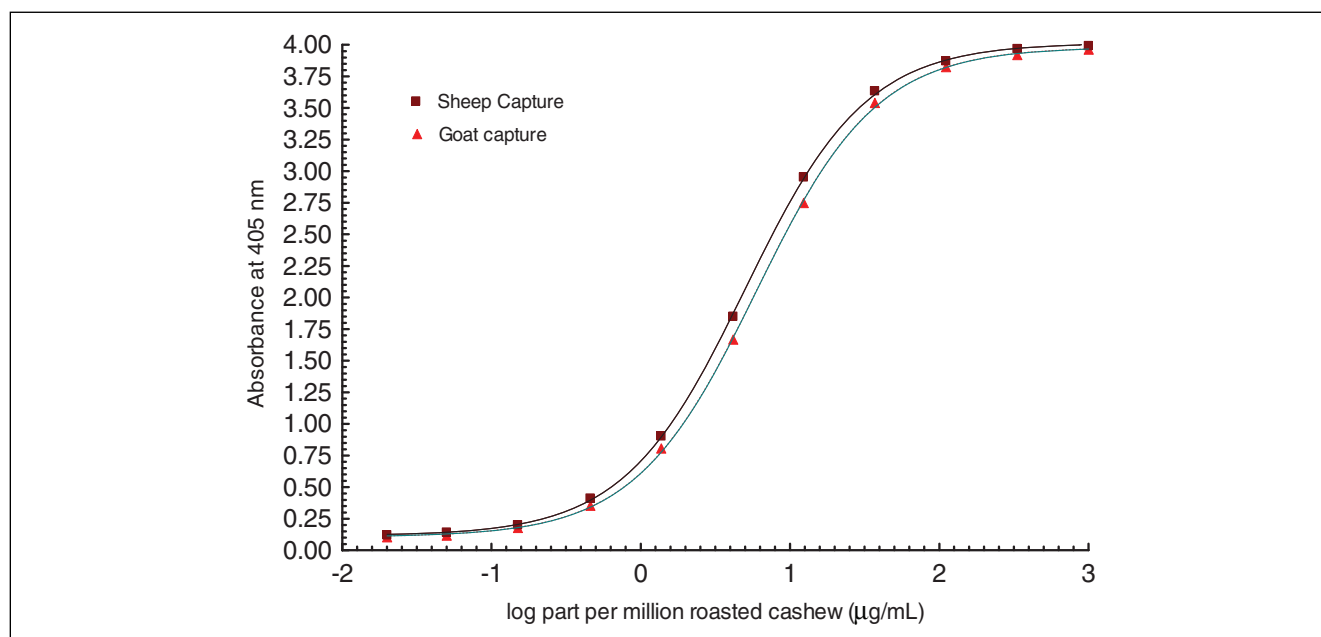


Figure 1—Standard curves obtained from the sheep capture cashew ELISA (■) and goat capture cashew ELISA (▲). The standard curve solutions were prepared in 0.01 M PBS with 1% NFDM by dilution of an extract containing 1000 ppm ground roasted cashew. Each data point represents 6 readings, with a mean standard error of < 0.02 absorbance unit.

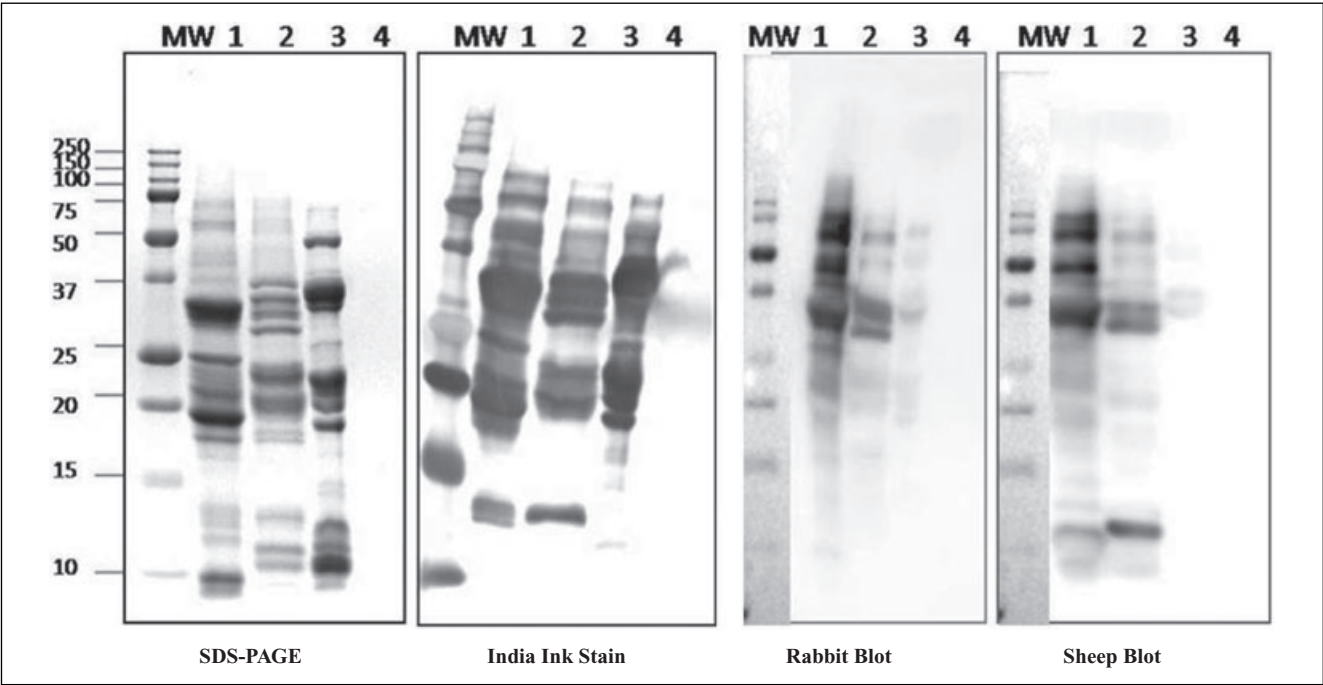


Figure 3—Cross-reactivity assessment of the specificity of the antisera: the first panel shows SDS-PAGE gel stained brilliant blue G-colloidal stained SDS-PAGE gel with separated roasted cashew (1), pistachio (2), hazelnut (3), and pectin (4) extracts. MW is the molecular weight standard for comparison purposes. Panel 2 shows the proteins transferred to PVDF membrane and stained with India ink. Panel 3 shows binding of rabbit anti-cashew sera to the proteins on the PVDF membrane. Panel 4 shows binding of sheep anti-cashew sera to the proteins on the PVDF membrane.

Table 2—Recovery and percent recovery of cashew residues from the manufactured model vanilla ice cream, as determined by the cashew ELISA.^a

Spike levels	Levels detected in ELISA (ppm ^b cashew)						Average ^c (ppm cashew)	% Recovery
	Extract A		Extract B		Extract C			
	Trial 1	Trial 2	Trial 1	Trial 2	Trial 1	Trial 2		
0 ppm	BLQ ^d	BLQ	BLQ	BLQ	BLQ	BLQ	NA ^e	NA
1 ppm	1.10	1.00	1.15	1.16	1.11	1.23	1.12 ± 0.02	112 ± 3.20
5 ppm	5.33	5.66	5.33	5.60	2.39	5.98	5.55 ± 0.07	111 ± 2.07
10 ppm	11.90	11.90	12.40	12.90	13.20	14.40	12.8 ± 0.22	128 ± 3.90
25 ppm	28.30	26.70	27.80	29.60	29.80	29.60	28.6 ± 0.50	114 ± 2.00
51 ppm	59.30	56.80	63.40	59.30	68.70	58.90	61.1 ± 1.10	119 ± 3.44
102 ppm	123.50	123.00	115.90	118.00	135.70	129.90	124 ± 1.82	124 ± 3.02

^aStandard used for the generation of standard curve in the ELISA was vanilla ice cream extracts containing 1000 ppm powdered roasted cashew.
^bppm = parts per million (mg of cashew per kg).
^cData are mean ± STD Error (*n* = 18 replicates).
^dBLQ = below the limit of quantification, <1.0 ppm.
^eNA = not applicable.
Mean percent recovery = 118 ± 2.94%.

Table 3—Recovery and percent recovery of cashew residues from the manufactured model sugar cookie, as determined by the cashew ELISA.^a

Spike levels	Levels detected in ELISA (ppm ^b cashew)						Average ^c (ppm cashew)	% Recovery
	Extract A		Extract B		Extract C			
	Trial 1	Trial 2	Trial 1	Trial 2	Trial 1	Trial 2		
0 ppm	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	NA ^c	NA
1 ppm	1.16	0.94	1.01	0.77	1.16	0.88	0.99 ± 0.06	98.7 ± 6.35
5 ppm	4.25	3.31	4.22	3.46	3.92	3.26	3.74 ± 0.18	74.7 ± 3.68
10 ppm	8.37	7.42	8.31	6.89	7.90	6.63	7.59 ± 0.30	75.9 ± 2.98
25 ppm	20.10	18.74	22.52	21.43	21.00	19.41	20.5 ± 0.57	82.1 ± 2.71
50 ppm	44.67	39.10	43.78	37.25	51.09	48.26	44.0 ± 2.15	88.1 ± 4.29
100 ppm	91.76	90.97	90.60	75.68	100.33	70.36	86.6 ± 4.59	86.6 ± 4.59

^aStandard used for the generation of standard curve in the ELISA was sugar cookie extracts containing 1000 ppm powdered roasted cashew.
^bppm = parts per million (mg of cashew per kg).
^cData are mean ± STD Error (*n* = 18 replicates).
^dBLQ = below the limit of quantification, <1.0 ppm.
^eNA = not applicable.
Mean percent recovery = 84.3 ± 4.03%.

Table 4–Recovery and percent recovery of cashew residues from the manufactured model milk chocolate, as determined by the cashew ELISA.^a

Spike levels	Levels detected in ELISA (ppm ^b cashew)						Average ^c (ppm cashew)	% Recovery
	Extract A		Extract B		Extract C			
	Trial 1	Trial 2	Trial 1	Trial 2	Trial 1	Trial 2		
0 ppm	BLQ ^d	BLQ	BLQ	BLQ	BLQ	BLQ	NA ^e	NA
1 ppm	1.07	1.2	1.02	1.12	1.03	1.16	1.10 ± 0.03	110 ± 2.96
5 ppm	5.22	6.18	4.86	5.61	4.73	5.22	5.30 ± 0.22	106 ± 4.32
10 ppm	11.9	12.60	9.80	10.2	10.2	10.9	10.9 ± 0.45	109 ± 4.47
25 ppm	26.4	27.8	22.4	24.6	24.1	25.1	25.1 ± 0.76	100 ± 3.06
50 ppm	52.0	55.8	48.1	51.8	46.2	50.9	50.8 ± 1.36	102 ± 2.73
100 ppm	104.0	105.0	99.3	102.0	97.4	107.0	102 ± 1.48	102 ± 1.48
250 ppm	272.0	275.0	248.0	230.0	275.0	263.0	260 ± 7.41	104 ± 2.96
500 ppm	569.0	502.0	493.0	479.0	544.0	502.0	514 ± 13.99	103 ± 2.80
1000 ppm	1074	979	1011	967	1099	1016	1024 ± 21.32	102 ± 2.13

^aStandard used for the generation of standard curve in the ELISA was milk chocolate extracts containing 1000 ppm powdered roasted cashew.

^bppm = parts per million (mg of cashew per kg).

^cData are mean ± STD Error (*n* = 18 replicates).

^dBLQ = below the limit of quantification, < 1.0 ppm.

^eNA = not applicable.

Mean percent recovery = 104 ± 2.99%.

Table 5–A limited survey of food items for cashew residues by the cashew ELISA.^a

Sample identification	Total nr tested	Range of cashew levels detected (ppm ^b)
Contains labeling	6	
Chocolate confections	5	96600
Cereal bars	1	54700
Nuts	1	31000
Nut butters	2	BLQ ^c – 45
Precautionary (advisory) labeling	36	
Chocolate confections	12	BLQ
Cereal bars	5	BLQ
Nuts	10	BLQ(8)
		1.25–21 (2)
Nut butters	6	BLQ(3)
		1.73–271 (3)
Cookies	3	BLQ
Potential for cashew but no labeling	18	
Ice cream	10	BLQ
Cereal bars	5	BLQ
Nut butters	2	BLQ
Cookies	1	BLQ

^aStandard used for the generation of the standard curve in the ELISA was PBS + NFDM extracts containing 1000 ppm powdered cashew.

^bppm = parts per million (mg of cashew per kg).

^cBLQ = below the limit of quantification, < 1 ppm.

All samples were extracted in 0.01 M PBS + NFDM and each extract was analyzed in triplicates in just one trial.

only one sample from this lot was tested so the results may not be representative. Positive results were obtained with foods having precautionary labeling that were likely manufactured on shared equipment with products containing cashews; examples include nuts and nut butters. Because the precautionary statements often refer to tree nuts and not specifically to cashews, some of these products may actually not have been at risk of cashew contamination. The surveyed products with no declaration of cashew or other nuts were selected because other similar products contained cashews, enhancing the possibility of cashew contamination. Thus, all products tested in this survey were compliant with FALCPA, although some products with precautionary labels apparently pose little risk to cashew-allergic consumers.

Conclusions

The sandwich-type cashew ELISA developed in this study can be used as an analytical means by the food industry and the reg-

ulatory agencies to provide evidence for the effectiveness of allergen control strategies, in the verification of undeclared cashew residues implicated as possible causes of reactions in tree-nut allergic consumers, and to guarantee compliance with food labeling guidelines. The developed cashew ELISA could eventually allow the improvement of allergen management in the food industry, which eventually will improve the quality of life of cashew-allergic consumers.

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