

Detection of Walnut Residues in Foods Using an Enzyme-Linked Immunosorbent Assay

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ABSTRACT: Tree nuts, including walnuts, can be responsible for allergic reactions. Food manufacturers have the responsibility to declare the presence of walnuts on packaged foods even when trace residues may be present from the use of shared equipment or the adventitious contamination of ingredients. The aim of this study was to develop a rapid, sensitive, and specific enzyme-linked immunosorbent assay (ELISA) method for the detection of walnut protein residues. Mixtures of raw and roasted English walnuts of several varieties were defatted, powdered, and used as separate antigens in sheep and New Zealand white rabbits. An ELISA was developed using the sheep antiroasted walnut serum as the capture reagent and rabbit antiroasted walnut serum as the detector reagent followed by addition of commercial goat anti-rabbit IgG antibody labeled with alkaline phosphatase and subsequent substrate addition. The performance of the ELISA was validated by testing known amounts of walnut (0 to 100 ppm) either spiked into or manufactured into milk chocolate, cookies, muffins, or ice cream. Recoveries of 1 to 100 ppm walnut-in-chocolate ranged from 71.6% to 119% $\pm$ 7% to 16.5%. The walnut ELISA has a detection limit of 1 ppm (1 μ g/g) walnut in several food matrices. Substantial cross-reactivity was observed with pecan while minimal cross-reactivity was noted for hazelnut, mustard, mace, and poppy seed among almost 100 foods and food ingredients tested. This walnut ELISA can be used to detect undeclared walnut residues in foods and ingredients and as a tool to validate the effectiveness of allergen control programs for walnuts.

Keywords: allergy, ELISA, immunoassay, walnut

Introduction

Two major types of walnuts, English walnuts (*Juglans regia*) and black walnuts (*Juglans nigra*), are marketed on a worldwide basis. Nearly all walnuts sold in the United States, are English walnuts consisting of 5 primary varieties (Chandler, Hartley, Serr, Vina, and Payne). More than 300,000 tons of walnuts are grown and marketed in the United States each year. Most walnuts (92%) in the U.S. market are sold in the shelled form.

Walnuts are considered to be a value-added ingredient because of consumer perceptions, sensory attributes, and beneficial health properties (Ros and others 2004; Blomhoff and others 2006). Shelled walnuts are widely used as ingredients in various foods including baked goods, salads, ice creams, confections and candy, and meat substitutes (vegetarian burgers). Walnut oil is also used as a food ingredient especially in salad dressings. Walnut oil is a good source of mono- and polyunsaturated fatty acids and tocopherols (Savage and others 1999). Furthermore, ground or pulverized walnut shells have a variety of industrial applications such as carriers, fillers, and extenders and abrasives such as jet cleaners (www.ecoshell.com). Walnut hull extract is used as a flavoring in the food industry and as a dietary supplement.

Tree nuts as a group are considered among the most common allergenic foods on a worldwide basis (FAO 1995). The overall prevalence of tree nut allergy in the United States was estimated at 0.5% in a telephone survey of 12,032 individuals in 4,374 households (Sicherer and others 1999). In the United States, walnut is the most commonly allergenic of the various tree nuts according to the telephone survey (Sicherer and others 1999), a clinical study of 54 pediatric patients with tree nut allergy (Sicherer and others 1998), and

a clinical study of 115 pediatric patients with tree nut allergy (Fleischer and others 2005). In a clinical study of 62 patients with peanut and/or tree nut allergy in the United Kingdom, walnut allergy occurred less frequently than Brazil nut, almond, or hazelnut allergy (Ewan 1996) but this likely reflects differences in consumption by comparison to the United States. The median age for the onset of tree nut allergies was 36 mo (Sicherer and others 2001) so susceptible individuals seem to be sensitized by exposure early in life. Allergic reactions to tree nuts can be severe and life threatening on occasion (Teuber and others 2003). Fatal (Bock and others 2001; Pumphrey 2004) and near-fatal (Sampson and others 1992; Kemp and others 1995; Macdougall and others 2002; Green and others 2006) reactions have been attributed to the unintended ingestion of walnuts by individuals with walnut allergies. Walnut-allergic individuals have also succumbed from the ingestion of foods made with other tree nuts (Boyd 1989; Flannagan and Wolf 2004), which may indicate cross-reactivity to other nuts or possibly contamination of the intended nut ingredient with walnuts. In a study of individuals in the Peanut/Tree Nut Registry of the Food Allergy & Anaphylaxis Network, 156 reactions were reported to have occurred in restaurants with 24% caused by tree nuts and 27% resulting in severe symptoms (Furlong and others 2001). The minimal eliciting dose for walnuts in walnut-allergic individuals is not known although Pumphrey (2004) notes that fatal reactions to peanuts and tree nuts involved estimated doses ranging from several milligrams to more than 10 g. Allergic reactions arising from residues of walnuts occurring in other foods have also not been widely reported, although our group investigated 1 case involving a walnut-contaminated cookie some years ago (Nordlee and others 1993).

The avoidance of walnuts in the diet is the only effective approach to the prevention of allergic reactions (Taylor and others 1986). The proper labeling of packaged foods is critical to the implementation of a successful walnut avoidance diet. The presence of undeclared walnuts in foods presents serious risks to

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walnut-allergic consumers. Undeclared walnut residues can occur in foods as a result of several circumstances including the use of shared processing equipment between walnut-containing and other formulations and the presence of undeclared walnuts in ingredients (Taylor and Hefle 2000). Effective sanitation of the shared equipment is necessary to prevent cross contact. Since low (milligram) amounts of allergenic proteins can elicit reactions in food-allergic individuals (Taylor and others 2002), sensitive and specific methods are needed to detect such residues. The enzyme-linked immunosorbent assay (ELISA) is the technique of choice because it has the potential to detect protein residues with great sensitivity and specificity (Taylor and Nordlee 1996), and the walnut allergens are found in the protein fraction (Teuber and others 2003). Several methods exist currently for the detection of walnut residues in other foods including polymerase chain reaction (PCR) methods (Brezna and others 2006; Yano and others 2007) and a recently reported ELISA method based on polyclonal antisera raised against a specific 2S albumin protein from walnut (Doi and others 2008). We report the development of a sensitive and specific ELISA for the detection of walnut residues in foods based upon the use of polyclonal antisera raised against a mixture of walnut proteins. The food industry will be able to use this ELISA to detect the presence of walnut residues in other ingredients and to assess the effectiveness of clean up of shared processing equipment.

Materials and Methods

Immunogen preparation

The immunogen consisted of a mixture of several lots and brands of English walnuts in an attempt to gather a mixture of varieties. The mixed walnuts were washed 6 times with deionized distilled water (1:8, w/v) and air-dried. A portion of the walnuts was dry-roasted at 270 °F for 10 min. The raw or roasted walnuts were chopped, frozen, and then ground to a fine uniform particle size using a 16-speed Oster (Niles, Ill., U.S.A.) blender equipped with pulse control. The ground roasted and ground raw walnuts were defatted by washing 1:5 (w/v) with ethyl ether (5 times) and then 1:5 (w/v) with acetone (2 times), and filtered each time with retention of the fines. Finally, the powdered raw or roasted walnuts were thoroughly air-dried. Both the defatted, powdered raw, and roasted walnuts were used as immunogens. Protein concentrations of the defatted powdered immunogens were determined by the Kjeldahl method (raw defatted walnut 46.4%, roasted defatted walnut 34.9%).

Polyclonal antibody production and titer determination

Polyclonal antibodies were produced at Covance Research Products Inc. (Denver, Pa., U.S.A.) in 1 sheep, 1 goat, and 3 New Zealand white rabbits using standard immunization protocols with each immunogen. The 10 animals (3 rabbits, 1 sheep, and 1 goat for each of the 2 immunogens) received initial subcutaneous injections to multiple sites (2 each for the rabbits, 4 sites each for the goats and sheep) with the defatted powdered immunogen and Freund's Complete Adjuvant. Scheduled 21-d interval boosts followed with rotating injection sites (rabbits subcutaneous, sheep and goats subcutaneous or intramuscular) of the defatted powdered immunogen and Freund's incomplete adjuvant. The initial boost for the rabbits consisted of 250 µg protein followed by 4 successive scheduled boosts of 125 µg protein and all subsequent scheduled boosts at 100 µg protein. The goats and the sheep received an initial injection of 1000 µg protein followed by 5 successive scheduled boosts at 500 µg protein and all following scheduled boosts at 250 µg protein. Antibodies were partially purified from sera using suc-

cessive precipitation with 50% and 35% ammonium sulfate (Harlow and Lane 1988), followed by redissolution and extensive dialysis (12000 to 14000 molecular weight cutoff, Spectrum, Gardena, Calif., U.S.A.) against excessive 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). Titer values of collected antisera were determined by a noncompetitive ELISA method in which log dilutions of serum were applied to plates coated with 0.1 µg of walnut protein per well from extracts of the appropriate raw or roasted immunogen in a coating buffer of 0.015 M Na₂CO₃, 0.035 M NaHCO₃, and 0.02% NaN₃, pH 9.6 at 100 µL per well, as previously described (Hefle and others 2001). The titer values of the antisera were calculated using Prism Windows-based computer software (GraphPad Software Inc., San Diego, Calif., U.S.A.) to determine the dilution of antibody needed to reach/achieve the midpoint of the linear portion of the dilution curve. Antisera with titer values quantified at 10000 and above were pooled, both by species and for raw and roasted walnut and used for development of the ELISA. The protein content of the pooled sera was determined by the method of Lowry and others (1951).

ELISA

The assay is a modification of the sandwich ELISA described previously by Hefle and others (1994). Sheep antiroasted walnut and rabbit antiroasted walnut antisera were used as the capture and detector antibodies, respectively. Each well of a 96-well microtiter plate (Maxisorp, Nalge Nunc Intl., Rochester, N.Y., U.S.A.) was coated with 100 µL of coating buffer (0.015 M Na₂CO₃, 0.035 M NaHCO₃, and 0.02% NaN₃, pH 9.6), containing 0.25 µg sheep antiroasted walnut antibody protein. Coated microtiter plate(s) were incubated for 16 h at 4 °C and then washed 4 times with PBS-T (0.05% Tween 20 [BioRad Laboratories Inc., Hercules, Calif., U.S.A.] in 0.01 M PBS and 0.02% NaN₃, pH 7.4) using a programmable automatic plate washer (Dynex MRW 35400 microplate washer, Dynex Technologies Inc., Chantilly, Va., U.S.A.). Blocking of unused binding sites was accomplished using 350 µL per well of 0.1% gelatin (300 bloom porcine [Sigma-Aldrich Co., St. Louis, Mo., U.S.A.] in 0.1M PBS, pH 7.4) with incubation for 1 h at 37 °C. Excess blocking agent was removed by washing 4 times with PBS-T. Then, 100 µL of each standard or sample extract were applied to wells in triplicate, and plates were incubated for 1 h at 37 °C. After washing 4 times with PBS-T, bound walnut was captured by the addition of 0.25 µg of rabbit antiroasted walnut antibody protein in 100 µL 0.1 M PBS-BSA (0.1% Bovine Serum Albumin [Fraction V-USB Corp. Inc., Cleveland, Ohio, U.S.A.]) in 0.01 M PBS and 0.02% NaN₃, pH 7.4) to each well with incubation for 1 h at 37 °C. After washing 4 times with PBS-T, the amount of walnut was detected by successive addition to each well of: 100 µL of commercial goat anti-rabbit immunoglobulin G labeled with alkaline phosphatase (Pierce Biotechnology Inc., Rockford, Ill., U.S.A.) diluted 1:5000 in 0.01 M PBS-BSA, pH 7.4 with incubation at 37 °C for 1 h, washing 4 times with PBS-T, and 100 µL of *p*-nitrophenyl phosphate substrate (Sigma-Aldrich Co.) with incubation for 30 min at room temperature. Color development was read on a Dynatech MR5000 microplate reader (Dynex Technologies Inc.) at 410 nm after stopping the reaction by addition of 100 µL of 1 N sodium hydroxide to each well. A series of standards with known concentrations of walnut-spiked extracts were included on each plate, and standard curves were developed using Prism Windows-based computer software (GraphPad Software Inc.).

Food samples. Retail food samples (40) were purchased locally in Lincoln (Nebraska, U.S.A.), including foods labeled as containing walnut (positive samples, [6], foods labeled "may contain

walnut" or similar advisory statements [28], and foods without any declaration of walnut content [6] but at possible risk for walnut because of other foods in the product line containing walnut). Walnut-containing ingredients were obtained from the manufacturers of those ingredients.

Preparation of walnut and pecan powders. Powders of English walnuts were prepared as described under section Immunogen Preparation. Finely ground roasted black walnut and finely ground roasted pecan were made in the same manner.

Extraction of food samples and spiked food standards. Food samples and walnut-spiked samples were ground to a fine, uniform powder using a blender (Sunbeam Corp., Delray Beach, Fla., U.S.A.). The ground samples, except chocolate, were extracted at a ratio of 1:20 in 0.1 M PBS pH 7.4 with 1% skim milk powder (Oxoid Ltd. from Fisher Scientific, Hampton, N.H., U.S.A.) for 2 h at 150 rpm in a shaking 60 °C waterbath (Julabo, Model SW22, Julabo U.S.A. Inc., Allentown, Pa., U.S.A.). Chocolate samples were extracted at a ratio of 1:20 in 0.1 M PBS, pH 7.4 with 1% skim milk powder, and 0.1% Tween-20 for 2 h at 150 rpm in a shaking 60 °C waterbath. Black walnut hull extract was extracted at a ratio of 1:40 in 0.1 M PBS, pH 7.4 with 5% fish gelatin (G7765; Sigma-Aldrich Co.), 1% skim milk powder, and 0.1% Tween 20, also extracted for 2 h at 150 rpm in a shaking 60 °C waterbath. Extracts were centrifuged at 2000 × g in a Sorvall RC 5B Plus Centrifuge (Kendro Laboratory Products, Newton, Conn., U.S.A.).

Preparation of spiked food standards. Spiked extracts of chocolate containing 1000 ppm walnut were prepared by adding 1000 µg of finely ground roasted English walnut (nondefatted) per gram of the appropriate powdered food matrix before extracting as described previously. Standard curves were prepared by serial dilution of the spiked extract with an extract of the food without added walnut (negative control extract). Test food-sample extracts were also diluted with the same negative control extract as was used to prepare the standard curve. The spiked food extracts and negative control extracts are prepared from the same/equivalent food as is the test food sample (that is, sugar cookie spike and sugar cookie negative control are used for the preparation of the standard curve and dilutions of the test food sample when a sugar cookie is the test food sample).

Finely ground roasted English and black walnuts, respectively, were used to spike butter cookies, as described below for muffins, to prepare standard curves for both types of walnut in the cookie matrix. Similarly, finely ground roasted English walnut and pecan, respectively, were used to spike vanilla ice cream as described below for muffins to prepare standard curves of walnuts and pecans in an ice cream matrix.

Preparation of naturally incurred standard. Walnut in milk chocolate reference standards were also prepared in a commercial chocolate manufacturing facility as previously described for almond standards (Hlywka and others 2000). The walnut used in preparing the chocolate standards was the same finely ground roasted walnut as used for the immunogen preparation before defatting. The walnut was commercially manufactured into a commercial recipe milk chocolate at concentrations of 0 and 100 ppm roasted walnut (wt/wt). The milk chocolate reference standards of 0, 0.5, 1, 2, 5, 10, 25, and 100 ppm roasted walnut were then prepared using appropriate amounts of the 100 ppm chocolate with the 0 ppm chocolate.

Cross-reactivity study

A variety of tree nuts, legumes, seeds, fruits, and food ingredients were evaluated for cross-reactivity in the walnut ELISA assay. The foods were extracted at a ratio of 1:20 in PBS with 1% skim milk

powder and mixed on a Labquake rocking platform mixer (Barnstead/ThermoLyne Dubuque, Iowa, U.S.A.) overnight followed by centrifuging as described previously. The protein concentrations of the supernatants were determined (less the protein concentration of the extraction buffer, which contains 1% skim milk powder) by the method of Lowry and others (1951). The extracts were tested as extracted and at 0.1 mg protein/mL, 1 mg/mL, and 10 mg/mL (as applicable/dependent upon the initial protein content of the extract).

Food matrix validation

To establish the statistical consistency of the assay, duplicate lots of 20 different retail samples (40 total) of prepackaged commercially processed muffins or muffin mixes or freshly baked muffins from local bakeries were obtained in a variety of flavors/ingredients (apple-cinnamon, banana, banana nut, blueberry, chocolate chip, cranberry-orange, honey-raisin bran, lemon poppy seed, raspberry, strawberry, and wild berry). Each muffin or muffin mix was ground to a powder and finely ground roasted walnut was spiked into the powdered muffin to achieve a walnut concentration of 1000 ppm before extraction. The 1000 ppm spiked extract of muffin matrix was diluted with the extract of the same muffin matrix (0 ppm) to achieve a standard curve of walnut in muffin from 0.0975 ppm walnut to 100 ppm walnut (the 1000 ppm spiked muffin extract was diluted with the negative control muffin extract to achieve a standard curve with the following concentrations: 100, 50, 25, 12.5, 6.25, 3.13, 1.56, 0.78, 0.39, 0.195, and 0.0975 ppm walnut). The walnut used for spiking the muffin matrix was the same finely ground walnut as was used for the immunogen before it was defatted. Each muffin or muffin mix was extracted in duplicate (80 extractions) as described previously for nonchocolate samples. Each extract was then analyzed in duplicate (each on a separate plate) by the walnut ELISA (160 total ELISA's), as described previously, to assess the statistical consistency of results obtained in a food matrix. For each of the 20 muffins, 2 lots, 2 extractions, and 2 plates, a 3-parameter logistic regression was run using the form $Y = \beta_0 e^{(-e^{-(\beta_1 (\ln X - \beta_2))})}$ where $Y = \text{response variable}$ and $\ln X$ is the natural log of predictor variable. β_0 , β_1 , and β_2 are parameters of the regression to be estimated through least squares. The parameter estimates were then used in a nested analysis of variance (ANOVA) program to assess the comparative variability attributable to muffins, lots, extractions and plates. Thus, there were 3 ANOVAs, 1 for each parameter.

Results and Discussion

The immunized rabbits and sheep were most responsive to the walnut immunogens with high titers for rabbit sera of 46000 and 59000, respectively, for raw and roasted walnut immunogen (sera protein contents of 16.6 mg/mL for both) and for sheep sera of 34000 and 33000, respectively, for raw and roasted walnut immunogen (sera protein contents of 40.8 and 54.4 mg/mL, respectively). The titers of the pooled goat sera were lower at 16000 and 13000, respectively, for raw and roasted walnut immunogen (sera protein contents of 36.8 and 52.2 mg/mL, respectively). Titers performed on sera obtained from animals before immunization indicated that none of the animals possessed preexisting antibodies to walnut.

Preliminary assay trials indicated that the optimal ELISA results were obtained using sheep antiroasted walnut antibody, at a protein concentration of 2.5 µg/mL, as the primary coating or capture antibody and rabbit antiroasted walnut antibody, at a protein concentration of 2.5 µg/mL, as the secondary or chase antibody. The lower limit of quantification (LOQ) is defined as the lowest level

of walnut that can be detected in the ELISA with acceptable accuracy and precision and was set at 1 ppm in several food matrices in which walnut would commonly be used, such as cookies, ice cream, muffins, and chocolate. The LOQ of 1 ppm yielded an average of 1.04 ppm with a standard deviation of 0.35 ppm and CV% of 33% when evaluated in a milk chocolate food matrix using 15 ELISA's (Table 1). At the 0.5 ppm level, this same approach yielded an av-

Table 1 – Reproducibility of extraction and ELISA of walnut manufactured at various levels into milk chocolate.

Extraction efficiency of walnut manufactured into milk chocolate			
Manufactured ppm	Average ^a concentration ppm	SD	CV%
100	103.50	17.97	17.36
25	23.99	5.18	21.58
10	9.54	1.31	13.73
5	4.75	1.04	21.98
2	1.97	0.61	31.13
1	1.04	0.35	33.19
0.5	0.56	0.23	41.20

^aAverage of 15 walnut ELISAs.

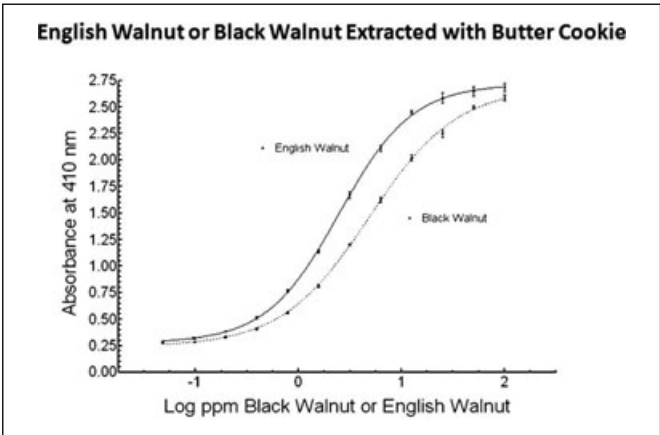


Figure 1 – Standard curve of butter cookies spiked with finely ground English and finely ground black walnut.

erage 0.56 ppm with a standard deviation of 0.23 ppm and CV% of 41%. Figure 1 shows that the antisera obtained with English walnut immunogen is capable of detecting residues of both roasted English and black walnut in butter cookies. As noted in Figure 1, the standard curves for English and black walnut differ although the ELISA has similar sensitivity for both types of walnuts. A similar standard curve was obtained for walnut in milk chocolate (Figure 2). To assess the effects of the chocolate manufacturing process on the ability to extract walnut from a chocolate matrix, the ELISA responses of walnut-spiked extracts of milk chocolate were compared to manufactured milk chocolate (naturally incurred standard) containing varying levels of walnut. Comparable standard curves were obtained (Figure 2) demonstrating that walnut protein was effectively extracted from the manufactured chocolate matrix with this extraction protocol. The extraction of proteins from chocolate can be difficult probably due to the presence of reactive phenolic substances in this matrix. However, a similar extraction technique has been successful on previous occasions with the analysis of casein residues in dark chocolate (Hefle and Lambrecht 2004) and almond residues in milk chocolate (Hlywka and others 2000). The extraction protocol and ELISA are also reproducible as shown by the results of the extraction and analysis of the walnut in milk chocolate standards in 15 successive trials (Table 1).

The antibodies produced and used in this ELISA were also very specific for walnut with 1 exception—pecan. The cross-reactivity of pecan is illustrated in Figure 3 where spiking of vanilla ice cream with walnuts and pecans is compared. Clearly, the ELISA detects walnut better than pecan (Figure 3) where 100 ppm of pecan in ice cream yields only 40% of the absorbance observed with 100 ppm of walnut. This ELISA would not be useful for the detection of walnut residues in a pecan-containing product or be able to discern between the identity of walnut and pecan when attempting to identify unknown pieces of material. The cross-reactivity of pecan in the walnut ELISA is not surprising given the close botanical relationship between the 2 tree nuts. Extracts of mustard seed, poppy seed, hazelnut, and mace displayed a much lesser degree of cross-reactivity in the walnut ELISA with extracts of the foods being detected at “apparent” walnut levels of 1 to 3 ppm. The weak reactivity of these foods is unlikely to be noticed when they are

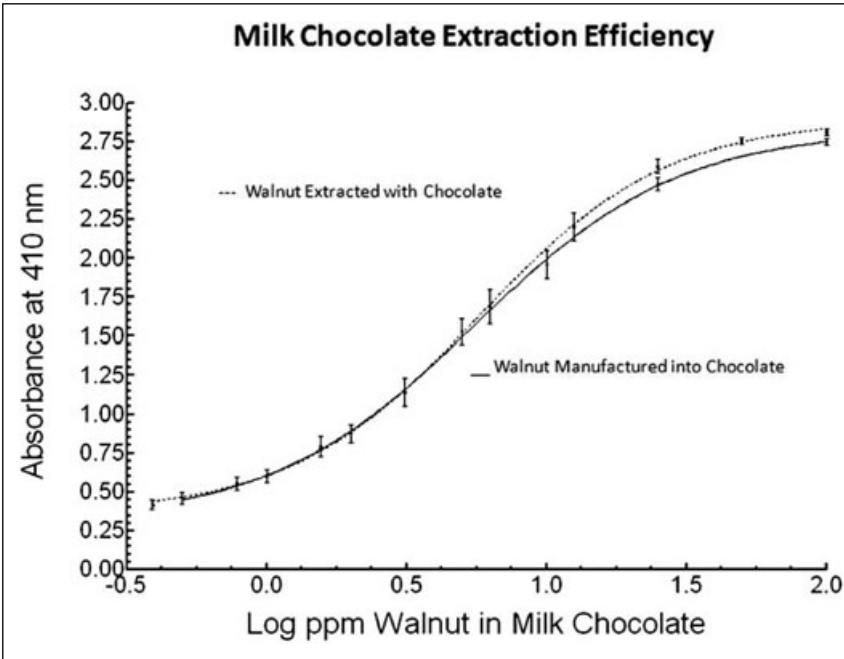


Figure 2 – Standard curve of milk chocolate either spiked with various levels of a 1000 ppm walnut-spiked milk chocolate or milk chocolate manufactured to contain various levels of walnut.

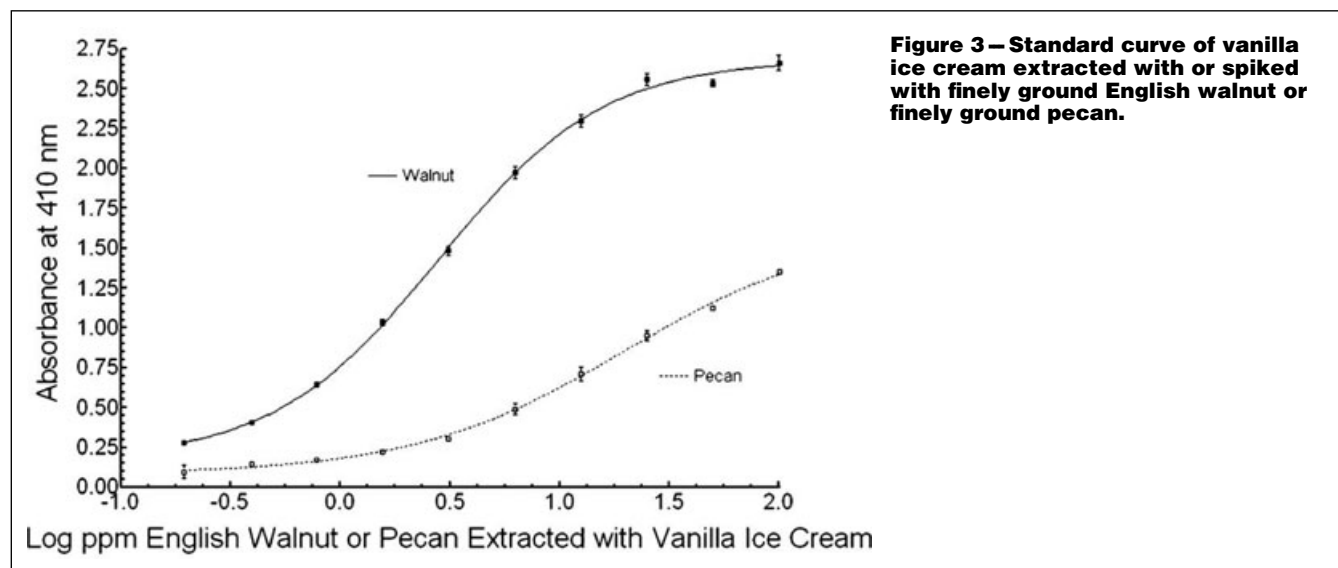


Figure 3—Standard curve of vanilla ice cream extracted with or spiked with finely ground English walnut or finely ground pecan.

present in lower amounts in typical food formulations. For example, poppy seed did not interfere when present in amounts contained in a variety of poppy seed-containing muffins. A group of more than 80 other foods or food ingredients showed no reaction in the walnut ELISA (Table 2). Other tree nuts including almond, cashew, pistachio, Brazil nut, macadamia nut, pine nut, coconut, and chestnut did not cross-react in the walnut ELISA.

The consistency of this walnut ELISA was further demonstrated using spiked samples of 20 different types of muffins. As shown in Figure 4, the extraction procedure and ELISA method were reproducible across the various muffin formulations. The results of the ANOVAs indicated that, for all 3 parameters, the extraction showed the greatest variability followed by the plate. The muffin-to-muffin variability was relatively small when compared to the extraction variability, being about one-half to one-tenth the size. Hence, the results were fairly consistent with the mean value for the 1st parameter, β_0 , of 125.7 with a muffin standard error of 3.59. For the 2nd and 3rd parameters, the overall means were 0.5116 and 0.2061 with muffin standard errors of 0.0164 and 0.1272, respectively.

This walnut ELISA method was applied to a range of retail food products that either contained walnuts or might have been manufactured on shared equipment with walnuts. Walnuts were detected in bakery products, muffins, cookies, brownies, breakfast cereal, snack and nutrition bars, and confectionery products where the presence of walnut was declared on the ingredient list (Table 3). Walnut was not detected in any of the related products (without precautionary labeling or bearing walnut on the label) indicating that no cross contact had occurred. However, the results obtained with muffins, cookies, ice cream, and chocolate spiked with walnuts indicates that the walnut ELISA is capable of detecting walnut residues in other products manufactured on shared equipment or in shared facilities.

Few food ingredients are derived from walnuts. The walnut ELISA was able to detect residual walnut in black walnut hull extract used as a flavoring. Due to the nature of the black walnut hull extract, perhaps the presence of unknown components in this material, the extraction efficiency of the procedure was reduced yielding a limit of quantitation of 10 ppm. The extraction efficiency was improved and the limit of quantitation was lowered to 5 ppm by extraction at a ratio of 1:40 with 0.1 M PBS containing 5% fish gelatin, 1% skim milk powder, and 0.1% Tween 20. No walnut protein was detectable in walnut oil in this ELISA. If the

Table 2—List of foods and food ingredients showing no cross-reactivity in the walnut ELISA.

Tree nuts	Seeds
Almond	Anise seed
Brazil nut	Caraway seed
Cashew	Celery seed
Chestnut (native and puree)	Fennel seed
Coconut	Mustard seed
Macadamia nut	Poppy seed
Pine nuts	Sesame seed
Pistachio	Sesame seed flour
Legumes	Sunflower seed
Chickpea (garbanzo bean)	Cereals
Green pea	Corn meal
Peanut	Rice
Soybean (raw and roasted)	Wheat flours (all-purpose, bread, cake)
Soy milk	Wheat, cracked
Soybean flour	Whole-wheat flour
Fruits and vegetables	Spices and flavorings
Apple (dried and fresh Delicious)	Allspice
Banana (dry chips and fresh)	Almond extract
Green bell pepper (fresh)	Basil
Red bell pepper (fresh and roasted)	Black pepper
Cherry (sweet)	Cinnamon
Cranberries (dried)	Cloves
Dates (dried)	Cumin
Kiwi (fresh)	Marjoram
Lemon (fresh)	Nutmeg
Mango (dried)	Oregano
Mushrooms (portabella and shiitake)	Paprika
Peach (fresh)	Vanilla extract
Prunes (dried)	Salt
Raisins	Other food ingredients
Raspberries (canned)	Sugar (refined and brown)
Strawberries (fresh)	Barley malt syrup
Other foods	Carob powder
Chocolate (dark and milk)	Caramel color
Egg substitute	Corn starch
Egg, whole	Corn syrup (light and dark)
Honey (clover)	Wheat germ
Cheese (parmesan and romano)	Yeast (active dry brewers)
Vegetable shortening	Molasses

walnut oil was highly refined, it might not contain walnut protein residues. However, the presence of allergen residues in gourmet tree nut oils has been established previously (Teuber and others 1997). Cold-pressed walnut oil might contain protein residues although it is unknown if these proteins would be detectable in this

ELISA. The ELISA systems are aqueous so the analysis of protein residues in oils is challenging and requires extraction into aqueous solvents.

ELISAs have advantages for the detection of trace residues of allergenic foods because these assays can specifically detect protein from the source material. The vast majority of food allergens are proteins, although only a few of the many thousands of proteins found in foods are allergens (Taylor 2002). ELISAs can be developed with antisera produced against either mixtures of proteins as in the case described here or to single proteins. Recently, Doi and others (2008) described the development of an ELISA to walnuts using antisera developed against the 2S albumin protein of walnut. This protein is a known walnut allergen called Jug r 1 (Teuber and others 1998). Alternatively, PCR assays offer another approach to the detection of residues of allergenic foods. PCR detects DNA residues from the source food as opposed to proteins. PCR assays for the detection of walnut residues in processed foods have previously been described (Brezna and others 2006; Yano and others 2007).

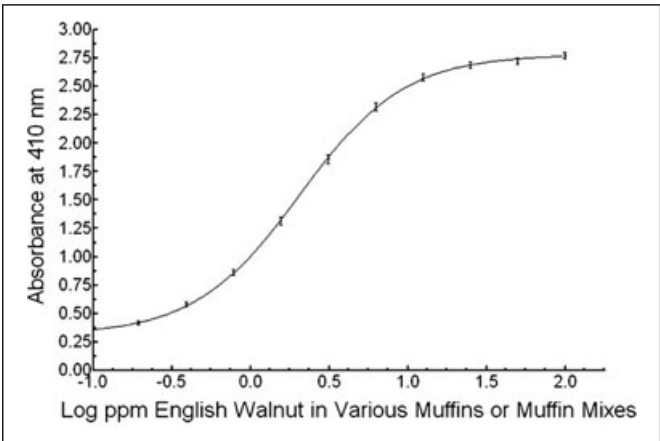


Figure 4—Standard curve composed of 20 different varieties of muffins spiked with finely ground English walnut powder.

Table 3—Retail survey of foods containing walnuts and similar foods without walnut listed on the label.

Sample identification	Total number tested	Concentration detected
Contains labeling		
Snack Bar	1	56000 ppm
Muffin	1	50000 ppm
Nutrition bar	1	160000 ppm
Brownie	1	9500 ppm
Cookie	1	47000 ppm
Cereal	1	4200 ppm
Precautionary labeling		
Bakery	7	1 sample at 2 ppm ^a
Cereal	3	BLQ
Confectionery	5	1 sample at 23 ppm ^a
Miscellaneous	3	BLQ
Nutrition bar	2	BLQ
Snack bar	6	1 sample at 49 ppm ^a
Snack food	2	BLQ
Potential for walnut with no precautionary labeling		
Brownie	1	BLQ
Muffin	1	BLQ
Baking ingredient	1	BLQ
Cereal	1	BLQ
Ice cream	1	BLQ
Snack food	1	BLQ

^aRemaining samples were BLQ.
BLQ = below the lower limit of quantitation of 1 ppm walnut.

This ELISA detects residues of walnut at concentrations that are pertinent to any health hazard assessment for walnut-allergic consumers. While regulatory consensus has not been achieved on minimal eliciting or threshold doses for allergenic foods, evidence from clinical oral challenge trials indicates that the minimal eliciting doses for individuals allergic to foods such as peanuts, milk, and eggs ranges from 0.5 mg to 8 g (Bindslev-Jensen and others 2002; Morisset and others 2002; Taylor and others 2002). A 20-g serving of cookie containing 1 ppm of walnut (the lower limit of quantitation of this ELISA) would provide only 0.02 mg of walnut, a level that is likely below the minimal eliciting dose for most, if not all, walnut-allergic individuals. Admittedly, no published evidence of minimal eliciting doses exists for walnuts so some uncertainty remains but evidence does indicate that minimal eliciting doses for hazelnut are well above 0.02 mg (Wensing and others 2002). Clearly, the food industry is implementing good manufacturing practices intending to prevent allergen cross contact (Taylor and others 2006). This walnut ELISA provides another tool to assess the effectiveness of such efforts.

Conclusions

This sandwich-type walnut ELISA is an important analytical tool for food manufacturers and regulatory agencies in sanitation validation and the evaluation of allergen control strategies. Walnut residues could be detected at a limit of quantitation of 1 ppm in a variety of food matrices including muffins, cookies, ice cream, and chocolate. However, an inter-laboratory validation study should be performed to define the statistical parameters of the assay. This walnut ELISA allows the food industry to detect potentially allergenic residues of walnut that might occur in other foods as a result of the use of shared facilities or equipment and to assess the adequacy of sanitation procedures aimed at removal of walnut residues from shared equipment.

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References

Bindslev-Jensen C, Briggs D, Osterballe M. 2002. Can we determine a threshold level for allergenic foods by statistical analysis of published data in the literature? *Allergy* 57:741–6.

Blomhoff R, Carlsen MH, Anderson LF, Jacobs DR Jr. 2006. Health benefits of nuts: potential role of antioxidants. *Br J Nutr* 94(Suppl 2):S52–60.

Bock SA, Munoz-Furlong A, Sampson HA. 2001. Fatalities due to anaphylactic reactions to foods. *J Allergy Clin Immunol* 107:191–3.

Boyd GK. 1989. Fatal nut anaphylaxis in a 16-year-old male: case report. *Allergy Proc* 10:255–7.

Brezna B, Hudecova L, Kuchta T. 2006. A novel real-time polymerase chain reaction (PCR) method for the detection of walnuts in food. *Eur Food Res Technol* 223:373–7.

Doi H, Touhata Y, Shibata H, Sakai S, Urisu A, Akiyama H, Teshima R. 2008. Reliable enzyme-linked immunosorbent assay for the determination of walnut proteins in processed foods. *J Agric Food Chem* 56:7625–30.

Ewan PW. 1996. Clinical study of peanut and nut allergy in 62 consecutive patients: new features and associations. *Br Med J* 312:1074–8.

Flannagan LM, Wolf BC. 2004. Sudden death associated with food and exercise. *J Forensic Sci* 49:543–5.

Fleischer DM, Conover-Walker MK, Matsui EC, Wood RA. 2005. The natural history of tree nut allergy. *J Allergy Clin Immunol* 116:1087–93.

[FAO] Food & Agriculture Organization of the United Nations. 1995. Report of the FAO technical consultation on food allergies, Rome, Italy, November 13–14, 1995. Rome, Italy: Food & Agriculture Organization of the United Nations.

Furlong TJ, DeSimone J, Sicherer SH. 2001. Peanut and tree nut allergic reactions in restaurants and other food establishments. *J Allergy Clin Immunol* 108:867–70.

- Green TD, Palmer KP, Burks AW. 2006. Delayed anaphylaxis to walnut following epinephrine administration. *J Pediatr* 149:733–4.
- Harlow E, Lane DP. 1988. *Antibodies: a laboratory manual*. Cold Spring Harbor, N.Y.: Cold Spring Harbor Laboratory.
- Hefle SL, Chu FS, Bush RK. 1994. A sandwich-type enzyme-linked immunosorbent assay (ELISA) for quantitation of selected peanut proteins in foods. *J Food Prot* 57:419–23.
- Hefle SL, Jeanniton E, Taylor SL. 2001. Development of a sandwich enzyme linked immunosorbent assay for the detection of egg residues in processed foods. *J Food Prot* 64:1812–6.
- Hefle SL, Lambrecht DM. 2004. Validated sandwich enzyme linked immunosorbent assay for casein and its application to retail and milk allergic complaint foods. *J Food Prot* 67:1933–8.
- Hlywka JJ, Hefle SL, Taylor SL. 2000. A sandwich enzyme-linked immunosorbent assay for the detection of almonds in foods. *J Food Prot* 63:252–7.
- Kemp SF, Lockey RF, Wolf BL, Lieberman P. 1995. Anaphylaxis—a review of 266 cases. *Arch Int Med* 155:1749–54.
- Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. 1951. Protein measurement with the Folin phenol reagent. *J Biol Chem* 193:265–75.
- Macdougall CF, Cant AJ, Colver AF. 2002. How dangerous is food allergy in childhood? The incidence of severe and fatal allergic reactions across the UK and Ireland. *Arch Dis Child* 86:236–9.
- Morisset M, Moneret-Vautrin DA, Kanny G, Guenard L, Beaudouin E, Flabbee J, Hatahet R. 2002. Thresholds of clinical reactivity of milk, egg, peanut and sesame in immunoglobulin E-dependent allergies: evaluation by double-blind or single-blind placebo-controlled challenges. *Clin Exp Allergy* 33:1046–51.
- Nordlee JA, Atkins FM, Bush RK, Taylor SL. 1993. Anaphylaxis from undeclared walnut in commercially processed cookies. *J Allergy Clin Immunol* 91:154.
- Pumphrey RSH. 2004. Fatal anaphylaxis in the UK, 1992–2001. In: *Anaphylaxis*, Novartis Foundation Symposium 257. Chichester, England: John Wiley & Sons Ltd., p 116–32.
- Ros E, Nunez I, Perez-Heras A, Serra M, Gilabert R, Casals E, Deulofeu R. 2004. A walnut diet improves endothelial function in hypercholesterolemic subjects: a randomized crossover trial. *Circulation* 109:1609–14.
- Sampson HA, Mendelson L, Rosen JP. 1992. Fatal and near-fatal anaphylactic reactions to food in children and adolescents. *N Engl J Med* 327:380–4.
- Savage GP, Datta PC, McNeil DL. 1999. Fatty acid and tocopherol contents and oxidative stability of walnut oil. *J Am Oil Chem Soc* 76:1059–63.
- Sicherer SH, Burks AW, Sampson HA. 1998. Clinical features of acute allergic reactions to peanuts and tree nuts in children. *Pediatrics* 102:E1–6.
- Sicherer SH, Munoz-Furlong A, Burks AW, Sampson HA. 1999. Prevalence of peanut and tree nut allergy in the U.S. determined by a random digit dial telephone survey. *J Allergy Clin Immunol* 103:559–62.
- Sicherer SH, Furlong TJ, Munoz-Furlong A, Burks AW, Sampson HA. 2001. A voluntary registry for peanut and tree nut allergy: characteristics of the first 5149 registrants. *J Allergy Clin Immunol* 108:128–32.
- Taylor SL. 2002. Protein allergenicity assessment of foods produced through agricultural biotechnology. *Ann Rev Pharmacol Toxicol* 42:99–112.
- Taylor SL, Nordlee JA. 1996. Methods for the detection of food allergens. *Food Technol* 50:231–4, 238.
- Taylor SL, Hefle SL. 2000. Good manufacturing practices for allergenic foods—use of shared equipment. *Food Allergy Intolerance* 1:114–7.
- Taylor SL, Bush RK, Busse WW. 1986. Avoidance diets—how selective should we be? *N Engl Reg Allergy Proc* 7:527–32.
- Taylor SL, Hefle SL, Bindslev-Jensen C, Bock SA, Burks AW, Christie L, Hill DJ, Host A, Hourihane JO'B, Lack G, Metcalfe DD, Moneret-Vautrin DA, Vadas PA, Rance F, Skrypec DJ, Trautman TA, Muhlmeden-Yman I, Zeiger RS. 2002. Factors affecting the determination of threshold doses for allergenic foods: how much is too much? *J Allergy Clin Immunol* 109:24–30.
- Taylor SL, Hefle SL, Farnum K, Rizk SW, Yeung J, Barnett ME, Busta F, Shank FR, Newsome R, Davis S, Bryant CM. 2006. Analysis and evaluation of food manufacturing practices used to address allergen concerns. *Comp Rev Food Sci Food Safety* 5:138–57.
- Teuber SS, Brown RL, Haapanen LAD. 1997. Allergenicity of gourmet tree nut oils processed by different methods. *J Allergy Clin Immunol* 99:502–7.
- Teuber SS, Dandekar AM, Peterson WR, Sellers CL. 1998. Cloning and sequencing of a gene encoding a 2S albumin seed storage protein precursor from English walnut (*Juglans regia*), a major food allergen. *J Allergy Clin Immunol* 101:807–14.
- Teuber SS, Comstock SS, Sathe SK, Roux KH. 2003. Tree nut allergy. *Curr Allergy Asthma Rpts* 3:54–61.
- Wensing M, Penninks AH, Hefle SL, Akkerdaas JH, Van Ree R, Koppelman SJ, Bruijnzeel-Koomen CAFM, Knulst AC. 2002. The range of minimum provoking doses in hazelnut-allergic patients as determined by double-blind, placebo-controlled food challenges. *Clin Exp Allergy* 32:1757–62.
- Yano T, Saki Y, Uchida K, Nakao Y, Ishihata K, Nakano S, Yamada T, Sakai S, Urisu A, Akiyama H, Maitani T. 2007. Detection of walnut residues in processed foods by polymerase chain reaction. *Biosci Biotechnol Biochem* 71:1793–96.