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A Murine Monoclonal Antibody Based Enzyme-Linked Immunosorbent Assay for Almond (*Prunus dulcis* L.) Detection

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ABSTRACT: A sandwich enzyme-linked immunosorbent assay (ELISA) using anti-almond soluble protein rabbit polyclonal antibodies as capture antibodies and murine monoclonal antibody 4C10 as the detection antibodies was developed. The assay is specific and sensitive (3−200 ng almond protein/mL) for almond detection. The standardized assay is accurate (<15% CV) and reproducible (intra- and inter assay variability <15% CV). The assay did not register any cross-reactivity with the tested food matrices, suggesting the assay to be almond amandin specific. The assay could detect the presence of declared almond in the tested matched commercial samples. Further, the assay reliably detected the presence of almonds in the laboratory prepared food samples spiked with almond flour.

KEYWORDS: *almond, ELISA, mAb 4C10, detection, assay*

INTRODUCTION

Food allergy is on the rise in the western countries and is therefore of concern from a consumer safety viewpoint. With ∼12 million Americans suffering from food allergy, up to 8% children and 2% adults, managing food allergy and improving food safety is of importance.[1] Recent FDA recall data (2012, fourth quarter)[2] analysis reported by Stericycle ExpertRECALL (2013)[3] indicate food allergy concerns are second, behind *Salmonella* food-poisoning. With over 170 foods reported to cause an allergic reaction[4] and with no available cure for food allergies, avoidance of the offending food allergen is the best choice for sensitive consumers.

Among the eight food groups that account for almost 90% of food-induced allergies,[5−8] edible tree nut seeds are one of the major food groups responsible for causing food allergies. Strict avoidance of the offending food is desirable to eliminate the unwarranted exposure of the offending food by the sensitive individuals. However, accidental ingestions and reactions to the offending food are continuing challenges.[9] Among tree nuts, almonds are ranked number one in production, export, and economic value.[10] Although safely enjoyed by most, almond-sensitive individuals need to know of the presence of almonds to eliminate the unwarranted almond exposure.

Several commercially sold almond detection assay kits, although useful, exhibit one or more limitations that may include lack of specificity, sensitivity, and robustness and possible interference by food matrices. In 1999, a sensitive (ppm detection) method using anti-almond major protein rabbit polyclonal antibody (pAb) based enzyme-linked immunosorbent assay (ELISA) was reported[11] from our laboratories. Although useful as a screening assay, this rabbit pAb-based ELISA lacked the desired specificity, as certain food matrices appeared to interfere in the assay under the tested assay conditions.[11]

This paper reports the results of efforts to develop a murine monoclonal antibody (mAb) based ELISA for specific, sensitive, and robust almond detection.

MATERIALS AND METHODS

Materials. Unless stated otherwise, Nonpareil almonds (kindly supplied by the Almond Board of California, Modesto, CA) were used to prepare almond flours and proteins and were used as the reference. Pecans (Desirable cultivar, Dr. T. Thompson, USDA-ARS, Pecan Breeding and Genetics, Somerville, TX), pistachios (Paramount Farms, Inc., Los Angeles, CA), walnuts (Blue Diamond Growers, Sacramento, CA), and Virginia peanuts (Dr. Sean O'Keefe, VPI & SU, Blacksburg, VA) were gifts. Edible seeds of Brazil nut, cashew, hazelnut, macadamia, pine nut, Spanish peanuts, sesame seeds (polished, white color), sunflower seeds, soybean, and navy bean; food ingredients; and commercially processed foods were purchased from local grocery stores and were processed as needed and as described in detail by Tiwari et al.[12] Almond-flour-spiked foods were prepared in the laboratory. Sources of chemicals, supplies, and reagents have been reported.[13]

Different types of 96-well microplates [2797 (Serocluster, not treated, nonsterile, "U" bottom, polyvinyl chloride), 9018 (EIA/RIA plate, high binding, nonsterile, flat, polystyrene), 3366 (EIA/RIA plate, high binding, nonsterile, round, polystyrene), 3797 (EIA/RIA plate, medium binding not treated, nonsterile, round, polystyrene), 3370 (assay plate with low evaporation lid not treated, sterile, flat, polystyrene), 3360 (assay plate, no lid, tissue culture treated, sterile, round, polystyrene)] were gifts [Corning Inc. (Lowell, MA)]. Extra 96-well 2797 microplates were purchased from Corning Inc. (Lowell, MA).

Electrophoresis and immunoblotting supplies were from Hoefer Scientific Co. (San Francisco, CA), Spectra/Por 6 dialysis membranes [approximate molecular weight cutoff (MWCO) of 1000 and 6000−8000, flat width 38 mm, diameter 24 mm, length 10 m] were from

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Spectrum Laboratories, Inc. (Rancho Dominguez, CA), and YM-30 Amicon centrifugal filter devices were from Millipore Corp., (Billerica, MA).

The Ultrospec 2100 pro UV/visible spectrophotometer was from GE Healthcare (Piscataway, NJ), the pH meter was from Corning Inc. (Lowell, MA), pipette tips were from USA Scientific, Inc. (Ocala, FL), and the BioTek PowerWave 200 microplate scanning spectrophotometer and KC4 software were from Bio-Tek Instruments, Inc. (Winooski, VT). PVDF membranes (0.2 μm, 20 × 20 cm sheet) were from Whatman, Inc. (Piscataway, NJ); cellulose extraction thimbles (25 mm ×100 mm), filter paper No. 4, and Whatman chromatography paper (3MM CHR 15 × 17.5 cm) were from Whatman International Ltd. (Maidstone, UK); Protran nitrocellulose membranes (NC, 0.2 μm, 200 × 3 m) were from Schleicher & Schuell Bioscience, Inc. (Keene, NH); and X-ray film (BioMax XAR film) was from Eastman Kodak Co. (Rochester, NY).

Horseradish peroxidase labeled goat anti-rabbit IgG, goat anti-mouse IgG (whole molecule) peroxidase conjugate antibody developed in goat (A4416 0.8 mg/mL), Ponceau S (P3504, practical grade), *p*-nitrophenyl phosphate (disodium salt), anti-mouse IgG (whole molecule)−alkaline phosphatase antibody produced in goat (A3652, Lot. 050M6016, 2.8 mg/mL), anti-rabbit IgG (whole molecule)−alkaline phosphatase antibody produced in goat, Folin−Ciocalteu's phenol reagent (2 N), luminol (97.0%), and bovine serum albumin (BSA) were from Sigma Chemical Co. (St. Louis, MO).

Chemzymes ultrapure acrylamide was from Polysciences, Inc. (Warrington, PA), TEMED (*N*,*N*,*N*′,*N*′-tetramethylenediamine) and bis-acrylamide were from Bio-Rad (Hercules, CA), and Western ReProbe for stripping and reprobing Western blots was from G-Biosciences (St. Louis, MO).

All other chemicals (ACS grade), plasticware/glassware, Isotemp* 650D gravity-convection standard lab incubators, Eppendorf pipettes (single and multichannel), and protein markers including Fisher's EZ-RUN Pre-Stained *Rec* Protein Ladder for SDS−PAGE (BP3603 is a mixture of 10 recombinant, highly purified, colored proteins with apparent molecular weights ranging from 10 to 170 kDa; BP3602 is a mixture of 14 highly purified recombinant proteins with the apparent molecular weights ranging from 10 to 200 kDa) were purchased from VWR Scientific (West Chester, PA) and Fisher Scientific Co. (Pittsburgh, PA).

Methods. *Preparation of Flours.* All high moisture content ingredients and foods were dried as described earlier.[12,14] Briefly, heat-sensitive foods were freeze-dried (e.g., ice cream, cheese), whereas fresh produce (e.g., fruits and vegetables) and high-sugar dried fruit (e.g., raisins) matrices were oven-dried for 24 h at 50−60 °C. All seeds, food ingredients, and processed foods, in their dried form, were ground in an Osterizer blender (speed setting "grind"; Galaxy model 869-18R, Jaden Consumer Solutions, Boca Raton, FL) to obtain uniform flours. As needed, the flours were defatted for 8 h using a Soxhlet apparatus and petroleum ether (boiling point range 38.2−54.3 °C, BDH, VWR Scientific, West Chester, PA) as the solvent. After overnight drying in a fume hood, the powder was passed through a 40 mesh sieve and then stored in screw-capped plastic bottle at −20 °C until further use.

Preparation of Protein Extracts. The desired dry and sieved samples (40 mesh), 100 mg each, were extracted with borate saline buffer (BSB, 0.1 M H_3BO_3, 0.025 M $Na_2B_4O_7$, 0.075 M NaCl, pH 8.45) (flour/solvent = 1:10 w/v) for 1 h at room temperature (RT, ∼25 °C), followed by centrifugation at 16 000*g* for 15 min at RT. Aliquots of the supernatant were analyzed (supernatants were stored at 4 °C prior to analysis) within 48 h of preparation, and the remainder was stored in plastic microcentrifuge tubes (1.5 mL capacity) at −20 °C until further use. Amandin [also known as prunin, almond major protein (AMP), Pru du 6] was prepared from defatted Nonpareil almond flour as described.[12]

Antibody Production and Screening of mAbs. Rabbit pAbs and murine mAbs were produced against the desired soluble almond proteins according to the standard procedures (McCullough and Spier)[15] and The Florida State University animal care and use committee (ACUC) approved original protocol #0207 in the Biomedical Research Facility at the Florida State University(FSU) and Hybridoma Core Facility (Department of Biological Science, FSU), respectively.

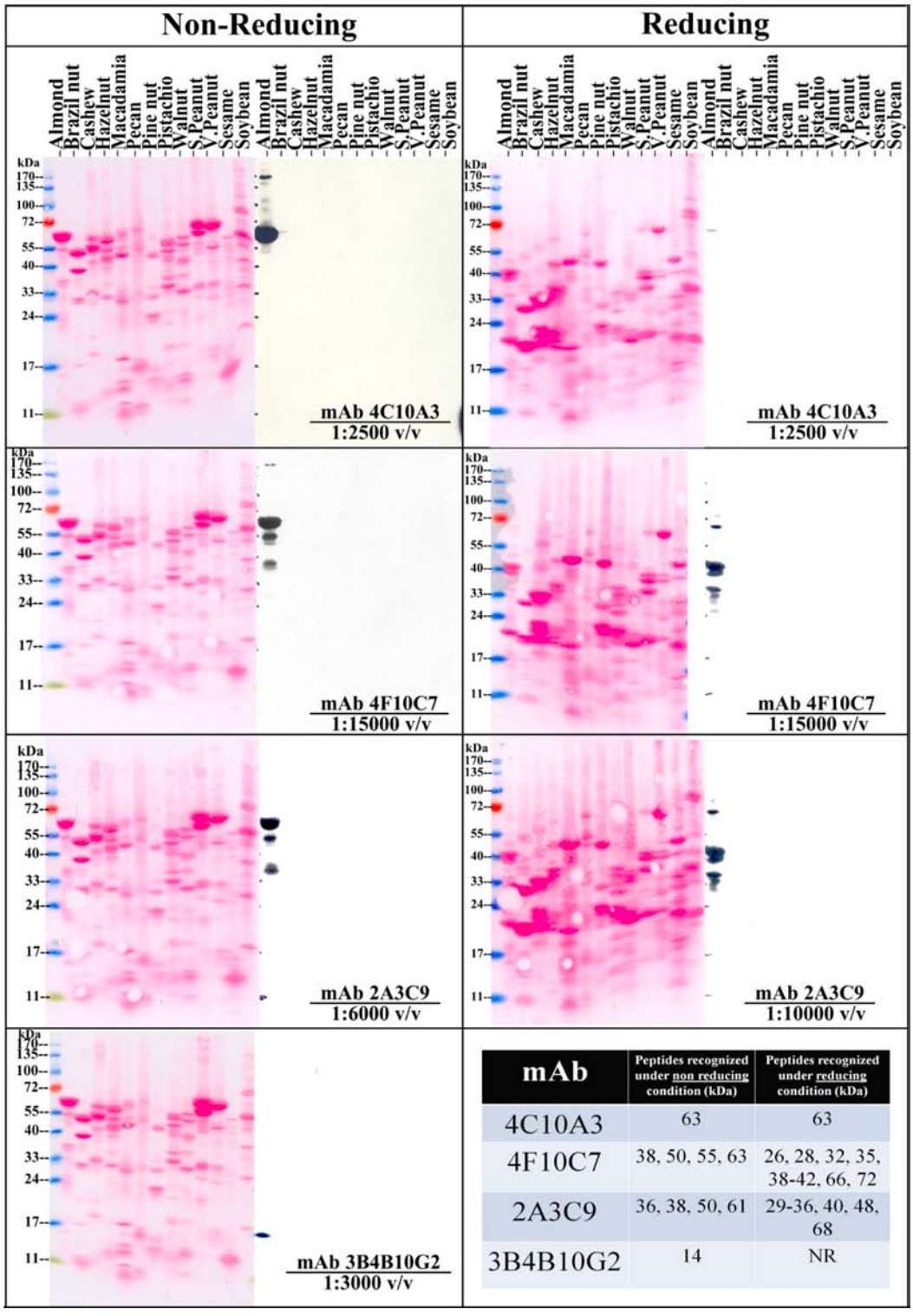
Whole almond (WA) flour was defatted as described in Preparation of Flours. The BSB solubilized proteins from the defatted WA flours were used for the production of rabbit pAbs. Two New Zealand white female rabbits were immunized each with BSB solubilized almond proteins (0.5 mg) in 0.5 mL of RiBi adjuvant as described.[11] Five booster doses (0.5 mg each) were administered in RiBi adjuvant each at 4 week intervals. Each rabbit was subsequently bled and the serum was collected and stored at −20 °C until further use. Preimmune serum was collected to serve as the control when determining the antibody titer.

For murine mAbs, BALB/c mice were immunized each with BSB extracted almond proteins (25 μg) in RiBi adjuvant. One booster dose (15 μg) was administered in RiBi after 3 weeks. The mice spleens were removed 1 week after the booster dose, and plasma cells were fused with myeloma cells (NS-1) to create hybridoma cells. The mAbs were screened and assayed for relative strength of reaction against almond proteins and various food proteins by direct binding ELISA and immunoblot.[16] The mAbs were screened for sensitivity (the amount of almond protein detected for the fixed dilution of the mAb) and specificity (no immunoreactivity against the tested seed proteins other than amandin). Among the screened mAbs, mAb 4C10 was selected for the assay development.

Antibody Purification. Antibodies were purified by affinity chromatography using protein G-Sepharose. Briefly, antiserum or mAb supernatant (one volume) was mixed with one volume of 0.1 M phosphate-buffered saline (PBS, pH 7.2) and one volume of protein G matrix (preswelled in 0.1 M PBS, pH 7.2) in a 15 mL conical tube at a final ratio of 1:1:1 (v/v/v). The tube was then incubated for 12 h in the cold room (4 °C) on a rocker (60 Hz, Rocker II model 260350, Boekel Scientific, Feasterville, PA). The mixture was packed into a 5 mL disposable polypropylene column (Pierce Inc., Rockford, IL). The flow-through (FT) fraction containing unbound protein was collected. Absorbance using optical density (OD) was read at 280 nm using PBS as the reference. The column was then rinsed with additional PBS buffer (three bed volumes), and the FT fractions were collected separately until the OD at 280 nm reached the baseline. Two bed volumes of 0.2 M glycine sulfate (GS, pH 2.3) (Fisher Scientific Co., Pittsburgh, PA) were added to elute the bound antibody from the protein G column, and the eluate was collected in 1.5 mL conical microtubes (polypropylene, VWR International, West Chester, PA) containing 0.1 mL of 1 M tris(hydroxymethyl)aminomethane buffer pH adjusted by 0.1 M HCl (Tris-HCl, pH 8.5). PBS was then added to wash the column, FT fractions were collected in separate conical microtubes, and the absorbance was read at 280 nm using GS as the reference. The tubes containing the IgG were pooled in a 15 mL conical tube and immediately neutralized with dropwise addition of 1 M Tris-HCl (pH 8.5). Sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) and Coomassie Brilliant Blue R (CBBR) staining or silver staining were performed to confirm IgG purification. The IgG fraction was then dialyzed against 10 mM PBS and concentrated using YM-3 Amicon centrifugal filtration devices. mAb IgG concentration was estimated by measuring the absorbance at 280 nm using a UV spectrophotometer and an extinction coefficient of 1.38 for IgG.[17] Sodium azide (NaN_3) was added to a final concentration of 0.1% (w/v), and aliquots were stored at −20 °C until further use.

ELISA Construction and Optimization. Sandwich ELISA was optimized for the type of plate, detection antibody, antibody titer, coating buffer, incubation temperature, and incubation time.

Plates. The six types of Corning Costar 96-well microtiter plates tested for capture antibody binding—(a) 2797 (Serocluster, not treated, nonsterile, "U" bottom, polyvinyl chloride), (b) 9018 (EIA/RIA plate, high binding, nonsterile, flat, polystyrene, (c) 3366 (EIA/RIA plate, high binding, nonsterile, round, polystyrene, (d) 3797 (EIA/RIA plate, medium binding not treated, nonsterile, round, polystyrene, (e) 3370 (assay plate with low evaporation, lid, not treated, sterile, flat, polystyrene, (f) 3360 (assay plate, no lid, tissue



mAb	Peptides recognized under non reducing condition (kDa)	Peptides recognized under reducing condition (kDa)
4C10A3	63	63
4F10C7	38, 50, 55, 63	26, 28, 32, 35, 38-42, 66, 72
2A3C9	36, 38, 50, 61	29-36, 40, 48, 68
3B4B10G2	14	NR

Figure 1. Immunoreactivity and specificity of anti-amandin mAbs using Western blotting. Please note that nitrocellulose membranes were visualized by brief staining (5 min) with 0.1% (w/v) Ponceau S (pink bands) after transferring the proteins on to the membranes or strips and prior to developing the Western blots. NR = 3B4B10G2 did not react with any polypeptides under reducing condition, indicative of nonaccessible epitope. Tree nut seed protein load was 30 μg each and mAb dilution was as indicated. This figure is a composite of seven Western blots and one Excel table.

culture treated, sterile, round, polystyrene)—were gifts [Corning Inc. (Lowell, MA)]. The plate with highest coating efficiency and uniformity was chosen for assay development.

Selection of mAb and Determination of Its Titer. Using Western blotting, mAbs 4C10, 4F10, 2A3, 4G2, and 3B4 were found to be specific for almond soluble proteins (Figure 1). These mAbs were further evaluated for titer and detection range using sandwich ELISA. Rabbit polyclonal antibodies [for details, please see 1. Sandwich ELISA] were used to capture the antigen in the checkerboard titration that was used to establish the optimal dilutions for two ELISA components (e.g., antigen concentration and antibody concentration) in a single experiment. In the procedure used, antigen was serially diluted down the plate and the primary antibody was serially diluted across the plate. Criteria for selection were OD_{405nm} = 0.8−1 for

positive control, $OD_{405nm} \leq 0.2$ for blank or negative control, and ratio of signal:noise ≥ 3 (ideally 10). Antibody dilution was selected initially according to the preliminary results from antibody characterization and narrowed down for checkerboard titration. Antigen concentration tested included 100 000, 10 000, and 8000 ng/mL based on previous experience. The mAbs and antigen were then diluted in two dimensions at a factor of 10, 5, 4, or 2 for determining the optimal mAb−antigen interaction. A four-parameter curve with a low antigen concentration for the 50% signal (in sandwich assay) and a wide linear range at the highest dilution of the detection mAb were used to determine the optimal interaction between the antigen and mAb.

Coating Buffer. Six coating buffers covering pH range 5−10 were tested for antibody coating efficiency. They were 0.1 M citrate phosphate buffer (48.5 mM citric acid, 103 mM Na_2HPO_4, pH 5.0), 0.10 M PBS (pH 7.2), 0.01 M PBS containing 0.02% Tween 20 (pH 7.4), 0.01 M Tris-HCl (pH 7.6), 0.1 M BSB (pH 8.45), and 0.1 M $NaHCO_3$ (pH 9.6). The buffer with the highest coating efficiency was used for subsequent assay development.

Incubation Conditions. Test conditions including incubation temperature and time and color development temperature and time were evaluated and optimized. Specifically, the following were evaluated: coating temperature [(a) 4, 8, 16, or 24 h at 8 °C (cold room); (b) 0.25, 0.5, 0.75, 1, 2, or 4 h at RT; (c) 0.25, 0.5, 0.75, 1, 2, or 4 h at 37 °C (constant temperature incubator with an accuracy of ±0.1 °C], coating time (1, 2, or 4 h), antigen incubation time (0.25, 0.5, 0.75, 1, 2, or 4 h), primary antibody incubation time (0.25, 0.5, 0.75, 1, 2, or 4 h), color development temperature and time [(a) 0.25, 0.5, 0.75, 1, or 2 h at 22 °C and (b) 0.25, 0.5, 0.75, 1, or 2 h at 37 °C].

Temperatures and durations that produce high OD_{405} nm and good reproducibility of antigen concentration at 50% maximum signal were selected.

ELISA Validation. 1. Limit of Detection (LOD). LOD is defined as the smallest quantity or concentration of an analyte that can be reliably distinguished from the background in the assay. The optimized assay was tested against a series of samples according to the recommendations of International Union of Pure and Applied Chemistry (IUPAC).[18] LOD was calculated using the formula $LOD_1 = 3\sigma/D$ ($\alpha = 0.05$) where σ is the standard deviation of the blank (mean) and D is the slope of the regression line for the linear range. LOD_2 was also calculated based on the method of Redl et al.[19] In this method, the LOD_2 was calculated by adding 3 times the standard deviation of the obtained absorbance to the mean absorbance and then converting the resulting absorbance to concentration using the equation of the calibration curve. According to this same IUPAC reference, limit of quantification (LOQ) was determined by adding 10 times (instead of 3 times) the standard deviation of the obtained absorbance to the mean absorbance and then converting to concentration, as was done for LOD_2. LOQ is the lowest level of analyte in a sample that can be reasonably quantified at a specified level of precision.

2. Sensitivity. Sensitivity is defined as the ability to detect positive samples as positive. This was done using almond-containing products and was computed using $A/B \times 100\%$, where B is the number of positive samples tested and A is the number of positive samples that the test was able to correctly identify as positive.[20]

3. Specificity. Specificity is defined as the ability to detect negative samples as negative. This was achieved by testing non-almond products. It was computed using $C/D \times 100\%$, where D is the number of negative samples tested and C is the number of negative samples that the test was able to correctly identify as negative.[20]

4. Cross-Teactivity. Cross-reactivity is defined as a positive response to a tested substance/sample that does not contain the targeted analyte (almond seed amandin). Both almond-containing and non-almond products were tested by the ELISA assay to assess possible cross-reactivity under the optimized assay conditions. To determine the possible cross-reactivity of mAb 4C10, dry matrices containing <1% fat (e.g., wheat flour) were used without any further treatments. Dry food matrices containing >1% fat were defatted using a Soxhlet apparatus as described under Preparation of Flours, air-dried, and powdered. Protein extracts were prepared as described under Preparation of Protein Extracts. The protein content of the supernatant was estimated as per the method of Lowry et al.[21] or Bradford[22] using bovine serum albumin fraction V (Sigma Chemical Co., St. Louis, MO) as the standard protein. The ratio (R) = concentration at the 50% of the maximum signal for the sample/the concentration at the 50% of the maximum signal for the reference (Nonpareil Supreme almond) was determined to assess cross-reactivity of the tested sample. The R value for Nonpareil proteins was 1 and was used as a reference to express the immunoreactivity of the tested samples.

5. Reproducibility. Reproducibility, also called precision, is defined as the ability of the assay to duplicate results in repeat determinations. Reproducibility was determined by a statistical measure of the variation—the coefficient of variation (% CV)—between replicate determinations in the same assay (intra-assay variability) and in different assays (inter-assay variability). Variation of at least five replicates of each sample was measured. At least three different samples within the plate for intra-assay variability and in different plates for inter-assay variability were measured.[23] Percent coefficient of variation (% CV) was calculated for the intra-assay or inter-assay variability by dividing the standard deviation (S_p) by the mean of the replicate determinations (X_p) and multiplying by 100.

$$\text{CV}\ (\%) = (S_p/X_p) \times 100$$

6. Accuracy. Accuracy describes the closeness of the mean test results obtained by the assay to the true value (concentration) of the targeted analyte. Recovery studies were performed by mixing an aliquot of non-almond sample extract and an almond standard. The almond content was measured and the percentage of recovery was determined.[23]

ELISA Format. ELISAs were typically conducted in triplicate. After selecting the assay parameters, two commonly used ELISA formats, sandwich and inhibition (described below), were compared for sensitivity. Regardless of the format, WA was the captured antigen and amandin was the targeted antigen using murine mAb for amandin detection. Amandin was selected as the targeted antigen for almond detection as amandin accounts for ∼65% of the total soluble proteins[24] and is a major allergen in almond seeds.[25−27]

1. Sandwich ELISA. Ninety-six-well microtiter plates were coated with the rabbit polyclonal antibody 99R20 (604 ng in 50 μL/well) in the coating buffer (48.5 mM citric acid, 103 mM sodium phosphate, pH 5.0) and incubated at 37 °C for 2 h. The plates were washed three times with Tris-buffered saline (TBS-T; 10 mM Tris, 0.9% w/v NaCl, 0.05% v/v Tween 20, pH 7.6) and blocked for 1 h at 37 °C with 200 μL/well of 5% (w/v) non-fat dry milk (NFDM) in TBS-T. The plates were washed three times with TBS-T and the standard protein or other sample protein extracts were added. For the protein standard, 100 μL of full fat Nonpareil almond seed flour BSB extracted proteins (8000 ng protein/mL) was added to the first row and 75 μL of 1% (w/v) NFDM in TBS-T was added to the rest. Twenty five microliters from the first row was pipetted into the second row and mixed thoroughly (i.e., the standard was diluted 1:4). This step was repeated for the rest of the rows, and 25 μL from the last row was discarded. For protein extracts, other than the standard proteins, 100 μL of original extracts or suitably diluted samples (8000 ng protein/mL) was added to the top row and 10× or 4× serially diluted samples were added to the rest. Standards and samples were then incubated for 1 h at 37 °C and again washed three times with TBS-T. The plate was then incubated for 1 h at 37 °C with the suitably diluted detection antibody (Table 1) recognizing the captured antigen. For example, 50 μL of the murine mAb 4C10 diluted 1800 times in TBS-T (containing 1% NFDM w/v) provided a detection mAb concentration of 4 ng/well. The plate was again washed three times with TBS-T and incubated with alkaline phosphatase (AP)-labeled goat anti-mouse antibody (Molecular Probes, Inc., 2003) diluted in 1% (w/v) NFDM in TBS-T (24 ng/well) for 1 h at 37 °C. After three subsequent washings with TBS-T, the colorimetric reaction was developed by adding 50 μL/well of phosphatase substrate [5 mg/mL *p*-nitrophenyl phosphate tablet dissolved in 5 mL of substrate buffer

Table 1. Detection Sensitivity of Anti-Amandin mAbs Using Indirect Sandwich ELISAs[a]

detection mAb (concn, μg/mL)	dilution factor (v/v)	antigen concn at 50% signal, ng/mL	detection range, ng/mL
4C10 (145.04)	1:1800	39.46 ± 0.54	3−200
4F10 (88.24)	1:100	37.08 ± 5.98	3−300
2A3 (28.68)	1:100	44.42 ± 3.29	10−200
4G2 (151.47)	1:100	44.08 ± 3.11	8−200
3B4 (236.62)		NA[b]	

[a]Capture antibody was rabbit anti-whole defatted almond flour protein extract (604 ng/well), antigen [whole almond protein extract, 8000 ng/mL in 0.1 M borate saline buffer (BSB, pH 8.45)] was loaded in the top well and diluted 4× in the successive seven rows. Detection mAbs were diluted using 1% (w/v) NFDM in TBS-T as indicated. Data are expressed as mean ± SEM (n = 9). [b]mAb did not register signal, indicating that the epitope was not accessible in the native protein.

(0.0049% w/v $MgCl_2$, 0.096% v/v diethanolamine, pH 9.8)]. The color development was typically allowed to proceed for 10 min and then the reaction was stopped by adding 50 μL of 3 M NaOH to each well and the absorbance (OD_{405nm}) was read by a BioTek PowerWave 200 microplate scanning spectrophotometer (Winooski, VT). On the same plate, standards (freshly prepared WA in triplicate), negative control (0.1% w/v BSA), and blanks (BSB, TBS-T only, no capturing antibody, no detecting antibody, no secondary antibody, and no substrate) were included. A four-parameter curve was generated by KC4 software and the antigen concentration for the 50% of the maximum signal was determined. Immunoreactivity of almond proteins present in the targeted samples was determined by comparing the antigen concentration in the sample to the antigen concentration that generated 50% of the maximum signal in the almond protein standard curve. The concentration (C) of the reactive protein in the food sample (e.g., food matrix or ingredient) was determined using the sample absorbance closest to the 50% signal of the almond standard curve using the formula

$$C = c[(a - \mathrm{OD})/(\mathrm{OD} - d)]^{1/b}$$

where a = minimum asymptote, b = slope factor, c = inflection point, and d = maximum asymptote.[28]

Amandin recovery (%) was calculated on the basis of the sample protein content determined and that amandin accounts for ∼65% of almond seed soluble proteins.[24]

2. Inhibition ELISA. Ninety-six-wells of a microtiter plate were coated with 50 μL of 10 μg/mL defatted almond flour BSB soluble proteins in the coating buffer (48.5 mM citric acid, 103 mM sodium phosphate, pH 5.0) for 1 h at 37 °C. The plates were washed three times with TBS-T and blocked for 1 h at 37 °C with 5% (w/v) NFDM in TBS-T (200 μL/well). The plates were again washed three times with TBS-T. Concurrently, 90 μL of mAb 4C10 (5.2 ng/well) in 1% (w/v) NFDM in TBS-T was added to the first row and 80 μL was added to the rest. Ten microliters of 0.1 mg protein/mL full fat Nonpareil almond seed flour BSB extracted protein standard or other sample protein extracts was added in the first row to achieve a final concentration of 10 μg protein/mL. Twenty microliters from the first row was pipetted into the second row and mixed thoroughly (i.e., the standard and sample proteins were diluted 1:5). This step was repeated for the rest of the rows and 20 μL from the last row was discarded. After incubation for 1 h at 37 °C, 50 μL aliquots/well preincubated mAb 4C10 were transferred to the corresponding wells of the almond protein coated plate and incubated for 1 h at 37 °C. The plate was washed three times with TBS-T and incubated with 50 μL of AP-labeled secondary antibody diluted in 1% (w/v) NFDM in TBS-T (24 ng/well) for 1 h at 37 °C. Color development was as described above. On the same plate, standards (freshly prepared WA in triplicate), negative control (0.1% BSA), and blanks (TBS-T only, no capturing Ab, no detecting Ab, no secondary Ab, and no substrate) were included. A four-parameter curve was generated by KC4 software, and the 50% inhibitory concentration (IC_{50}) of the analyte was determined.

Analytical. Moisture, protein, fat, ash, and carbohydrate content of the samples were determined by the AOAC procedures[29] as described earlier.[30] Soluble proteins were determined by the Lowry et al.[21] and Bradford[22] methods. Bovine serum albumin (BSA) fraction V (Sigma Chemical Co., St. Louis, MO) was used as the standard protein. BSA standard curves (0−200 μg for the Lowry method and 0−600 μg for the Bradford method) were prepared for each assay in appropriate buffer, and suitable blanks were used in all assays. SDS-PAGE[31] and Western blotting were done as described earlier.[27] Briefly, the SDS−PAGE gels were run as follows. The separating gels were 8−25% linear monomer acrylamide gradient, 14.5 cm × 16.5 cm × 1.5 mm with 1.0 cm × 16.5 cm × 1.5 mm stacking gels (5% monomer acrylamide). The separating gel contained 0.075 M Tris-HC1 (pH 8.8), 0.1% (w/v) SDS, while the stacking gel consisted of 0.125 M in Tris-HC1 (pH 6.8) and 0.1% (w/v) SDS. The gel running buffer was 0.05 M Tris/0.19 M glycine (pH 8.5) containing 0.1% (w/v) SDS. Gels were run at 8−10 mA/gel while being cooled with running tap water (15 °C) until the dye migrated to the gel edge. Gels were stained in 50% (v/v) methanol containing 10% (v/v) acetic acid and 0.25% (w/v) Coomassie Brilliant Blue R for 16 h and destained with 50% (v/v) methanol containing 10% (v/v) acetic acid for 2−4 h followed by 5% (v/v) methanol containing 7.5% (v/v) acetic acid. Silver staining of the gels was done as described by Westermeier.[32] Gels were first fixed in 0.7 M trichloroacetic acid [ACS grade, BDH through VWR Scientific (West Chester, PA)] in 30% (v/v) methanol containing 0.16 M sulfosalicylic acid (ACS Reagent, Mallinckrodt Baker Inc. Phillipsburg, NJ) for 2 h. After washing with excess distilled deionized (DD) H_2O five times for 5 min each, the gels were treated with 25% (v/v) methanol containing 8% (v/v) acetic acid for 1 h followed by 10% (v/v) glutaraldehyde [Fisher Scientific Co. (Pittsburgh, PA)] for 2 h. The gels were thoroughly washed with DD H_2O overnight (three or four changes) before treating with 0.8% silver nitrate [ACS grade, Fisher Scientific Co. (Pittsburgh, PA)] solution for 20 min (1.6 g $AgNO_3$ was dissolved in 8 mL of DD H_2O before adding into 42 mL of DD H_2O containing 0.15 g of NaOH and several drops of concentrated NH_4OH, to prepare a clear solution, and DD H_2O was added to a final volume of 200 mL). The gels were rinsed three times with excess DD H_2O for 5 min each and then incubated in the developer solution [50 μL formaldehyde per 100 mL 0.005% (w/v) citric acid] until the bands were adequately developed. The developer was decanted, and 200 mL of 0.05% (w/v) citric acid containing 0.035 g of methylamine [min. 98%, Fisher Scientific Co. (Pittsburgh, PA)] was added and kept for 5 min to stop the color development. Kodak fixer (Kodak, Rochester, NY) was subsequently added to remove the white background from the gels, and then the stained gels with clear background were washed and stored in DD H_2O.

Western blotting was used to determine the reactive sample proteins probed by the desired antibody. Briefly, proteins from the SDS−PAGE gels were transferred onto 0.22 μm Protran nitrocellulose (NC) paper (Schleicher and Schuell Biosciences Inc., Keene, NH) using a Hoefer TE22 (for 10 × 8 cm small gels) or TE52 (for 14 × 18 cm large gels) transverse electrophoresis unit as described by Towbin et al. (1979).[33] The blotted membranes or strips (3 mm wide, 20 μg each) were visualized by brief staining (5 min) with 0.1% (w/v) Ponceau S before blocking with 5% (w/v) NFDM powder in Tris-buffered saline containing Tween 20 [10 mM Tris, 0.9% (w/v) NaCl, and 0.05% (v/v) Tween 20 (TBST, pH 7.6)] for 1 h at RT. The membrane was washed with two changes of fresh TBS-T for 2 min each. The membrane was then incubated with suitably diluted protein G-purified IgG mAbs (v/v) 10−12 h at 4 °C on a rocker. The membrane was then washed three times with TBS-T for 15 min each. The membrane was incubated with diluted secondary antibody [1 × 10^4, v/v, horseradish peroxidase (HRP) labeled goat anti-mouse for 1 h at RT on a rocker (Lab-line Thermal Rocker, model 4637, Lab-line Instruments Inc., Melrose Park, IL). The membrane was washed again as described above. Bands reactive to mouse mAbs were visualized by using the luminol/p-coumaric acid system. The luminol and

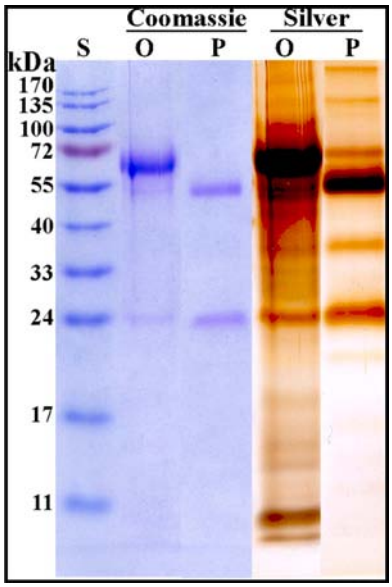


Figure 2. SDS−PAGE for the original (O) and protein G purified (P) mAb 4C10 stained with Coomassie Brilliant Blue R and silver stain. S = protein standards with molecular weights (kDa) indicated in the left margin. Protein load was 3 μg for mAb and 5 μL for the S.

Table 2. Comparison of Indirect Sandwich and Inhibition ELISA Using mAb 4C10[a]

	antigen (ng/mL)		
format	50% of max signal	IC_{50}	linear detection range
sandwich	39.5 ± 0.5		8−200
inhibition		328 ± 46.8	80−1000

[a]Data are expressed as mean ± SEM (n = 4); IC_{50} = inhibitor concentration for 50% of the maximum signal.

Table 3. Detection Sensitivity of Anti-Amandin mAbs Using Indirect Sandwich ELISAs[a]

detection mAb(s)[b]	fraction, v/v	antigen concn at 50% signal, ng/mL	detection range, ng/mL
4C10	1	37.27 ± 2.40	3−200
4C10 + 4F10	1:1	45.4 ± 12.4	5−300
4C10 + 2A3	1:1	48.9 ± 10.7	8−1000
4C10 + 4G2	1:1	39.2 ± 6.0	5−300
4F10 + 2A3	1:1	27.8 ± 1.0	5−200
4F10 + 4G2	1:1	26.4 ± 0.0	5−300
2A3 + 4G2	1:1	36.0 ± 2.5	5−500
4C10 + 4F10 + 2A3	1:1:1	30.9 ± 0.3	5−300
4C10 + 4F10 + 4G2	1:1:1	42.50 ± 1.9	5−300
4C10 + 2A3 + 4G2	1:1:1	39.0 ± 0.4	8−300
4F10 + 2A3 + 4G2	1:1:1	43.3 ± 11.7	5−400
4C10 + 4F10 + 2A3 + 4G2	1:1:1:1	31.44 ± 1.0	5−200
LSD ($P \leq 0.05$)		19.02	

[a]Capture antibody was rabbit anti-whole almond protein extract (604 ng/well); antigen (whole almond protein extract, 8000 ng/mL in 0.1 M borate saline buffer (BSB, pH 8.45) was loaded in the top well and diluted 4× in the successive seven rows. Detection mAbs were diluted using 1% (w/v) NFDM in TBS-T as indicated. Data are expressed as mean ± SEM (n = 2). [b]Diluted mAbs 4C10 (1:1000), 4F10 (1:100), 2A3 (1:50), and 4G2 (1:50) in 1% (w/v) NFDM in TBS-T were mixed in the ratios indicated. Antigen concentration for 50% signal data are expressed as mean ± SEM (n = 2).

Table 4. Accuracy of the Indirect Sandwich ELISA Using mAb 4C10 as the Detection Antibody

		amandin amount (ng/mL) in the sample		amandin content	
sample[a]	replicate no.	predicted	amandin amount determined, ng/mL	%[b]	% mean ± SEM
A	1	125	114.8	91.8	96.7 ± 2.3
	2		125.4	100.3	
	3		118.6	94.9	
	4		122.4	97.9	
	5		128.7	102.9	
	6		118.3	94.6	
	7		124.4	99.6	
	8		136.6	109.3	
	9		136.2	109.9	
	10		106.9	85.5	
	11		107.5	86	
	12		110.2	88.2	
B	1	31.3	31.2	99.9	107.4 ± 1.6
	2		31.6	101	
	3		34	108.7	
	4		32	102.4	
	5		36.3	116	
	6		37	118.3	
	7		34.8	111.3	
	8		32.9	105.3	
	9		33.3	106.7	
	10		33.4	106.9	
	11		33.2	106.2	
	12		33.3	106.5	

[a]Whole almond protein extract in 0.1 M BSB (pH 8.45) buffer after suitable dilutions as indicated by the predicted amandin concentration was used. Predicted amount of amandin content in the protein extract was estimated on the basis of amandin accounting for the 65% of soluble seed proteins.[24] [b]Amandin content determined is expressed as a percent of the predicted amandin content.

Table 5. Intra- and Inter-Assay Variability for the mAb-4C10-Based Indirect Sandwich ELISA[a]

		amandin concn, ng/mL				
		1	2	3	mean ± SEM	% CV
		Intra-Assay				
assay 1	sample A	114.7	125.4	118.6	119.6 ± 5.4	4.5
	sample B	31.2	31.5	34	32.3 ± 1.5	4.7
		Inter-Assay				
sample A	assay a	122.4	128.7	118.3	121.3 ± 11.5	9.5
	assay b	124.4	136.6	136.2		
	assay c	106.9	107.5	110.2		
sample B	assay a	32	36.3	37	34 ± 1.7	4.9
	assay b	34.8	32.9	33.3		
	assay c	33.4	33.2	33.3		

[a]Whole almond protein extracts in 0.1 M BSB (pH 8.45) buffer after suitable dilutions were used.

p-coumaric solutions were mixed together and spread evenly to cover the entire area of the blot. The solution was left for 5 min on the membrane at RT. The membrane was dried, placed in a translucent plastic cover, and exposed to X-ray film (Kodak X-OMAT AR Film, Eastman Kodak Co., Rochester, NY) for autoradiographic visualization.

Statistics. All ELISA experiments were performed at least in duplicate, and data are reported as mean ± standard error of the mean (SEM). One-way ANOVA was performed with SPSS software

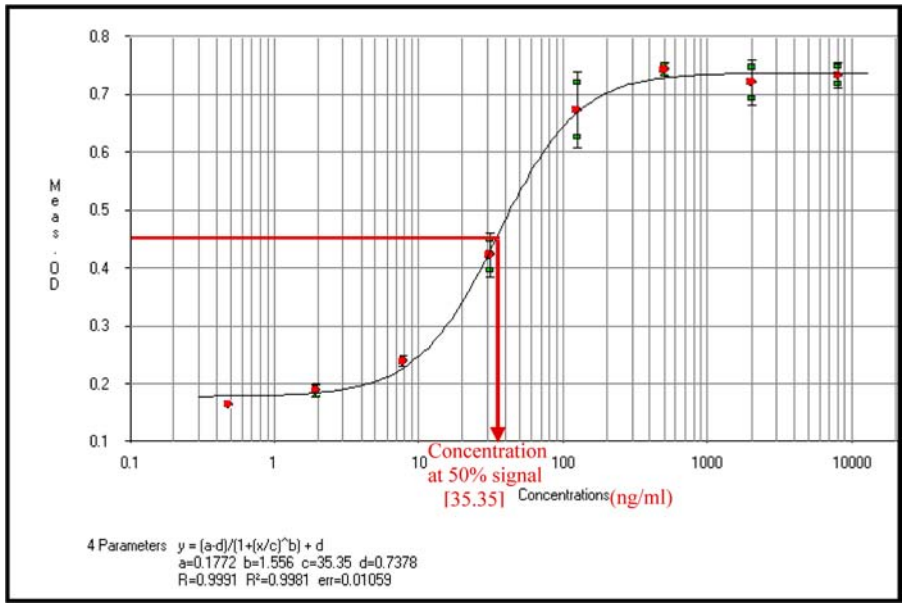


Figure 3. Optimized sandwich ELISA using mAb 4C10 as the detection mAb (n = 33).

Table 6. Food Matrices Used To Determine the Cross-Reactivity of the mAb-4C10-Based Indirect Binding Sandwich ELISA[a]

#	Food Additives		Breakfast cereal		Beverages		Plant Foods
	Colors/pigments (natural)	22	Raisin bran whole mix	47	Black tea		Dry beans/legumes/seeds
1	Annato	23	Corn flakes	48	Green tea	65	Black bean
2	Grape seed tannin	24	Multigrain cereal	49	Cocoa	66	Chickpea
	Colors/pigments (synthetic) FD&C		Flour		Dairy Products	67	Lentil
3	Blue No. 1	25	All-purpose wheat flour		Cheese	68	Navy bean
4	Green No. 3	26	Whole wheat flour	50	Cottage cheese	69	Soybean
5	Red No. 40		Confectionary	51	Swiss cheese	70	Spanish peanut
6	Yellow No. 5	27	Baker's sweetened chocolate		Milk products	71	Virginia peanut
	Thickeners	28	Baker's unsweetened chocolate	52	Non-fat dry milk	72	Sesame seed
7	Pectin	29	Dark chocolate	53	Vanilla ice cream	73	Sunflower seed
8	Microcrystalline cellulose	30	Milk chocolate	54	Yogurt (plain)		Tree nuts
9	Corn starch	31	Sugar		Animal-Based Foods	74	Brazil nut
	Spices/Seasoning	32	Brown sugar		Poultry	75	Cashew nut
10	All-purpose spice mix	33	Baking powder	55	Chicken	76	Hazelnut
11	Black pepper		Fruits		Eggs/egg products	77	Macadamia
12	Salt	34	Apple	56	Egg white	78	Pecan
13	Cinnamon	35	Banana	57	Egg yolk	79	Pine nut
14	Onion	36	Cherry		Meat/meat products	80	Pistachio
15	Nutmeg	37	Orange	58	Beef	81	Walnut
	Cereals	38	Mango	59	Lamb		Vegetables
	Grains	39	Peach	60	Pork	82	Asparagus
16	Amaranth	40	Pear		Seafoods	83	Green pepper
17	Barley	41	Pineapple		Vertebrate	84	Broccoli
18	Corn	42	Strawberry	61	Salmon	85	Carrot
19	Oat	43	Plum	62	Tuna	86	Red potato
20	Rice	44	Apricot		Crustacean	87	Spinach
21	Rye	45	Cranberry	63	Crab	88	Green salad mix
		46	Raisin		Mollusks		Fungi
				64	Oyster	89	Mushroom

[a]Commercially sold foods/ingredients were purchased at local grocery stores or were gifts as described in the Materials and Methods.

(19.0 for Windows, SPSS Inc., Chicago, IL) to compare means for difference among three or more treatment groups. Posthoc analysis was performed using Fisher's least significant difference (LSD) at $P \leq 0.05$. Paired t test was used to compare two means.

RESULTS AND DISCUSSION

Antibody Selection and Properties. On the basis of initial screening, anti-WA protein rabbit pAbs were selected as the capture antibody. Earlier investigations[12] indicated that the anti-WA protein rabbit pAbs exhibited cross-reactivity against certain food matrices. Therefore, several mAbs were screened using Western blotting under non-reducing and reducing conditions (Figure 1). The results indicated that, among the tested murine mAbs, 4C10, 4F10, 2A3, and 3B4 were specific for almond detection. In further testing using ELISAs (Table 1), mAb 4C10 was selected as the detection antibody for the assay development. The selection of mAb 4C10 was based on several criteria which included (a) sensitive and specific recognition of amandin under nonreducing and reducing conditions, (b) good

Table 7. Robustness of the mAb-4C10-Based Indirect Sandwich ELISA

	food	description	manufacturer[a]	mg amandin detected[b]/g sample
1	dark chocolate	control (no declared almond)	I	ND[c]
2		with declared almond		1.99 ± 0.09
3		spiked with 10% (w/w) almond FF[d] flour		1.55 ± 0.07 (15.91% ± 0.68%)
4		control (no declared almond)	II	ND
5		with declared almond		2.83 ± 0.36
6		special dark with declared almond		1.76 ± 0.07
7		spiked with 10% (w/w) almond FF flour		6.54 ± 0.47 (67.08% ± 4.84%)
8	milk chocolate	control (no declared almond)	II	ND
9		with declared almond		11.26 ± 0.13
10		with declared toffee and almond		3.38 ± 0.15
11		spiked with 10% (w/w) almond FF flour		9.51 ± 1.19 (97.49% ± 12.23%)
12		control (no declared almond)	III	ND
13		with declared raisin and almond		6.49 ± 1.49
14		spiked with 10% (w/w) almond FF flour		10.63 ± 1.57 (109.03% ± 16.10%)
15	granola bar	control, contained declared oats and honey	IV	ND
16		with declared roasted almond		3.52 ± 0.20
17		with declared almond	V	3.80 ± 0.70
18		spiked with 10% (w/w) almond FF flour	IV	5.61 ± 0.90 (57.52% ± 9.19%)
19	cereal	control, honey roasted, no declared almond	VI	ND
20		with declared almond		5.42 ± 0.17
21		multigrain cereal with declared almond	VII	7.39 ± 0.66
22		spiked with 10% (w/w) almond FF flour	VI	7.18 ± 0.45 (73.63% ± 4.62%)
23	ice-cream	control, French vanilla, no declared almond	VIII	ND
24		with declared almond		7.35 ± 2.30
25		spiked with 10% (w/w) almond FF flour		8.50 ± 0.25 (87.21% ± 2.57%)
26	trail mix	control (no declared almond)	IX	ND
27		with declared almond		6.87 ± 0.38
28		with declared almond, variety 1	V	2.60 ± 0.32
29		with declared almond, variety 2		4.66 ± 1.64
30		spiked with 10% (w/w) almond FF flour	IX	4.35 ± 0.51 (44.62% ± 5.19%)
		LSD ($P \leq 0.05$)		1.23

[a]I = Dove (Mars), II = Hershey, III = Cadbury, IV = Nature Valley (General Mills), V = Planters (Kraft Foods), VI = Post Foods, VII = Winn Dixie, VIII = Haagen-Dazs, IX = Emerald (Diamond Foods). [b]Values in the parentheses represent the percent recovery of amandin in the spiked samples. Amandin content in the protein extract was estimated on the basis of amandin accounting for the 65% of soluble seed proteins.[24] [c]ND = not detected. [d]FF = full fat. The indicated sample was spiked with full fat Nonpareil almond flour such that the final sample contained 10% by weight full fat Nonpareil almond flour prior to subjecting the sample to protein extraction and subsequent analysis of the extracted proteins.

titer value for immunoassay formats tested, (c) the mAb 4C10 being highly reactive to native prunin and appearing to recognize a conformational epitope,[34] and (d) the mAb being well-characterized with respect to its molecular properties and relevance to human allergies.[35] On the basis of completed research we found that the mAb 4C10 to be interesting not only because of its sensitivity, specificity, and good titer but also because this mAb recognized amandin, the major allergen in almond seeds.[25,27] Amandin accounts for ∼65% of the total soluble proteins,[24,27] is highly soluble in aqueous solvents,[27] and is stable toward commonly encountered food processing methods.[36,37] Amandin is a well characterized[27,38] molecular marker for almond detection as it is the major storage protein in tested almond marketing varieties[39] and genotypes and hybrids.[26] More recently,[35] hydrogen−deuterium exchange (HDX)−mass spectrometry (MS) determined that mAb 4C10 is a good surrogate mAb to investigate amandin immunoreactivity, as the epitope recognized by mAb 4C10 overlaps with a subset of almond allergic patient sera IgE binding epitopes on recombinant amandin (prunin 6.01). The results of the study[35] revealed that three discontinuous strands, amino acids 21−45, 320−328, and 460−465, of the prunin amino acid sequence (507 amino acids) are held in close proximity due to the secondary, tertiary, and/or quaternary structure forming the epitope recognized by mAb 4C10. Further, disulfide bond reduction of the prunin and amandin demonstrated that the epitope structure, although substantially destroyed, was not completely eliminated, as probing with mAb 4C10 as the detection antibody registered quantitative signal, indicating that the epitope contains conformational and linear character.

Assay Optimization. Among the tested plates, plate 2797 (Corning Inc., Lowell, MA) registered good antigen binding capacity (66.06 ng/mL antigen for 50% of maximum ELISA signal) when a 50 μL volume of the rabbit pAbs was used for antigen capture, were lightweight, were easy to handle, and were therefore selected for assay development. For WA proteins, among the tested variables, the following were judged to be optimal for the developed assay. Incubation temperature was 37 °C (all steps), coating buffer was citrate-phosphate buffer (pH 5.0), plate coating incubation time was 2 h, and incubation time was 1 h. Color was developed for 30 min and color development was stopped by adding 50 μL of 3 M NaOH.

Sandwich ELISA. Several commercial assays currently available for detecting almonds,[13] although useful, exhibit

limitations that include (a) lack of data for cross-reactivity, (b) assay being cross-reactive with non-almond proteins, (c) not targeting specific allergenic protein(s) in almond seeds, (d) antibody used in the assay not being well-defined and characterized, (e) lack of assay validation, or a combination thereof. In the current investigation, amandin was the marker protein, and the protein G purified (Figure 2) mAb 4C10 was selected as the detection antibody. On the basis of the known detection limits of Coomassie Blue R (~100 ng) and silver staining (~5−10 ng) for protein staining in the SDS−PAGE gels (University of Missouri−Columbia Proteomic Center, 2006) and the protein load (3 μg) used in Figure 2, we estimated the purity of 4C10 to be ≥99%. Comparison of the sandwich and inhibition ELISAs (Table 2) indicated the former to be more sensitive than the latter and therefore was the selected format for further use. Attempts to use mAbs singly in sandwich or inhibition format (Table 2) or in combination (Table 3) did not improve assay sensitivity over the one registered by the mAb-4C10-based sandwich ELISA. A typical sandwich ELISA using mAb 4C10 as the detection antibody is shown in Figure 3. The LOD of the assay was 3 ng of soluble almond seed proteins/mL and the assay sensitivity was 15 ng of full fat almond flour/mL. Under the optimized assay conditions (detection range 3−200 ng of soluble almond protein/mL), the assay permits detection of the presence of 0.15−10 ppm of full fat almond flour present in 100 mg of food, and the food was extracted with 1 mL 0.1 M BSB (pH 8.45) buffer.

Assay Validity. The optimized assay was accurate (Table 4) and reproducible as indicated by CV < 15% of intra- and inter-assay variability (Table 5). The LOD_1 (6.9 ng/mL) and LOD_2 (2.5 ng/mL) for soluble almond protein detection demonstrated that the assay is sensitive. The sandwich ELISA was tested for possible cross-reactivity using several commercially sold foods and food ingredients (Table 6). These foods/ingredients were selected to represent a wide variety of foods/ingredients that may come in contact with almonds (in various forms) during food manufacture and/or processing. The tested foods/ingredients did not exhibit cross-reactivity. In a recent paper[40] it was indicated that published protein based assays for almond detection and quantification "should be highly specific to avoid false-positive testing". These investigators expressed their concern as false-positive results may result in "expensive food recalls or dietary restrictions due to experimentally based mislabeling". The authors opined that the protein-based methods "were either cross-reactive to other food ingredients or the studies did not include closely related species like apricot, peach, or plum in specificity verification. Thus, data on specificity are incomplete. Moreover, polyclonal antibodies used in ELISA may not be generally or commercially available". In the current investigation, apple, peach, pear, plum, and strawberry (Table 6), all of which belong to the *Rosaceae* family that includes almonds,[41] were included when testing for cross-reactivity using the sandwich ELISA. The results indicated that the BSB protein extracts prepared from the tested foods/ingredients did not exhibit cross-reactivity. Although commonly consumed fruits from the *Rosaceae* family were included in the current investigation, the *Rosaceae* family is known to be quite diverse with approximately 90 genera and 3000 species.[42,43] *Rosaceae* family is traditionally divided into four subfamilies according to the fruit type: Rosoideae (Rosa, Fragaria, Potentilla, and Rubus; fruit, achene), Prunoideae (Prunus; fruit, drupe), Spiraeoideae (Spiraea; fruit, follicle or capsule), and Maloideae (Malus, Pyrus, and Cotoneaster; fruit, pome).[44] Reported poor resolution in the phylogenetic tree backbone of the *Rosaceae* family is attributed to rapid evolutionary divergence within the family.[42] This lack of resolution may result in unanticipated cross-reactivity and therefore additional testing for cross-reactivity of additional edibles from the *Rosaceae* family is needed.

To assess applicability and robustness, the assay was evaluated for its ability to detect the presence of almonds in select, commercially sold, matched samples (Table 7). The results demonstrated that the sandwich ELISA detected the presence of almond. The assay did not register any false positive or negative results among the tested commercial samples. The amandin recovery range for the spiked (10% w/w level) samples was 15.91%−109.03%.

Since chocolate is one of the food matrices that uses almonds for manufactured products enjoyed by consumers, laboratory samples of chocolate (white, dark, and milk) spiked with known amounts of Nonpareil WA flour were also assessed by the sandwich ELISA. The results (Table 8) confirmed that the ELISA can detect the presence of almond (i.e., no false positives/negatives) in all the tested samples. Dark chocolate seemed to decrease amandin recovery, especially at 0.5% (w/w) spiking level, an observation consistent with previously reported low recovery of amandin[12] and Brazil nut proteins[14] in chocolate matrices. Although the cause(s) for such low recoveries of spiked samples is not known, protein−polyphenol (from chocolate) interactions that may result in the formation of insoluble complexes may contribute to low protein extraction efficiency.[14] These results indicate the need for careful investigations of the effect of food matrices on the recovery of targeted allergens.

Table 8. Amandin Recovery from Nonpareil Almond Full Fat Flour Spiked Chocolate As Determined by the mAb-4C10-Based Indirect Sandwich ELISA

chocolate	manufacturer[a]	% spiking level (w/w)	% amandin recovery[b]
white	X	0	ND[c]
		0.5	63.03 ± 0.98
		1	97.67 ± 11.01
		2	182.12 ± 27.84
		5	190.83 ± 28.63
		10	169.32 ± 31.67
dark	II	0	ND
		0.5	0.02 ± 0.00
		1	0.11 ± 1.28
		2	5.50 ± 0.96
		5	19.13 ± 1.97
		10	81.28 ± 5.58
milk	II	0	ND
		0.5	18.45 ± 2.68
		1	120.60 ± 33.31
		2	143.63 ± 33.48
		5	149.15 ± 18.61
		10	169.35 ± 27.87
	LSD ($P \leq 0.05$)		33.68

[a]X = Baker's (Kraft Foods), II = Hershey. [b]Amandin content in the protein extract was estimated based on amandin accounting for the 65% of soluble seed proteins.[24] [c]ND = not detected.

In summary, under the tested assay conditions, the murine mAb-4C10-based sandwich ELISA assay is specific, sensitive, and robust and is therefore suitable for the detection of almond traces. More work is needed to further assess the assay applicability in detecting amandin in almond varieties, processed almonds, and the presence of almond in diverse food systems.

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Notes

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