



Effects of processing and storage on almond (*Prunus dulcis* L.) amandin immunoreactivity



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ARTICLE INFO

Keywords:

Almond
Amandin
ELISA
Processing
Storage
Immunoreactivity

ABSTRACT

A murine monoclonal antibody (mAb)-based enzyme-linked immunosorbent assay (ELISA) was used to assess amandin immunoreactivity in processed and long-term stored almonds. The results demonstrated that amandin immunoreactivity is stable in variously processed almond seeds. Using the ELISA, amandin immunoreactivity could be detected in commercial whole raw and processed (blanched, sliced, dry roasted, and indicated combinations thereof) almond seeds stored for eleven years and eight months, defatted almond seed flours from several almond varieties/hybrids and their borate saline buffer-solubilized protein extracts stored for ten years and seven months, and several almond varieties grown in different California counties (full fat flours and their defatted flour counterparts). Roasting Nonpareil whole full fat almond seeds, full fat flour, and defatted flour at 170 °C for 20 min each with 2, 5, 10, and 20% w/w corn syrup or sucrose did not prevent amandin detection by ELISA. Similarly, amandin detection in select food matrices spiked with Nonpareil almond protein extract was not inhibited. In conclusion, amandin is a stable target protein for almond detection under the tested processing and storage conditions.

1. Introduction

With rising incidence of food-induced allergies in the western world and the increased global awareness of food allergies, efforts are needed to insure safety of sensitive consumers (Sicherer & Sampson, 2009; Wang & Sampson, 2012). The eight major food groups accounting for ~90% food-induced allergies in the US include milk, eggs, fish, crustacean shellfish, peanuts, soybeans, tree nuts, and wheat. The Food Allergen Labeling and Consumer Protection Act (FALCPA) became effective in the US in 2006 (<http://www.fda.gov/Food/GuidanceRegulation/GuidanceDocumentsRegulatoryInformation/Allergens/ucm106187.htm>) and was designed to protect consumers from unintended exposure to the offending allergen. FALCPA specifically requires declaration of the source of ingredients derived from common allergenic foods. Despite such continued progress, presence of undeclared allergens in food and food ingredients often requires recalls (Gendel & Zhu, 2013) that are expensive. High medical costs as a result of exposure to undeclared allergen by sensitive individuals further exacerbates the situation. Although milk allergies are ranked number one among the major food groups causing food allergies, milk-induced

allergies may be overcome during growing years (Sicherer & Sampson, 2014). On the other hand, peanut- and tree nut- induced allergies are not only severe, they are often not outgrown (Sicherer & Sampson, 2014) and their presence in food is therefore of concern.

Among the commonly consumed tree nuts, almonds are ranked number one in production, export, and economic value (ABC, 2016). Although safely enjoyed by most, almond sensitive individuals need to know the presence of almonds to eliminate unwarranted almond exposure. Many commercially sold assay kits are currently available for almond detection. Although useful for screening purposes, commercial kits that often use polyclonal antibodies (pAb) as detecting agent may lack assay specificity due to cross-reactivity with non-target proteins. A sensitive (ppm detection) method using anti-almond major protein rabbit pAb-based ELISA (Acosta, Roux, Teuber, & Sathe, 1999) was demonstrated to lack specificity (Tiwari et al., 2010). To address the food matrix interference in the rabbit pAb-based ELISA (Tiwari et al., 2010), amandin, the major storage protein (Wolf & Sathe, 1998) that is a major allergen (Roux, Teuber, Robotham, & Sathe, 2001; Sathe et al., 2002) in almond seeds, was used to produce monoclonal antibody (mAb). Amandin is a biochemically characterized protein (Gaur,

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Sethi, & Salunke, 2008; Kshirsagar, Fajer, Sharma, Roux, & Sathe, 2010; Sathe et al., 2002; Willison et al., 2011; Willison et al., 2013) relevant to human allergies as it is recognized by the almond allergic patients' serum IgE (Willison et al., 2011; Willison et al., 2013). Using murine anti-amandin mAb-based sandwich ELISA specific for sensitive (0.15–10 ppm) amandin detection has recently been reported (Su et al., 2013). The detection mAb 4C10 used in this ELISA recognized an amandin epitope that exhibited conformational character. Further, epitope mapping investigations revealed that the almond allergic patients' serum IgE recognized an amandin epitope with an overlap with the murine mAb 4C10-recognized amandin epitope (Willison et al., 2013).

In food, amandin is often in contact with a milieu of food components/ingredients and therefore amandin immunoreactivity may differ from that of isolated and biochemically purified amandin. Almond seeds are also often stored prior to being shipped or subjected to processing and/or use. Almond seed protein that may be used as a reference protein in assessing almond allergies, is likely to be stored. Antigen stability and shelf-life are of importance in allergy testing (van Ree et al., 2012; Vieths et al., 1998) as handling and storage of the reference protein may influence protein immunoreactivity. Amandin may be subjected to possible interactions with food components such as lipids, carbohydrates, proteins, and minerals as well as a variety of food ingredients often used in the production and processing of almonds and almond-containing products. In addition, almond seeds, almond containing-products, and almond protein extracts may experience a variety of environmental conditions during transport and storage that may affect amandin structure and the resultant change in protein structure may affect the amandin immunoreactivity. Together, amandin interactions with food components and the structural changes during transport and storage may affect amandin immunoreactivity and therefore possibly influence amandin detection that use anti-amandin antibody-based immunoassays. For amandin detection in such assays, false positive results may result in unwarranted restriction (or recalls) while false negative results may reduce/eliminate the ability to avoid undesirable amandin exposure of sensitive individuals.

Investigating amandin stability and detection in almond seeds and almond flours subjected to a variety of thermal processing methods commonly used in food industry was therefore of interest. In addition, effect of storage on almond seeds and almond protein extract on amandin immunoreactivity was also assessed.

2. Experimental

2.1. Materials

Nonpareil, Butte, Carmel, Mission, Monterey, Padre, Price, and Sonora almond (*Prunus dulcis* L.) seeds grown in different locations of California were provided by the Almond Board of California (Modesto, CA) and were used in preparing laboratory processed almonds and almond proteins. Commercially processed almond samples were obtained from Blue Diamond® Growers (Sacramento, CA, USA). Experimental almond germplasms were collected at the University of California-Davis, CA. Unprocessed Nonpareil almond seeds were used as the reference for all immunoassays. Recombinant amandin isoforms Pru du 6.01 and Pru du 6.02 were prepared as described (Willison et al., 2011). Sources of chemicals, reagents, and supplies have been reported earlier (Su et al., 2013).

2.2. Processing

Whole Nonpareil Supreme almond seeds were subjected to processing as described below.

2.2.1. Autoclaving

Almonds were autoclaved in a SterileMax bench top autoclave

(Barnstead International, Inc., Dubuque, IA, USA) at 121 °C, 15 psi, for 5 and 30 min. Autoclaved samples were air dried at room temperature (~25 °C, RT) in a fume hood until constant weight.

2.2.2. Blanching

Almonds were blanched (seed to water ratio = 1:10 w/v) at 100 °C for 3 and 10 min. Samples were air dried at RT in a fume hood until constant weight.

2.2.3. Frying

Almonds were fried in vegetable oil (seed to oil ratio = 1:10 w/v) at 191 °C for 1 and 3 min. Samples were air dried at RT in a fume hood until constant weight.

2.2.4. Microwaving

Almonds were microwaved in a Panasonic microwave oven (Sears, Roebuck and Co., Hoffman Estates, IL, USA) at 50% power (500 W) for 1 and 3 min.

2.2.5. Dry roasting

Almonds were roasted in a TempCon oven (American Scientific Products, McGaw Park, IL, USA) at 140 °C and 160 °C for 30 min each, and at 168 °C and 177 °C for 12 min each.

2.2.6. Dry roasting with sugars

Whole Nonpareil almond seeds and full fat flour, each separately, were roasted at 170 °C for 20 min each with 2, 5, 10, and 20% w/w corn syrup or sucrose. Half of the roasted full fat almond flours were defatted. The roasted whole almond seeds were ground and half of the resulting flours were defatted. Both full fat and defatted almond flours were analyzed for amandin immunoreactivity.

2.3. Preparation of flours

Foods and ingredients were dried as described earlier (Sharma, Roux, & Sathe, 2008; Tiwari et al., 2010). 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, USA) 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) as the solvent. After overnight drying in a fume hood at RT, the defatted flours were passed through a 40-mesh sieve and then stored in screw capped plastic bottles at –20 °C until further use.

2.4. Preparation of protein extracts

Almond seed flours were extracted in borate saline buffer (BSB, 0.1 M H₃BO₃, 0.025 M Na₂B₄O₇, 0.075 M NaCl, pH 8.45) (flour to solvent ratio = 1:10 w/v) at RT for 1 h, followed by centrifugation at 16,100 g for 15 min at RT. Aliquots of supernatant were stored in plastic microcentrifuge tubes, with caps closed, at –20 °C until further use.

2.5. pH exposure

Nonpareil defatted almond flour was extracted for 1 h in distilled water at flour to water ratio of 1:10 w/v and the pH of the slurry adjusted with 1.0 M NaOH or 1.0 M HCl to the desired pH value (pH 1 through 13, each at 1 pH unit interval). After holding the slurry at the adjusted pH for 1 h, half of each sample was neutralized to pH 7 and the other half was used as such. Samples exposed to different pHs were assessed quantitatively by sandwich ELISA and qualitatively by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting to visualize protein polypeptide profile and immunoreactivity.

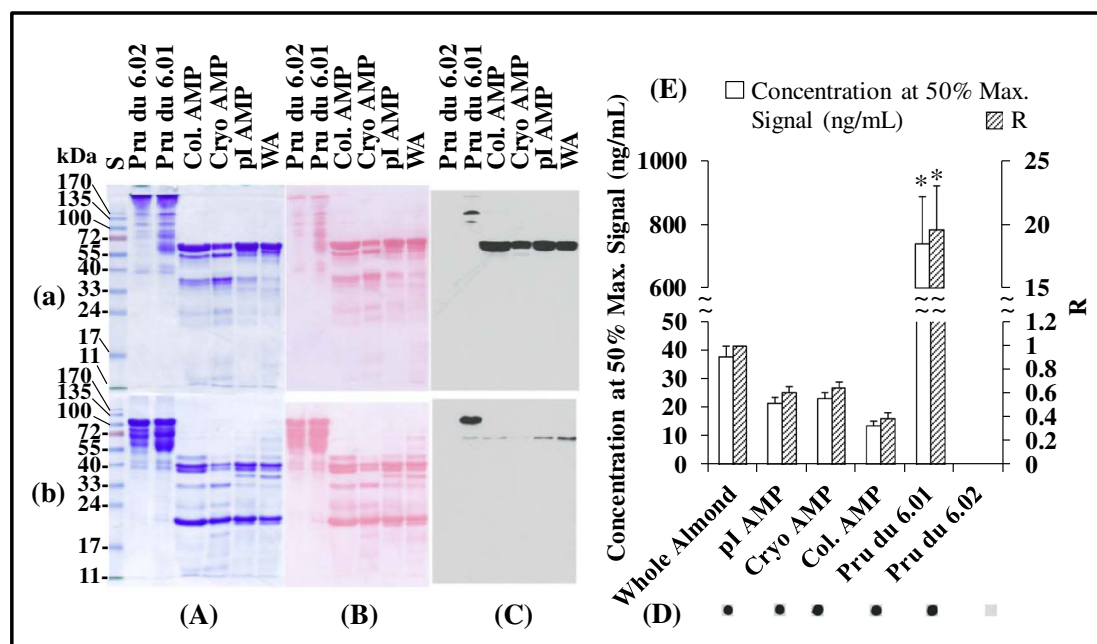


Fig. 1. SDS-PAGE, Western blots, dot blots, and ELISA of amandins in the absence (a) and presence (b) of 2% (v/v) β -mercaptoethanol. (A) 12% SDS-PAGE (B) Ponceau S staining of proteins transferred to 0.2 μ m nitrocellulose membrane (NC) from 12% SDS-PAGE (C) Western blots probed by mAb 4C10 (protein load was 15 μ g each) (D) Dot blots probed by mAb 4C10 (protein load was 1 μ g each). (E) ELISA probed by mAb 4C10. S: protein standards, Pru du 6.01 and 6.02: isoforms of recombinant amandins, Col. AMP: Column purified almond major protein (AMP), Cryo AMP: Cryo-precipitated AMP, pI AMP: Isoelectric precipitated AMP, WA: Whole almond BSB-soluble protein extract. *Results differ significantly from whole almond BSB-soluble protein extract ($P \leq 0.05$).

Table 1

Effects of thermal processing on Nonpareil almond amandin immunoreactivity as assessed by the ELISA.^a

Process	Mean $\pm$ SEM
Unprocessed	1.00 $\pm$ 0.00
Autoclaving 121 °C, 15 psi, 5 min	0.91 $\pm$ 0.07
Autoclaving 121 °C, 15 psi, 30 min	1.11 $\pm$ 0.07
Blanching 100 °C, 3 min	0.74 $\pm$ 0.08
Blanching 100 °C, 10 min	0.72 $\pm$ 0.07
Frying 191 °C, 1 min	0.93 $\pm$ 0.10
Frying 191 °C, 2 min	1.25 $\pm$ 0.16
Microwave 500 W, 1 min	0.91 $\pm$ 0.04
Microwave 500 W, 3 min	0.99 $\pm$ 0.01
Roasting 140 °C, 30 min	1.20 $\pm$ 0.28
Roasting 160 °C, 30 min	1.42 $\pm$ 0.34
Roasting 168 °C, 12 min	0.94 $\pm$ 0.15
Roasting 172 °C, 12 min	0.97 $\pm$ 0.12
LSD ^b	0.42

^a R value = ratio (mean $\pm$ SEM) of concentration at 50% maximum signal for processed to unprocessed, n = 6 each.

^b LSD = Fisher's Least Significant difference, differences between two means exceeding LSD value are significant ($P \leq 0.05$).

Table 2

Effects of long-term storage (11 years and 8 months) on amandin immunoreactivity as assessed by the ELISA.^a

Samples	Mean $\pm$ SEM
Nonpareil (control)	1.00 $\pm$ 0.00
Nonpareil (W ^b , N ^c)	1.13 $\pm$ 0.04
Nonpareil (W, B ^d)	0.97 $\pm$ 0.14
Nonpareil (S ^e , B)	0.69 $\pm$ 0.07
Nonpareil (W, N, DR ^f)	1.22 $\pm$ 0.07
Nonpareil (W, B, DR)	1.16 $\pm$ 0.10
California (W, N)	0.64 $\pm$ 0.03*
California (W, B)	0.65 $\pm$ 0.10*
California (S, B)	0.85 $\pm$ 0.07
California (W, N, DR)	2.46 $\pm$ 0.16*
California (W, B, DR)	0.82 $\pm$ 0.11
Carmel (W, N)	1.32 $\pm$ 0.21
Carmel (W, B)	0.72 $\pm$ 0.04
Carmel (S, B)	0.69 $\pm$ 0.06
Carmel (W, N, DR)	1.30 $\pm$ 0.18
Carmel (W, B, DR)	1.32 $\pm$ 0.22
Mission (W, N)	1.29 $\pm$ 0.12
Mission (W, N, DR)	0.96 $\pm$ 0.11
LSD ^g	0.33

^aR value = ratio of concentration at 50% maximum signal for stored almonds to fresh Nonpareil almond control, n = 3 each. ^bW = whole.

^cN = natural. ^dB = blanched. ^eS = sliced. ^fDR = dry roasted.

^gLSD = least significant difference. Differences between two means exceeding LSD value are significant ($P \leq 0.05$). *Results differ significantly from unprocessed Nonpareil control.

2.6. Amandin recovery from food matrix extracts spiked with soluble almond proteins

Select food matrices were extracted in 0.1 M BSB buffer (1:10 w/v) for 1 h at RT with constant mechanical shaking followed by centrifugation (16,100 g, 10 min, RT) and the supernatant, after suitable dilution with 1% (w/v) nonfat dry milk (NFD) in Tris-buffered saline-Tween 20 (TBS-T; 10 mM Tris, 0.9% w/v NaCl, 0.05% v/v Tween 20, pH 7.6), was spiked with 50 ng/mL (final concentration) BSB-solubilized almond proteins prepared from Nonpareil whole almond flour. The spiked food matrices were subjected to 4C10 sandwich ELISA to determine amandin recovery.

2.7. Storage

Flours prepared from several varieties (California, Carmel, and Mission from Blue Diamond® Growers, Sacramento) of unprocessed and commercially processed almonds (whole blanched, blanched sliced, dry roasted whole, and dry roasted whole blanched) were stored for 11 years 8 months at RT. Defatted flour and BSB solubilized protein extracts prepared from the Nonpareil variety and the genotypes and interspecies hybrids (fifty-eight types from the experimental almond

Table 3Effect of roasting in presence of sugar on amandin immunoreactivity as assessed by the ELISA.^a

	Processing	Defatted (Mean $\pm$ SEM)	Full fat (Mean $\pm$ SEM)
Full fat almond flour processed with sugar	Unprocessed	1.00 $\pm$ 0.00	
	2% syrup	16.53 $\pm$ 1.63*§	21.00 $\pm$ 1.46*‡§
	5% syrup	56.95 $\pm$ 5.49*‡§	41.25 $\pm$ 5.73*‡§
	10% syrup	69.71 $\pm$ 5.42*‡§	59.76 $\pm$ 7.73*‡§
	20% syrup	69.24 $\pm$ 9.60*‡§	47.03 $\pm$ 1.33*‡§
	2% sucrose	10.6 $\pm$ 1.12*	3.70 $\pm$ 0.30
	5% sucrose	9.83 $\pm$ 0.76*	2.97 $\pm$ 0.36
	10% sucrose	6.10 $\pm$ 0.26	4.97 $\pm$ 0.13
	20% sucrose	4.48 $\pm$ 0.37	3.38 $\pm$ 0.08
	2% syrup	1.78 $\pm$ 0.16	1.51 $\pm$ 0.05
	5% syrup	5.27 $\pm$ 0.72	7.38 $\pm$ 1.54
Whole almond seed processed with sugar	10% syrup	5.11 $\pm$ 0.13	4.51 $\pm$ 0.62
	20% syrup	3.60 $\pm$ 0.21	7.65 $\pm$ 0.29
	2% sucrose	5.67 $\pm$ 0.50	7.12 $\pm$ 0.66
	5% sucrose	3.91 $\pm$ 0.38	5.19 $\pm$ 0.15
	10% sucrose	3.60 $\pm$ 0.04	3.32 $\pm$ 0.40
	20% sucrose	2.74 $\pm$ 0.05	3.19 $\pm$ 0.84
	LSD ^b	8.74	7.00
LSD			7.90

^aR value = ratio of concentration at 50% maximum signal for Nonpareil almonds roasted with sugars to unprocessed defatted Nonpareil almond control, n = 3 each. ^bLSD = least significant difference. Differences between two means exceeding LSD value are significant ($P \leq 0.05$). Whole Nonpareil almond seeds or full fat flours were roasted with 2, 5, 10, or 20% (w/w) corn syrup or sucrose at 170 °C for 20 min. Roasted almond seeds and full fat flours were ground and defatted. Soluble almond proteins were extracted from both full fat and defatted almond flours and analyzed by ELISA. *Results differ significantly from unprocessed control. †Results differ significantly between defatted and full fat samples. ‡Results differ significantly between samples treated with syrup and sucrose. §Results differ significantly between processed flour and whole seeds.

germplasm collection at University of California, Davis) were stored for 10 years 7 months at -20 °C. For the storage studies of BSB solubilized proteins, protein extracts were prepared from Nonpareil variety used as the reference. The BSB soluble protein aliquots were adjusted to 20 mg protein/mL, glycerol was added to the BSB extracts (final concentration 50% v/v) prior to the storage and the samples were stored for two years and four months at 4 °C or -20 °C, either with or without 0.02% (w/v) sodium azide (NaN_3). Stored samples were assessed by the mAb 4C10-based sandwich ELISA.

2.8. Effects of lipids

Immunoreactivity of protein extracts prepared using full fat and defatted almond seed flours was assessed. Almond varieties harvested in the years 2003 to 2006 from 12 counties throughout the California Central Valley were provided by the Almond Board of California (Modesto, CA, USA).

2.9. Preparation of native and recombinant amandin

Since native or recombinant amandin may be used as a reference standard for immunoassays, immunoreactivity of both forms was assessed. Native amandin (also known as prunin, Pru du 6, almond major protein or AMP) was prepared using aqueous extracts of the defatted almond (Nonpareil) flours by three separate methods as described earlier (Sathe et al., 2002). The three methods used were: a) a combination of anion exchange and gel filtration chromatography (Col. AMP), b) cryo-precipitation (cryo-AMP), and c) isoelectric precipitation (pI AMP), respectively. Recombinant amandins (Pru du 6.01 and 6.02) were prepared as described earlier (Willison et al., 2011).

2.10. Analytical

2.10.1. Soluble protein

Soluble protein content of the samples was determined by the Lowry (Lowry, Rosebrough, Farr, & Randall, 1951) and Bradford (Bradford, 1976) methods using bovine serum albumin fraction V (Sigma Chemical Co., St. Louis, MO) as the standard protein.

2.10.2. ELISA

Ninety-six well microtiter plates were coated with the rabbit anti-whole defatted almond flour soluble proteins (0.1 M BSB, pH 8.45) pAb 99R20 (604 ng/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 plate was washed three times with TBS-T and blocked for 1 h at 37 °C with 200 μL /well of 5% (w/v) NFDM in TBS-T. The plate was washed three times with TBS-T and the reference protein or sample protein extracts (0.04–600 ng protein/well) were added and 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 detection mAb 4C10 (4–6 ng/well) in TBS-T (containing 1% w/v NFDM). The plate was again washed three times with TBS-T and incubated with alkaline phosphatase (AP)-labeled goat anti-mouse antibody (Molecular Probes®, Life Technologies Corp., Grand Island, NY) 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 AP substrate [5 mg/mL p-nitrophenyl phosphate tablet dissolved in 5 mL substrate buffer (0.0049% w/v MgCl_2 , 0.096% v/v diethanolamine, pH 9.8)]. The reaction was stopped by adding 50 μL of 3 M NaOH to each well and the absorbance ($\text{OD}_{405 \text{ nm}}$) was read by BioTek PowerWave™ 200 Microplate Scanning Spectrophotometer (Winooski, VT). On the same plate, references (freshly prepared Nonpareil whole almond BSB protein extracts in triplicate), negative control (0.1% w/v BSA), and blanks (TBS-T only, no capturing antibody, no detecting antibody, no secondary antibody, and no substrate) were included. The ratio (R) = sample protein concentration for the 50% of the maximum signal of the reference/protein concentration for the 50% of the maximum signal of the reference. For all immunoassays 0.1 M BSB (pH 8.45) buffer extracted proteins from the Nonpareil whole almond flour were used as the reference ($R = 1.0$).

2.10.3. SDS-PAGE

SDS-PAGE was performed using the Fling and Gregerson (1986) procedure as described earlier (Sathe et al., 2002).

2.10.4. Western blot and dot blot

Following SDS-PAGE, the proteins were transferred onto a 0.22 μm nitrocellulose membrane as described by Towbin, Staehelin, and Gordon (1979) at 4 °C for 4 h. The transferred polypeptides were

Table 4Effects of long-term storage (10 years and 7 months) on amandin immunoreactivity as assessed by the ELISA.^a

Samples	Defatted flour	BSB-extract
	Mean $\pm$ SEM	Mean $\pm$ SEM
Nonpareil (control)	1.00 $\pm$ 0.00	
SB1,16-75	1.40 $\pm$ 0.19	1.17 $\pm$ 0.26
SB1,16-76	2.03 $\pm$ 0.37*	1.79 $\pm$ 0.25*
F5,10-2	4.22 $\pm$ 0.38**†	2.53 $\pm$ 0.47*
F5,4-4	2.29 $\pm$ 0.16**†	1.39 $\pm$ 0.11
F5,4-10	3.17 $\pm$ 0.44*	3.43 $\pm$ 0.23*
F5,6-22	2.53 $\pm$ 0.16**†	1.01 $\pm$ 0.03
F5,6-13	1.75 $\pm$ 0.04	1.26 $\pm$ 0.05
F5,6-1	3.03 $\pm$ 0.22**†	1.73 $\pm$ 0.03*
F5,13-54	1.86 $\pm$ 0.06**†	1.17 $\pm$ 0.21
F5,10-9	1.70 $\pm$ 0.41	1.66 $\pm$ 0.07*
F5,20-38	1.99 $\pm$ 0.41*	1.71 $\pm$ 0.12*
F5,20-42	3.14 $\pm$ 0.13**†	1.04 $\pm$ 0.04
F5,20-44	1.99 $\pm$ 0.06*	1.36 $\pm$ 0.07
F5,20-52	2.80 $\pm$ 0.27*	2.56 $\pm$ 0.15*
F8N,6-68	6.12 $\pm$ 0.40**†	2.16 $\pm$ 0.06*
F5,19-82	4.54 $\pm$ 0.96*	3.86 $\pm$ 0.43*
F8N,7-4	2.57 $\pm$ 0.26*	2.04 $\pm$ 0.24*
F8N,7-16	5.95 $\pm$ 0.28**†	2.59 $\pm$ 0.16*
F8,7-13	2.81 $\pm$ 0.29**†	1.50 $\pm$ 0.04
F8N,7-11	2.24 $\pm$ 0.30**†	1.24 $\pm$ 0.02
Padre	2.16 $\pm$ 0.15**†	1.00 $\pm$ 0.27
Thompson	3.68 $\pm$ 0.06**†	2.47 $\pm$ 0.13*
Trusito	1.76 $\pm$ 0.16	1.76 $\pm$ 0.24*
Sonora	2.21 $\pm$ 0.07**†	1.49 $\pm$ 0.16
Mission	2.21 $\pm$ 0.12**†	1.02 $\pm$ 0.01
Nonpareil	2.20 $\pm$ 0.04**†	1.05 $\pm$ 0.12
Carmel	1.16 $\pm$ 0.07	0.79 $\pm$ 0.04
Fritz	2.57 $\pm$ 0.32**†	1.33 $\pm$ 0.21
Ruby	2.03 $\pm$ 0.04**†	0.85 $\pm$ 0.04
Ne Plus Ultra	1.72 $\pm$ 0.19	1.15 $\pm$ 0.12
Butte	1.81 $\pm$ 0.16†	0.86 $\pm$ 0.06
Price	1.47 $\pm$ 0.12	0.89 $\pm$ 0.06
Peerless	2.76 $\pm$ 0.04**†	1.02 $\pm$ 0.03
Livingston	2.39 $\pm$ 0.06**†	1.17 $\pm$ 0.06
LeGrand	2.24 $\pm$ 0.31*	2.42 $\pm$ 0.20*
SB13,45-8	1.41 $\pm$ 0.17	0.84 $\pm$ 0.05
SB13,28-21	3.48 $\pm$ 0.33*	3.14 $\pm$ 0.44*
SB13,54-39E	2.56 $\pm$ 0.08**†	0.83 $\pm$ 0.02
8010-22	2.82 $\pm$ 0.09**†	1.08 $\pm$ 0.02
SB3,56-89	2.40 $\pm$ 0.12*	1.78 $\pm$ 0.14*
F10C,12-28	2.26 $\pm$ 0.11*	2.43 $\pm$ 0.09*
F10C,9-22	1.01 $\pm$ 0.03	0.65 $\pm$ 0.01
F10C,15-1	2.38 $\pm$ 0.30*	1.82 $\pm$ 0.18*
F10C,25-10	2.19 $\pm$ 0.34*	1.83 $\pm$ 0.18*
F10C,20-51	3.70 $\pm$ 0.04**†	2.20 $\pm$ 0.07*
F8S,51-12	2.22 $\pm$ 0.23*	2.02 $\pm$ 0.14*
F8S,51-36	1.56 $\pm$ 0.13	1.36 $\pm$ 0.04
SB16,14-16	2.04 $\pm$ 0.19*	2.91 $\pm$ 0.10**†
SB16,14-13	3.08 $\pm$ 0.19**†	1.84 $\pm$ 0.05*
LG-OP1	3.28 $\pm$ 0.30**†	1.76 $\pm$ 0.01*
Nemared	2.60 $\pm$ 0.33*	2.11 $\pm$ 0.32*
F5,22-14	3.44 $\pm$ 0.22**†	1.41 $\pm$ 0.56
SB13,25-75	1.77 $\pm$ 0.23	1.21 $\pm$ 0.09
F5,14-75	4.01 $\pm$ 0.22**†	2.81 $\pm$ 0.23*
F5,16-60	6.41 $\pm$ 1.03**†	1.97 $\pm$ 0.35*
F5,8-33	3.58 $\pm$ 0.21**†	2.55 $\pm$ 0.10*
F5,8-29	2.59 $\pm$ 0.15**†	1.65 $\pm$ 0.11*
F5,8-31	3.58 $\pm$ 0.10**†	1.81 $\pm$ 0.16*
LSD ^b	0.81	0.53
LSD		0.68

^aR value = ratio of concentration at 50% maximum signal for stored almonds (in defatted flour form vs in BSB-extract) to fresh Nonpareil almond control, n = 3 each. ^bLSD = least significant difference. Differences between two means exceeding LSD value are significant ($P \leq 0.05$). *Results differ significantly from freshly extracted Nonpareil control. †Results differ significantly between defatted flour and BSB-extracts.

visualized by Ponceau S staining. For dot blot, samples containing 1 μ g protein were dotted on the nitrocellulose membrane and allowed to air dry. The unbound sites on the nitrocellulose membranes were blocked

with TBS-T containing 5% (w/v) NFDM for 1 h at RT. Subsequently, the membranes were washed with three changes of TBS-T for 5 min each. The membranes were then incubated with suitably diluted mAb 4C10 (~72 ng/mL) overnight at 4 °C on a rocker. After washing three times with TBS-T for 15 min each, the membranes were incubated with horseradish peroxidase (HRP)-labeled goat anti-mouse antibody (80 ng/mL; Sigma-Aldrich Co. LLC., St. Louis, MO, USA) for 1 h at RT on a rocker. The membranes were washed again as described above. Polypeptide bands or sample dots reactive to mAb 4C10 were visualized by mixing luminol with p-coumaric acid and hydrogen peroxide. The mixture was spread evenly to cover the entire membrane and left for 5 min. The membranes were dried, covered by a transparent plastic wrap, and exposed to X-ray film for auto-radiographic visualization (Venkatachalam, Teuber, Roux, & Sathe, 2002).

2.11. Statistics

All ELISA experiments were performed at least in triplicate and data are reported as mean $\pm$ standard error of mean (SEM). One-way ANOVA was performed with SPSS software (19.0 for Windows, SPSS Inc., Chicago, IL) to determine differences among treatment means. Post-hoc analysis was performed using Fisher's Least Significant Difference (LSD) at $P \leq 0.05$.

3. Results and discussion

3.1. Native and recombinant amandin immunoreactivity

Amandin being the major storage protein and being the major allergenic protein in almond seeds, may be used as a reference protein (i.e., a marker protein) for detecting traces of almond. Either a recombinant or native protein may serve as a reference protein. To be a reliable reference protein, the protein needs to be obtained in a consistent form using a specified method (e.g., recombinant amandin produced by a defined expression system/vector, native amandin purified from a specific almond variety using a specified method). Ideally, the recombinant reference protein of interest should exhibit same molecular properties as its native counterpart. However, depending on the clone used and the manner in which the recombinant protein is prepared, the targeted expressed recombinant protein may or may not display such character. For example, due to the lack of disulfide bonds, recombinant amandin registered barely detectable immunoreactivity compared to its native counterpart, which contains disulfide bonds (Willison et al., 2013). Such lack of disulfide bond(s) in protein may alter protein secondary, tertiary, quaternary structure or a combination thereof inherently present in its native counterpart. Alteration in the secondary, tertiary, and/or quaternary protein structure(s) may lead to change(s) in conformational epitope(s) on the targeted allergen. For these reasons although the recombinant protein can serve as a consistent, reliable, and relatively inexpensive reference protein, its utility to serve as a reference protein may decrease when it lacks thermally stable epitope(s) and/or human allergy relevant dominant conformational epitope(s) known to be present on its native counterpart. Therefore it was of interest to determine whether native and recombinant amandins are suitable as reference proteins for the purpose of almond detection. Immunoassay results (Fig. 1) demonstrate that native and recombinant (Pru du 6.01) amandin can be used as a reference protein as both are detectable by mAb 4C10. The data also suggest that whole almond flour protein extract may be used as a reference for the immunodetection of trace amounts of almond seeds. Column purified amandin (Col. AMP) exhibited the most and the recombinant amandin Pru du 6.01 the least immunoreactivity in the ELISA used. The lower immunoreactivity of Pru du 6.01 compared to its native counterpart assessed by murine mAb 4C10-based ELISA is consistent with the lack of disulfide bonds in the tested amandin molecules (Willison et al., 2013). Using a detection mAb that recognized an

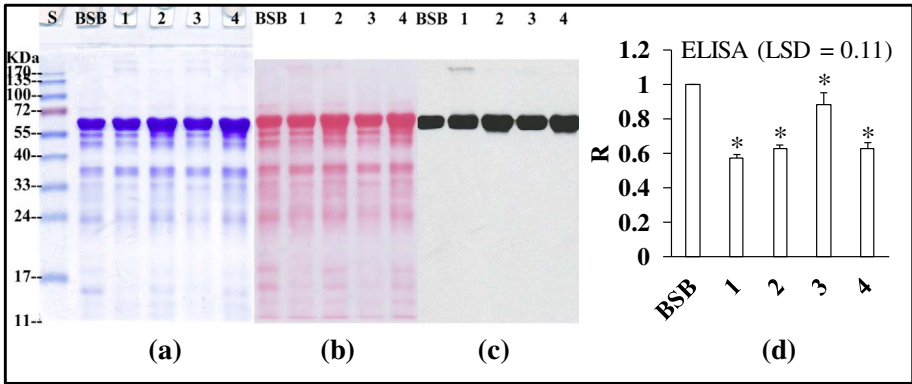


Fig. 2. SDS-PAGE and Western blots for stored almond BSB-extracts in the absence of 2% (v/v) β -mercaptoethanol. Left: 12% SDS-PAGE, middle: Ponceau S staining of proteins transferred to 0.2 μ m nitrocellulose membrane (NC) from 12% SDS-PAGE, right: Western blots probed by mAb 4C10 (protein load was 15 μ g each). S: protein standards, BSB: fresh whole almond BSB-extract (control), 1: BSB-extract stored without NaN_3 at 4 $^{\circ}\text{C}$, 2: BSB-extract stored without NaN_3 at -20 $^{\circ}\text{C}$, 3: BSB-extract stored with NaN_3 at 4 $^{\circ}\text{C}$, 4: BSB-extract stored with NaN_3 at -20 $^{\circ}\text{C}$. *Results differ significantly from fresh whole almond BSB-extract ($P \leq 0.05$).

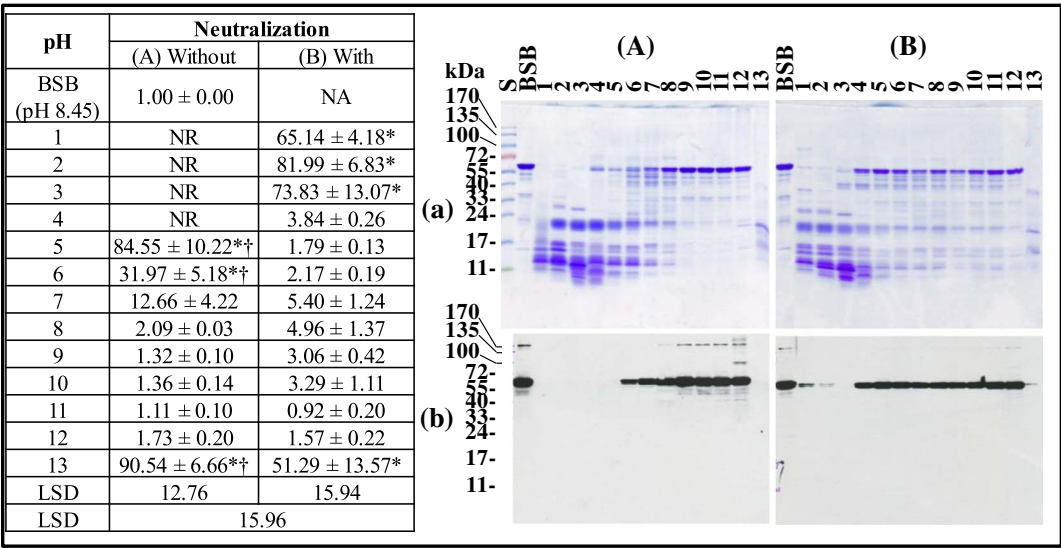


Fig. 3. ELISA (table), SDS-PAGE, and Western blots of almond proteins on pH (1–13) exposure with no neutralization (A) and with neutralization (B). (a) 8–25% SDS-PAGE (non-reducing) (b) Western blots of proteins transferred to 0.2 μ m nitrocellulose membrane (NC) from 8 to 25% SDS-PAGE and probed by mAb 4C10. S: protein standards, BSB: almond proteins extracted in BSB, pH 1–13: almond proteins extracted in pH 1–13 respectively. Protein load was 15 μ g each. NR = no reaction. LSD = Fisher's Least Significant difference, differences between two means exceeding LSD value are significant ($P \leq 0.05$). *Results differ significantly from BSB protein extract. †Results differ significantly between samples with and without neutralization.

epitope comprised of both conformational and linear portions, the lack of conformational portion in the recombinant amandin may account for the need of higher amount, for the same signal, compared to its native counterpart.

3.2. Effects of processing on amandin immunoreactivity

Results of immunoreactivity of variously processed Nonpareil almond seeds are summarized in Table 1. As can be seen from this table, with the exception of roasting at 168 $^{\circ}\text{C}$ and 172 $^{\circ}\text{C}$ for 12 min each, none of the processes caused significant decrease in amandin immunoreactivity. In view of the mAb 4C10 being an antibody recognizing a conformational and linear epitope, any change in amandin structure would reflect on the amandin immunoreactivity. The results suggested that amandin is stable towards the tested processing methods and therefore is a useful marker protein for almond seed detection.

Commercially processed samples along with their unprocessed counterparts of the selected marketing varieties of stored almonds were therefore assessed for their immunoreactivity using the mAb 4C10-based sandwich ELISA (Table 2). The results indicated that amandin immunoreactivity could be determined in all the tested seeds regardless of whether the seeds were processed and/or stored. Thermal stability of amandin observed in the current investigations is in agreement with previously reported findings (Su, Venkatachalam, Teuber, Roux, & Sathe, 2004; Venkatachalam et al., 2002).

Nonpareil almonds, in the form of whole seeds or full fat flour, were subjected to thermal processing in the presence of sucrose or corn syrup. Immunoreactivity of the processed whole almond seeds was not affected by the thermal processing (Table 3). Amandin immunoreactivity of processed full fat flour was not affected in presence of sucrose but was significantly decreased in the presence of syrup. Such reduction in immunoreactivity was likely due to the chemical modification (e.g., Maillard reaction) of amandin instead of conformational changes due to thermal processing alone. It has been reported that Maillard reaction may decrease the immunoreactivity of certain allergens (Gruber, Vieths, Wangorsch, Nerkamp, & Hofmann, 2004; Iwan et al., 2011; Yang, Li, Li, & Wang, 2013; Zhao et al., 2017). Syrup, being a possible source of reducing sugars and with a higher water activity than sucrose, may promote the Maillard reaction.

3.3. Effect of storage on amandin immunoreactivity

Almond seeds often experience storage under a variety of conditions of temperature, humidity, and other environmental factors that may influence protein structure and stability. These storage and environmental factors may depend on the variety of almond, and the locations where almonds are grown, transported, processed, and consumed. For these reasons in addition to commercially processed almonds, almonds of different genotypes were also stored and subsequently evaluated for immunoreactivity using the ELISA (Table 4). The results indicated that,

Table 5
Effects of almond lipids on amandin immunoreactivity as assessed by the ELISA.^a

Samples	DF	FF
	Mean $\pm$ SEM	Mean $\pm$ SEM
Nonpareil (control)	1.00 $\pm$ 0.00	
Butte (Butte)	3.23 $\pm$ 0.02*†	1.02 $\pm$ 0.07
Butte (Central CA)	1.22 $\pm$ 0.08†	0.95 $\pm$ 0.07
Butte (Fresno)	1.02 $\pm$ 0.06	1.79 $\pm$ 0.09*†
Butte (Kern)	1.84 $\pm$ 0.03*	2.82 $\pm$ 0.28*†
Butte (Madera)	1.58 $\pm$ 0.02*	1.65 $\pm$ 0.13*
Butte (Merced)	0.87 $\pm$ 0.03	2.24 $\pm$ 0.15*†
Butte (Northern CA)	1.11 $\pm$ 0.11†	0.66 $\pm$ 0.07*
Butte (Northern CA)	0.84 $\pm$ 0.09	0.93 $\pm$ 0.11
Butte (San Joaquin)	1.47 $\pm$ 0.03*†	0.85 $\pm$ 0.06
Butte (Southern CA)	0.95 $\pm$ 0.09	0.68 $\pm$ 0.07*
Butte (Southern CA)	1.54 $\pm$ 0.12*†	0.80 $\pm$ 0.03
Butte (Southern CA)	0.78 $\pm$ 0.10	0.89 $\pm$ 0.08
Butte (Stanilaus)	1.14 $\pm$ 0.05†	0.83 $\pm$ 0.02
Butte (Tulare)	1.23 $\pm$ 0.06	1.11 $\pm$ 0.08
Carmel (Butte)	2.46 $\pm$ 0.19*†	1.24 $\pm$ 0.06
Carmel (Central CA)	1.25 $\pm$ 0.04†	0.76 $\pm$ 0.04
Carmel (Central CA)	1.18 $\pm$ 0.12	1.09 $\pm$ 0.04
Carmel (Central CA)	0.88 $\pm$ 0.10	1.14 $\pm$ 0.19
Carmel (Central CA)	1.00 $\pm$ 0.09	1.61 $\pm$ 0.06*†
Carmel (Colusa)	1.27 $\pm$ 0.05	1.24 $\pm$ 0.13
Carmel (Fresno)	1.38 $\pm$ 0.06*	1.21 $\pm$ 0.06
Carmel (Glenn)	1.19 $\pm$ 0.00	1.44 $\pm$ 0.04*
Carmel (Kern)	0.77 $\pm$ 0.10	1.05 $\pm$ 0.02
Carmel (Madera)	1.37 $\pm$ 0.02*	1.31 $\pm$ 0.05
Carmel (Merced)	1.98 $\pm$ 0.07*†	1.26 $\pm$ 0.08
Carmel (Northern CA)	1.21 $\pm$ 0.06†	0.75 $\pm$ 0.04
Carmel (San Joaquin)	3.19 $\pm$ 0.10*†	1.39 $\pm$ 0.06*
Carmel (Southern CA)	1.14 $\pm$ 0.09	0.91 $\pm$ 0.05
Carmel (Southern CA)	0.98 $\pm$ 0.07†	0.67 $\pm$ 0.03*
Carmel (Stanilaus)	2.38 $\pm$ 0.13*†	1.79 $\pm$ 0.07*
Carmel (Tulare)	1.45 $\pm$ 0.07*†	0.88 $\pm$ 0.04
Mission (Butte)	2.64 $\pm$ 0.03*†	1.63 $\pm$ 0.13*
Mission (Central CA)	1.34 $\pm$ 0.10*	1.11 $\pm$ 0.14
Mission (Colusa)	2.75 $\pm$ 0.02*†	1.31 $\pm$ 0.03
Mission (ETH-SWTZ)	1.27 $\pm$ 0.02	1.96 $\pm$ 0.31*†
Mission (Fresno)	1.52 $\pm$ 0.05*	1.52 $\pm$ 0.02*
Mission (Glenn)	3.06 $\pm$ 0.02*†	1.33 $\pm$ 0.14*
Mission (Kern)	4.48 $\pm$ 0.05*†	1.86 $\pm$ 0.07*
Mission (Merced)	2.23 $\pm$ 0.21*†	1.88 $\pm$ 0.10*
Mission (San Joaquin)	5.31 $\pm$ 0.01*†	1.41 $\pm$ 0.06*
Mission (Stanilaus)	1.20 $\pm$ 0.16	3.30 $\pm$ 0.48*†
Monterey (Central CA)	2.43 $\pm$ 0.03*†	1.31 $\pm$ 0.04
Monterey (ETH-SWTZ)	1.84 $\pm$ 0.06*†	1.51 $\pm$ 0.14*
Nonpareil (Butte)	1.54 $\pm$ 0.06*	1.49 $\pm$ 0.01*
Nonpareil (Central CA)	1.47 $\pm$ 0.13*†	0.85 $\pm$ 0.11
Nonpareil (Central CA)	1.10 $\pm$ 0.10	0.95 $\pm$ 0.10
Nonpareil (Central CA)	1.64 $\pm$ 0.02*†	1.09 $\pm$ 0.02
Nonpareil (Central CA)	0.99 $\pm$ 0.04	1.45 $\pm$ 0.11*†
Nonpareil (Colusa)	1.64 $\pm$ 0.10*	1.41 $\pm$ 0.10*
Nonpareil (ETH-SWTZ)	1.62 $\pm$ 0.01*†	0.97 $\pm$ 0.07
Nonpareil (ETH-SWTZ)	1.91 $\pm$ 0.06*†	0.96 $\pm$ 0.01
Nonpareil (Fresno)	1.30 $\pm$ 0.13*	1.42 $\pm$ 0.07*
Nonpareil (Glenn)	2.00 $\pm$ 0.15*	2.34 $\pm$ 0.14*†
Nonpareil (Kern)	2.82 $\pm$ 0.05*†	1.35 $\pm$ 0.02*
Nonpareil (Madera)	1.83 $\pm$ 0.12*†	1.50 $\pm$ 0.10*
Nonpareil (Merced)	0.83 $\pm$ 0.05†	1.21 $\pm$ 0.02
Nonpareil (Northern CA)	1.31 $\pm$ 0.17*†	0.72 $\pm$ 0.03
Nonpareil (Northern CA)	1.45 $\pm$ 0.09*†	1.15 $\pm$ 0.04
Nonpareil (San Joaquin)	4.36 $\pm$ 0.14*†	1.53 $\pm$ 0.09*
Nonpareil (Southern CA)	1.24 $\pm$ 0.03	1.09 $\pm$ 0.15
Nonpareil (Southern CA)	1.14 $\pm$ 0.12	1.40 $\pm$ 0.11*
Nonpareil (Stanilaus)	1.03 $\pm$ 0.00	1.59 $\pm$ 0.04*†
Nonpareil (Tulare)	3.45 $\pm$ 0.26*†	1.80 $\pm$ 0.16*
Padre (Butte)	1.22 $\pm$ 0.01	1.24 $\pm$ 0.03
Padre (Fresno)	1.31 $\pm$ 0.09*	1.42 $\pm$ 0.05*
Padre (Kern)	1.30 $\pm$ 0.19*†	0.89 $\pm$ 0.06
Padre (Merced)	1.45 $\pm$ 0.06*	1.82 $\pm$ 0.09*†
Padre (San Joaquin)	1.97 $\pm$ 0.05*†	1.21 $\pm$ 0.12
Padre (Stanilaus)	0.72 $\pm$ 0.11	0.98 $\pm$ 0.13
Padre (Tulare)	1.21 $\pm$ 0.26	1.05 $\pm$ 0.02
Price (Butte)	2.09 $\pm$ 0.04*†	1.32 $\pm$ 0.06*

Table 5 (continued)

Samples	DF	FF
	Mean $\pm$ SEM	Mean $\pm$ SEM
Price (ETH-SWTZ)	2.19 $\pm$ 0.04*†	1.70 $\pm$ 0.19*
Price (ETH-SWTZ)	1.54 $\pm$ 0.06*	1.46 $\pm$ 0.02*
Price (Fresno)	2.03 $\pm$ 0.10*†	1.27 $\pm$ 0.09
Price (Kern)	0.62 $\pm$ 0.07*	1.23 $\pm$ 0.04†
Price (Merced)	0.96 $\pm$ 0.04	1.08 $\pm$ 0.09
Price (San Joaquin)	1.99 $\pm$ 0.06*†	1.43 $\pm$ 0.04*
Price (Stanilaus)	1.85 $\pm$ 0.15*†	1.13 $\pm$ 0.02
Price (Tulare)	4.42 $\pm$ 0.24*†	1.62 $\pm$ 0.18*
Sonora (Central CA)	1.63 $\pm$ 0.13*	1.44 $\pm$ 0.02*
LSD ^b	0.28	0.31
LSD		0.29

^aR value = ratio of concentration at 50% maximum signal for defatted (DF) or full fat (FF) marketing variety of almonds to fresh defatted (DF) Nonpareil almond control, n = 3 each. ^bLSD = least significant difference. Differences between two means exceeding LSD value are significant ($P \leq 0.05$). *Results differ significantly from Nonpareil control. †Results differ significantly between defatted flour and full fat flour.

Table 6
Effects of food matrices on amandin recovery as assessed by the ELISA.^a

Samples	Mean $\pm$ SEM
Nonpareil (control)	100.00 $\pm$ 2.06
Grape seed tannin (1:1000)	65.49 $\pm$ 1.24*
Corn starch (1:100)	108.33 $\pm$ 1.50
All-purpose spice mix (1:1000)	82.75 $\pm$ 1.13*
Salt (1:1000)	108.92 $\pm$ 1.58
Cinnamon (1:1000)	60.45 $\pm$ 1.73*
Corn (1:100)	115.02 $\pm$ 4.09*
Raisin bran whole mix (1:1)	114.32 $\pm$ 8.64*
Multigrain cereal (1:1)	93.43 $\pm$ 11.24
All-purpose wheat flour (1:1)	107.39 $\pm$ 2.34
Milk chocolate (1:100)	77.46 $\pm$ 3.86*
Sugar (1:100)	106.34 $\pm$ 7.87
Baking powder (1:1000)	97.30 $\pm$ 3.64
Apricot (1:100)	94.25 $\pm$ 7.22
Soybean (1:1)	172.54 $\pm$ 3.17*
Macadamia (1:100)	108.80 $\pm$ 1.33
Green salad mix (1:1)	97.07 $\pm$ 1.55
LSD ^b	13.84

^aData expressed as mean $\pm$ SEM of % recovery, n = 6 each; 50 ng/mL almond protein was loaded in each well of the food matrices (final volume = 75 μ L). ^bLSD = least significant difference. Differences between two means exceeding LSD value are significant ($P \leq 0.05$). *Results differ significantly from Nonpareil control spiked in TBS-T.

despite the variation in immunoreactivity between different almond varieties and genotypes as we reported earlier (Su et al., 2015), storage did not adversely affect the ability to detect amandin presence in the tested seeds. When the seeds are not used as the reference material and protein extracts prepared from the seeds are used as the reference, stability of the protein extracts is of interest. We therefore used the 0.1 M BSB buffer (pH 8.45) extracted proteins for the storage studies. The results (Fig. 2) indicate that the immunoreactivity of the protein extracts was stable over the investigated storage period in the presence or absence of NaN₃ stored at 4 °C and – 20 °C. Taken together, amandin immunoreactivity is stable for the investigated storage conditions and period. The assay is therefore useful for the purpose of amandin detection regardless of the almond variety/cultivar/genotype (full fat and defatted flours) as well as whether the seeds were processed or processed and stored and whether the seed proteins were solubilized and stored under the indicated conditions.

3.4. Effect of pH on amandin immunoreactivity

Almond may be subjected to various pH conditions in food

preparation. To assess the effect of pH on amandin immunoreactivity, Nonpareil almond whole protein extracts were adjusted to pH 1–13 and were tested by ELISA, SDS-PAGE, and Western blot with and without neutralization (Fig. 3). Amandin immunoreactivity evaluated by ELISA was not affected by pH in the range of 7–12 without neutralization and 4–12 with neutralization. The SDS-PAGE and Western blot results illustrated that the loss in immunoreactivity at low pH is likely due to the decreased amandin solubility at pH close to its isoelectric point (4.55–6.3) (Sathe et al., 2002). Neutralization could recover amandin immunoreactivity at pH 4–6. Reduced amandin immunoreactivity at pH 13 or pH ≤ 3 is likely due to irreversible protein aggregate formation and/or amandin denaturation.

3.5. Effect of lipid on amandin detection

The defatted Nonpareil almond samples registered concentration (mean $\pm$ SEM) at 50% of the maximum possible signal at 39.46 ± 0.54 ng/mL ($n = 510$) which was significantly lower ($P \leq 0.05$) than that of the full fat counterpart (60.27 ± 2.54 ng/mL, $n = 123$) and thus indicative of increased amandin immunoreactivity in the defatted almond. The high fat content in full fat almond flour may interfere with amandin extraction and/or antibody-amandin interaction thereby decrease amandin immunoreactivity for full fat almond samples. However, this difference does not preclude amandin detection by an order of magnitude and both defatted and full fat almond samples registered signals within the detection range for the optimized assay (Table 5).

3.6. Amandin recovery from food matrix extracts spiked with soluble almond proteins

The results from recovery experiments indicate the ELISA was able to detect 3.75 ng/well of whole almond protein (equivalent to 2.44 ng/well of amandin) in all the tested properly diluted food matrix extracts (Table 6). However, similar to what was observed by Tiwari et al. (Tiwari et al., 2010), significant matrix effects caused amandin over- or under-estimation in the spiked samples due to unveiling or masking of epitopes by food matrix components and/or interaction between food matrix components and assay reagents.

4. Conclusions

Thermal stability of the reference protein is important for the development of a robust assay that can reliably detect food allergens subjected to a variety of processing conditions. Amandin that is stable against heat treatments in both laboratory and commercial settings is therefore a good marker protein for almond detection. Versatility and shelf-life are also important parameters for the selection of a reference protein. Recombinant amandin, purified native amandin, and soluble amandin extract from defatted and full fat almond flours can all be used as reference proteins. Soluble amandin extract did not exhibit appreciable change in immunoreactivity over a long-term storage period in presence or absence of NaN_3 at 4 °C or -20 °C indicating a long shelf life of the reference protein. In conclusion, amandin is a stable marker protein for almond detection under the tested processing and storage conditions. This information is useful for developing sensitive and robust immunoassays for almond detection.

Acknowledgments

This work was supported by USDA grants (No. 9901530, No. 2004-35503-14117, and No. 2006-35503-17229, S.K.S. and K.H.R.); USDA-AFRI (2009-65503-05797 & 2011-67017-20079, S.K.S. and K.H.R.); USDA NIFA-AFRI (2010-04213, K.H.R. and S.K.S.); Almond Board of California, Modesto, CA (S.K.S. and K.H.R.); and the College of Human Sciences (Dissertation Award Program, M.S.). Dr. LeAnna Willison is

thanked for providing Pru du 6.01 and Pru du 6.02.

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