

Effects of the Maillard Reaction on the Immunoreactivity of Amandin in Food Matrices

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Abstract: Amandin is the major storage protein and allergen in almond seeds. Foods, containing almonds, subjected to thermal processing typically experience Maillard browning reaction. The resulting destruction of amino groups, protein glycation, and/or denaturation may alter amandin immunoreactivity. Amandin immunoreactivity of variously processed almond containing foods was therefore the focus of the current investigation. Commercial and laboratory prepared foods, including those likely to have been subjected to Maillard browning, were objectively assessed by determining Hunter L*, a*, b* values. The L* values for the tested samples were in the range of 31.75 to 85.28 consistent with Maillard browning. Three murine monoclonal antibodies, 4C10, 4F10, and 2A3, were used to determine the immunoreactivity of the targeted samples using immunoassays (ELISA, Western blot, dot blot). The tested foods did not exhibit cross-reactivity indicating that the immunoassays were amandin specific. For sandwich ELISAs, ratio (*R*) of sample immunoreactivity to reference immunoreactivity was calculated. The ranges of *R* values were 0.67 to 15.19 (4C10), 1.00 to 11.83 (4F10), and 0.77 to 23.30 (2A3). The results of dot blot and Western blot were consistent with those of ELISAs. Results of these investigations demonstrate that amandin is a stable marker protein for almond detection regardless of the degree of amandin denaturation and/or destruction as a consequence of Maillard reaction encountered under the tested processing conditions.

Keywords: almond, amandin, food matrices, immunoreactivity, mAb

Practical Application: Foods containing almond are often subjected to processing prior to consumption. Amandin, the major allergen in almond, may experience Maillard reaction. Understanding the change in amandin immunoreactivity as a result of Maillard reaction is important for amandin detection and production of hypoallergenic food products.

Introduction

Almonds are used as an ingredient in various foods including baked goods, confectionery items, granola bars, breakfast cereals, and snack mixes (Wolf and Sathe 1998; Chen and others 2006). It is currently the most consumed (1.8 lbs/capita/year) tree nut in the United States (USDA 2016). Although safely enjoyed by most, almonds may induce allergic reactions in sensitive individuals. Almond allergy is ranked as the fourth most frequent tree nut allergy in the United States (Sicherer and others 2010). Adverse reactions to almond may range from mild oral allergy syndrome to fatal anaphylaxis (Tawde and others 2006; Bock and others 2007). Amandin, also known as almond major protein, 11S legumin, prunin, and Pru du 6, is the major seed storage protein (~65%) and major allergen in almond (Roux and others 2001; Sathe and others 2002; Willison and others 2011).

Almonds are often subjected to heat processing prior to or during their incorporation into foods (Venkatachalam and others 2002). Foods, containing almonds, subjected to thermal processing typically experience Maillard reaction. The resulting destruction of amino groups, protein glycation, and/or denaturation may alter amandin immunoreactivity. Maillard reaction-induced decrease in immunoreactivity has been reported for soybean (Babiker and others 1998; van de Lagemaat and others 2007), cherry

(Gruber and others 2004), squid (Nakamura and others 2006), buckwheat (Nakamura and others 2008; Yang and others 2013), milk (Bu and others 2009), hazelnut (Iwan and others 2011), and silver carp (Zhao and others 2017) allergens. In contrast, Maillard reaction increases immunoreactivity of peanut lectin (Chung and Champagne 1999), 2S albumin (Gruber and others 2005), 7S vicilin (Maleki and others 2000), and scallop tropomyosin (Nakamura and others 2005). The effect of Maillard reaction on amandin immunoreactivity is unknown.

The objectives of this study were 2-fold: (1) to assess the effects of Maillard reaction on the immunoreactivity of amandin in commercial and laboratory prepared food matrices containing almonds, and (2) to determine the specificity, sensitivity, and robustness of the murine monoclonal antibody (mAb) based immunoassays used for its detection.

Materials and Methods

Materials

Nonpareil almond seeds were provided by the Almond Board of California (Modesto, Calif., U.S.A.). Food ingredients and commercial foods were purchased from local grocery stores. Sources of chemicals, reagents, and supplies have been reported (Su and others 2013).

Methods

Sample preparation. Preparation of thermally processed almond seeds and almond containing food have been reported (Su and others 2004; Liu and others 2015).

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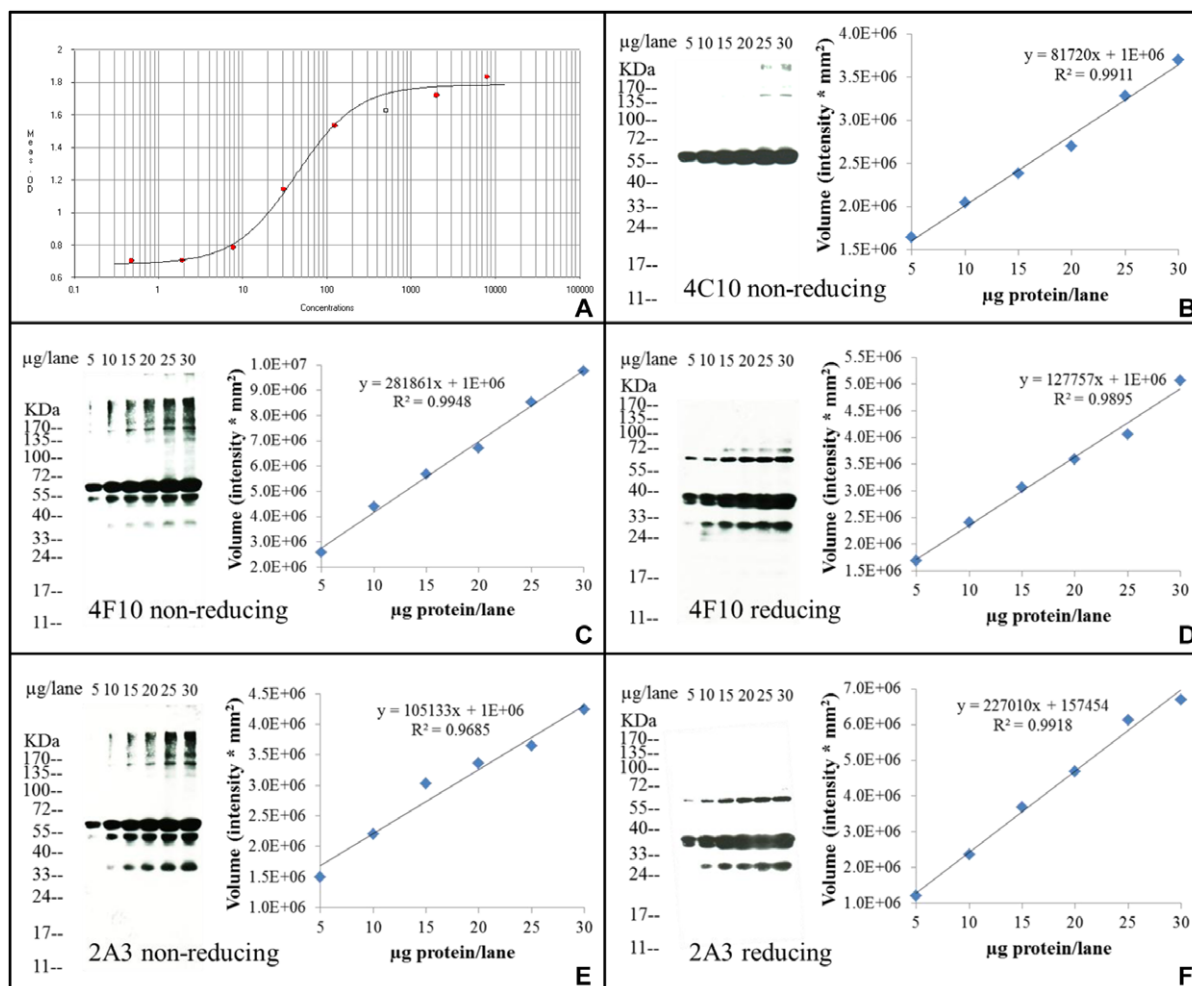


Figure 1—A: a typical sandwich ELISA 4-parameter curve for almond protein quantification. B to F: standard curve for amandin band intensity in Western blot using murine anti-amandin monoclonal antibodies (mAb) 4C10, 4F10, and 2A3 as detection antibodies. The protein load ranged from 5 to 30 µg/lane. Films were analyzed by the ChemiDoc™ XRS+ system. B, C, E are under nonreducing condition, D, F are under reducing condition. The mAb 4C10 did not register any positive signal in Western blot under reducing condition.

Flour preparation. Almond seeds, food ingredients, and processed foods were ground in an Osterizer blender (speed setting “Grind”; Galaxy model 869-18R, Jaden Consumer Solutions, Boca Raton, Fla., U.S.A.) to obtain uniform flours. As needed, the flours were defatted for 8 h using a Soxhlet apparatus (Thermo Fisher Scientific Inc., Waltham, Mass., U.S.A.) with petroleum ether as the solvent. Defatted flours were passed through a 40-mesh sieve and stored in screw capped plastic bottles at −20 °C until further use.

Hunter L*a*b* color scans. Colors of the defatted sample flours were determined according to Sharma and others (Sharma and others 2010). Samples were placed in the glass sample cup (2.5 inch diameter) of the LabScan XE spectrophotometer (Hunter Associates Laboratory, Reston, VA) set at 1 inch (diameter) view with 0°/45° geometry and 10° observer. Care was taken to ensure the sample cup bottom was completely covered with the sample. The L*, a*, and b* values were measured with EasyMatch QC software (version 3.90, Hunter Associates Laboratory, Reston, Va., U.S.A.). Four measurements were taken for each sample and averages of the duplicate preparations were reported.

Water activity (Aw). Water activity of the samples was measured at room temperature (25 °C, RT) using a Pawkit water

activity meter (Decagon Devices, Pullman, Wash., U.S.A.). All samples were measured in triplicates on the same day.

Isolation of amandin. Amandin was prepared by isoelectric precipitation as described by Wolf and Sathe (1998). Briefly, 3 g of defatted almond flour was dispersed in 100 mL of water with constant magnetic stirring. The pH of the dispersion was adjusted to 5.0 with 1 N HCl. After stirring for 1 h at RT, the extracts were centrifuged. The precipitate was collected, redispersed in distilled water, dialyzed against distilled water (48 h, 6 changes of water), lyophilized, and stored in screw capped plastic bottles at −20 °C until further use.

Soluble protein extraction. Each sample (100 mg) was extracted in 1.0 mL of 0.1 M borate saline buffer (BSB, 0.1 M H₃BO₃, 0.025 M Na₂B₄O₇, 0.075 M NaCl, pH 8.45) for 1 h at RT with constant mechanical shaking (Vortex Genie 2, VWR Scientific, Atlanta, Ga., U.S.A.) followed by centrifugation (16100 × g, 10 min, RT) as described previously (Su and others 2013).

Soluble protein determination. The method of Bradford (Bradford 1976) was used to determine the soluble protein content. Bovine serum albumin fraction V (Sigma Chemical Co., St. Louis, Mo., U.S.A.) was used as the standard protein.

Table 1–Water activity (Aw at 25 °C) and Hunter color scale L*, a*, b* values of food matrices containing almonds.

Food matrix		Aw	L*	a*	b*
No.	Description				
2	Whole raw Nonpareil almond seed	0.51 ± 0.01	66.65 ± 0.43	5.66 ± 0.10	22.57 ± 0.04
5	Cookie without almond	0.37 ± 0.02	70.80 ± 0.18	5.26 ± 0.13	30.56 ± 0.21
6	Cookie with 0.5% (w/w) almond	0.37 ± 0.01	69.56 ± 0.09	5.61 ± 0.04	29.42 ± 0.08
7	Cookie with 1.0% (w/w) almond	0.37 ± 0.02	62.13 ± 0.07	9.75 ± 0.03	33.05 ± 0.06
8	Cookie with 2.0% (w/w) almond	0.39 ± 0.01	69.62 ± 0.04	6.28 ± 0.03	30.55 ± 0.02
9	Cookie with 5.0% (w/w) almond	0.39 ± 0.02	66.58 ± 0.05	7.82 ± 0.02	31.23 ± 0.03
10	Sponge cake without almond	0.70 ± 0.02	74.19 ± 0.24	1.77 ± 0.30	26.06 ± 0.88
11	Sponge cake with 0.5% (w/w) almond	0.76 ± 0.02	74.36 ± 0.15	1.52 ± 0.63	28.31 ± 1.45
12	Sponge cake with 1.0% (w/w) almond	0.79 ± 0.01	73.23 ± 0.05	0.91 ± 0.07	27.64 ± 0.13
13	Sponge cake with 2.0% (w/w) almond	0.79 ± 0.01	73.60 ± 0.06	1.51 ± 0.06	28.06 ± 0.14
14	Sponge cake with 5.0% (w/w) almond	0.75 ± 0.03	75.63 ± 0.11	3.17 ± 0.02	26.71 ± 0.04
15	White cake without almond	0.43 ± 0.01	83.30 ± 0.09	3.14 ± 0.09	19.08 ± 0.17
16	White cake with 1.0% (w/w) almond	0.45 ± 0.01	75.91 ± 0.12	4.92 ± 0.02	23.71 ± 0.21
18	Dark chocolate without almond	0.54 ± 0.01	32.88 ± 1.25	6.87 ± 0.38	11.02 ± 0.79
19	Dark chocolate with 0.5% (w/w) almond	0.56 ± 0.00	31.99 ± 2.75	8.66 ± 0.87	12.56 ± 2.13
20	Dark chocolate with 1.0% (w/w) almond	0.62 ± 0.01	34.49 ± 0.03	8.85 ± 0.01	13.09 ± 0.01
21	Dark chocolate with 2.0% (w/w) almond	0.58 ± 0.02	32.55 ± 2.00	8.59 ± 0.83	12.25 ± 1.75
22	Dark chocolate with 5.0% (w/w) almond	0.57 ± 0.01	32.53 ± 2.17	8.71 ± 0.79	11.77 ± 1.94
23	Dark chocolate with 10.0% (w/w) almond	0.58 ± 0.01	31.75 ± 1.48	8.62 ± 0.83	11.53 ± 1.56
24	Milk chocolate without almond	0.50 ± 0.01	39.24 ± 2.44	8.95 ± 0.62	14.50 ± 2.00
25	Milk chocolate with 0.5% (w/w) almond	0.52 ± 0.00	38.86 ± 1.49	9.76 ± 0.41	15.00 ± 1.06
26	Milk chocolate with 1.0% (w/w) almond	0.51 ± 0.01	42.01 ± 1.47	9.77 ± 0.10	15.80 ± 0.84
27	Milk chocolate with 2.0% (w/w) almond	0.52 ± 0.02	40.90 ± 0.95	9.79 ± 0.27	15.62 ± 0.61
28	Milk chocolate with 5.0% (w/w) almond	0.53 ± 0.01	39.77 ± 1.78	9.91 ± 0.42	15.03 ± 1.25
29	Milk chocolate with 10.0% (w/w) almond	0.54 ± 0.01	41.14 ± 1.66	10.40 ± 0.30	16.78 ± 0.99
30	White chocolate without almond	0.48 ± 0.01	85.06 ± 0.16	-2.10 ± 0.03	22.89 ± 0.25
31	White chocolate with 0.5% (w/w) almond	0.48 ± 0.01	85.28 ± 0.90	-2.02 ± 0.08	17.62 ± 0.31
32	White chocolate with 1.0% (w/w) almond	0.48 ± 0.01	82.13 ± 1.10	-2.29 ± 0.06	14.49 ± 0.19
33	White chocolate with 2.0% (w/w) almond	0.48 ± 0.00	84.06 ± 0.31	-1.95 ± 0.01	16.90 ± 0.12
34	White chocolate with 5.0% (w/w) almond	0.49 ± 0.01	85.26 ± 0.33	-1.20 ± 0.14	17.50 ± 0.68
35	White chocolate with 10.0% (w/w) almond	0.50 ± 0.01	82.01 ± 1.76	-0.97 ± 0.06	16.54 ± 1.05
38	Brittle with 30.0% (w/w) almond	0.41 ± 0.01	73.81 ± 0.06	3.22 ± 0.05	16.10 ± 0.07
39	Granola bar without almond	0.48 ± 0.01	48.60 ± 0.19	12.17 ± 0.11	31.88 ± 0.20
40	Granola bar with 2.8% (w/w) almond	0.45 ± 0.02	49.15 ± 0.04	9.30 ± 0.03	23.99 ± 0.07
47	Shortbread cookie without almond	0.39 ± 0.01	67.75 ± 0.05	7.18 ± 0.00	29.45 ± 0.05
48	Dark chocolate almond cookie	0.43 ± 0.02	41.39 ± 0.13	9.63 ± 0.01	20.37 ± 0.02
55	Almond bar with cashew	0.57 ± 0.02	54.49 ± 0.08	9.26 ± 0.04	30.69 ± 0.08
56	Almond bar	0.52 ± 0.01	53.11 ± 0.11	9.71 ± 0.03	28.37 ± 0.07
57	Granola bar with oats and honey	0.49 ± 0.00	76.88 ± 0.16	4.67 ± 0.05	17.79 ± 0.12
59	Granola bar with roasted almond	0.56 ± 0.02	82.05 ± 0.04	2.49 ± 0.01	12.78 ± 0.03
LSD ($P \leq 0.05$)		0.02	11.68	3.00	4.45

Enzyme-linked immunosorbent assay (ELISA). The ELISA procedure has been reported (Su and others 2013). Ninety-six-well “U” bottom polystyrene microtiter plates (Corning Inc., Lowell, Mass., U.S.A.) were coated with 50 μ L/well of rabbit anti-whole almond polyclonal antibody (pAb) 99R20 (604 ng/well) diluted in coating buffer (48.5 mM citric acid, 103 mM Na_2HPO_4 , pH 5.0) and incubated at 37 °C for 2 h. The unbound sites were blocked with 200 μ L/well of 5% (w/v) of non-fat dry milk (NFDM) in Tris-buffered saline-Tween 20 (TBS-T, 10 mM Tris-HCl, 0.9% w/v NaCl, 0.05% v/v Tween 20, pH 7.6) and incubated for 1 h at 37 °C. The plates were washed 3 times with TBS-T and incubated with 90 μ L/well of reference almond protein (0.5 to 8000 ng/mL), sample protein extract (125 ng/mL), or amandin solutions (81.25 ng/mL corresponding to 65% of 125 ng/mL of whole almond protein) for 2 h at 37 °C. After washing with TBS-T for 3 times, 50 μ L/well of murine anti-amandin mAbs 4C10 (6.0 ng/mL), 4F10 (8.8 ng/mL) or 2A3 (14.34 ng/mL) in TBS-T containing 1% (w/v) of NFDM were added and incubated for 1 h at 37 °C. The plates were again washed for 3 times and 50 μ L/well of alkaline phosphatase-labeled goat anti-mouse IgG pAb (28 ng/well; Sigma Chemical Co., St. Louis, Mo., U.S.A.) in TBS-T containing 1% (w/v) NFDM was

added and incubated for 1 h at 37 °C. Following 3 subsequent washings with TBS-T, 50 μ L/well of *p*-nitrophenyl phosphate (pNPP) in substrate buffer (0.0049% w/v MgCl_2 , 0.096% w/v diethanolamine, pH 9.8) was added and incubated for 30 min at 37 °C in the dark. To stop the reaction, 50 μ L/well of aqueous 3 M NaOH was added. The absorbance was read at 405 nm using a PowerWave 200 microplate scanning spectrophotometer (BioTek Instruments, Inc., Vt., U.S.A.). Immunoreactivity (*R*) was defined as the ratio of protein concentration of sample required to register 50% of the maximum signal for the corresponding standard curve (S_{50}) to the protein concentration of reference that register 50% of the maximum signal for the standard curve (R_{50}).

$$R = S_{50}/R_{50}$$

$$S_{50} = (R_{OD} - \text{Blank}) / (S_{OD} - \text{Blank}) \times 125$$

R_{OD} = 50% maximum signal of the standard curve

S_{OD} = signal for sample at 125 ng/mL

Blank = Average signal from the blanks in plate

A larger *R* value means a lower immunoreactivity as more protein is required to register 50% of the maximum signal.

Table 2—Sample numbering and immunoreactivity determined by sandwich ELISA using murine anti-amandin monoclonal antibodies 4C10, 4F10, and 2A3 as detection antibodies.

No.	Food Matrix Name	ELISA (R)			Western blot non-reducing (V)			Western blot reducing (V)	
		4C10	4F10	2A3	4C10	4F10	2A3	4F10	2A3
Laboratory prepared samples									
1	Nonpareil almond major protein	0.95 ± 0.02	1.97 ± 0.02	4.03 ± 0.17	4.96	2.41	2.41	2.62	5.58
2	Whole raw Nonpareil almond seed	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	0.98	1.02	1.00	1.02	1.12
3	Roasted whole almond (375 °F 12 min)	3.00 ± 0.28	4.37 ± 0.24	8.36 ± 0.38	NID	0.46	NID	2.94	4.65
4	Roasted whole almond (325 °F 20 min)	0.67 ± 0.01	2.23 ± 0.02	4.04 ± 0.12	1.79	1.50	1.10	NID	NID
5	Cookie without almond	NID	NID	NID	NID	NID	NID	NID	NID
6	Cookie with 0.5% (w/w) almond	4.26 ± 0.41	4.58 ± 0.58	7.45 ± 0.23	NID	0.33	NID	0.52	NID
7	Cookie with 1% (w/w) almond	1.37 ± 0.23	2.61 ± 0.20	5.47 ± 0.18	NID	0.49	0.51	0.93	1.45
8	Cookie with 2% (w/w) almond	1.28 ± 0.02	2.17 ± 0.05	4.17 ± 0.07	0.07	0.59	0.59	1.09	1.97
9	Cookie with 5% (w/w) almond	0.77 ± 0.01	2.05 ± 0.07	3.71 ± 0.06	0.55	0.73	0.75	1.62	2.95
10	Sponge cake without almond	NID	NID	NID	NID	NID	NID	NID	NID
11	Sponge cake with 0.5% (w/w) almond	6.10 ± 0.18	2.52 ± 0.03	9.24 ± 0.20	0.50	0.44	0.47	0.75	NID
12	Sponge cake with 1% (w/w) almond	3.43 ± 0.10	1.94 ± 0.10	6.10 ± 0.20	0.49	0.45	0.56	1.01	0.85
13	Sponge cake with 2% (w/w) almond	2.63 ± 0.11	1.73 ± 0.07	4.71 ± 0.17	0.48	0.47	0.55	1.01	0.85
14	Sponge cake with 5% (w/w) almond	2.14 ± 0.01	1.49 ± 0.00	3.97 ± 0.02	0.68	0.55	0.62	1.23	0.96
15	White cake without almond	NID	NID	NID	NID	NID	NID	NID	NID
16	White cake with 1% (w/w) almond	2.62 ± 0.03	1.87 ± 0.02	4.79 ± 0.12	0.40	0.21	NID	NID	NID
17	Marzipan with 33% (w/w) almond	2.42 ± 0.03	1.66 ± 0.05	6.67 ± 0.22	0.72	0.55	0.61	1.16	0.84
18	Dark chocolate without almond	NID	NID	NID	NID	NID	NID	NID	NID
19	Dark chocolate with 0.5% (w/w) almond	10.05 ± 0.38	11.83 ± 0.62	23.30 ± 1.53	0.33	0.18	NID	0.38	NID
20	Dark chocolate with 1% (w/w) almond	9.87 ± 0.49	7.09 ± 0.58	14.77 ± 0.79	0.32	0.18	0.39	0.46	0.32
21	Dark chocolate with 2% (w/w) almond	7.18 ± 0.28	3.20 ± 0.58	11.47 ± 0.55	0.44	0.26	0.41	0.58	0.51
22	Dark chocolate with 5% (w/w) almond	4.92 ± 0.4	2.47 ± 0.03	7.42 ± 0.19	0.50	0.33	0.49	0.73	0.67
23	Dark chocolate with 10% (w/w) almond	4.56 ± 0.29	2.17 ± 0.1	7.00 ± 0.23	0.63	0.45	0.56	0.94	0.75
24	Milk chocolate without almond	NID	NID	NID	NID	NID	NID	NID	NID
25	Milk chocolate with 0.5% (w/w) almond	5.64 ± 0.05	5.96 ± 0.17	1.86 ± 0.27	NID	NID	NID	NID	NID
26	Milk chocolate with 1% (w/w) almond	5.51 ± 0.11	5.24 ± 0.18	1.47 ± 0.25	0.43	NID	NID	NID	NID
27	Milk chocolate with 2% (w/w) almond	6.03 ± 0.33	5.13 ± 0.42	1.59 ± 0.24	0.52	0.36	0.51	0.43	0.41
28	Milk chocolate with 5% (w/w) almond	4.56 ± 0.09	4.83 ± 0.06	1.57 ± 0.22	0.62	0.51	0.64	0.64	0.63
29	Milk chocolate with 10% (w/w) almond	3.72 ± 0.05	4.81 ± 0.25	1.46 ± 0.08	0.62	0.56	0.65	0.65	0.72
30	White chocolate without almond	NID	NID	NID	NID	NID	NID	NID	NID
31	White chocolate with 0.5% (w/w) almond	3.58 ± 0.01	3.48 ± 0.11	0.67 ± 0.95	0.37	0.26	0.44	NID	NID
32	White chocolate with 1% (w/w) almond	3.77 ± 0.08	4.11 ± 0.10	1.28 ± 1.07	0.50	0.34	0.50	0.43	0.38
33	White chocolate with 2% (w/w) almond	2.99 ± 0.03	3.60 ± 0.06	0.84 ± 0.87	0.57	0.54	0.65	0.69	0.67
34	White chocolate with 5% (w/w) almond	2.57 ± 0.04	3.43 ± 0.17	0.73 ± 0.76	0.61	0.62	0.67	0.74	0.71
35	White chocolate with 10% (w/w) almond	2.52 ± 0.09	3.33 ± 0.16	0.77 ± 0.73	0.64	0.68	0.74	0.78	0.73
36	Cashew brittle	NID	NID	NID	NID	NID	NID	NID	NID
37	Coconut brittle	NID	NID	NID	NID	NID	NID	NID	NID
38	Almond brittle with 30% (w/w) almond	1.48 ± 0.03	5.55 ± 0.21	9.45 ± 2.10	1.10	1.68	1.32	1.29	1.36
39	Granola bar without almond	NID	NID	NID	NID	NID	NID	NID	NID
40	Granola bar with 2.8% (w/w) almond	1.59 ± 0.04	5.4 ± 0.2	9.05 ± 0.56	0.62	0.94	0.71	0.75	0.64
41	Cornflake without almond	NID	NID	NID	NID	NID	NID	NID	NID
42	Cornflake without almond	NID	NID	NID	NID	NID	NID	NID	NID
43	Cornflake without almond	NID	NID	NID	NID	NID	NID	NID	NID

(Continued)

Table 2-Continued.

No.	Food Matrix Name	ELISA (R)			Western blot non-reducing (V)			Western blot reducing (V)	
		4C10	4F10	2A3	4C10	4F10	2A3	4F10	2A3
44	Cornflake with 1% (w/w) almond	3.80 ± 0.10	4.62 ± 0.43	8.78 ± 0.68	0.79	0.94	ND	0.80	0.60
45	Trail mix	1.88 ± 0.13	5.77 ± 0.46	8.87 ± 0.94	0.40	0.72	0.59	0.69	0.62
46	Trail mix with 10% (w/w) almond	1.50 ± 0.06	5.27 ± 0.11	6.71 ± 0.08	1.02	0.95	0.93	1.38	ND
47	Keebler shortbread cookie with no declared almond	ND	ND	ND	ND	ND	ND	ND	ND
48	Keebler dark chocolate cookie with declared almond	1.98 ± 0.13	2.82 ± 0.12	4.73 ± 0.03	0.37	0.66	0.72	0.97	1.81
49	Dove dark chocolate with no declared almond	ND	ND	ND	ND	ND	ND	ND	ND
50	Hershey dark chocolate with no declared almond	ND	ND	ND	ND	ND	ND	ND	ND
51	Dove dark chocolate with declared almond	1.45 ± 0.02	5.46 ± 0.32	9.69 ± 1.18	0.93	0.50	0.68	1.08	0.94
52	Hershey dark chocolate with declared almond	1.42 ± 0.01	4.66 ± 0.04	9.90 ± 0.94	0.94	0.50	0.69	0.93	0.98
53	Hershey milk chocolate with no declared almond	ND	ND	ND	ND	ND	ND	ND	ND
54	Hershey milk chocolate with declared almond	1.53 ± 0.04	6.21 ± 0.63	11.10 ± 0.24	0.61	0.52	0.63	0.52	0.60
55	Planters Nut-riton bar with declared almond and cashew	1.79 ± 0.14	5.41 ± 0.42	7.40 ± 0.18	0.60	0.58	0.67	0.75	0.74
56	Planters Nutrition bar with declared almond	1.54 ± 0.04	5.06 ± 0.26	8.10 ± 1.11	ND	0.65	0.66	0.77	0.75
57	Nature Valley granola bar with no declared almond	ND	ND	ND	ND	ND	ND	ND	ND
58	Nature Valley granola bar with declared roasted cashew	ND	ND	ND	ND	ND	ND	ND	ND
59	Nature Valley granola bar with declared roasted almond	1.72 ± 0.05	5.54 ± 0.59	8.54 ± 0.25	0.91	1.30	1.08	1.31	1.11
60	Shan kheer mix	ND	ND	ND	ND	ND	ND	ND	ND
61	Shan badam kheer mix	1.39 ± 0.04	6.24 ± 0.12	7.79 ± 0.14	0.61	0.61	0.56	0.53	0.46
62	Emerald tropical trail mix with no declared almond	ND	ND	ND	ND	ND	ND	ND	ND
63	Emerald breakfast trail mix with declared almond	1.41 ± 0.04	5.79 ± 0.38	6.70 ± 0.15	1.48	1.46	1.20	1.49	1.28
64	Planters Alaskan trail mix with declared almond	2.10 ± 0.17	6.11 ± 0.39	7.94 ± 0.38	0.69	0.70	0.70	0.80	0.69
65	Planters Appalachian trail mix with declared almond	1.62 ± 0.12	7.21 ± 0.08	7.21 ± 0.43	0.97	1.09	0.95	1.37	1.17
66	Planters raisin trail mix with declared almond	15.19 ± 4.44	8.55 ± 0.66	10.17 ± 0.26	ND	ND	ND	ND	ND

R is the ratio of protein concentration of sample required to register 50% of the maximum signal for the corresponding standard curve to the protein concentration of reference that register 50% of the maximum signal for the standard curve.

V is the ratio of reactive almond protein concentration in sample to reactive almond protein concentration in reference.

ND = not detected.

The monoclonal antibody 4C10 did not register any positive signal in Western blot under reducing condition.

Table 3–Samples tested for specificity of the murine anti-amandin monoclonal antibodies 4C10, 4F10, and 2A3.

Bakery/confectionery	Dairy	Walnut
Baking soda	Nonfat dry milk	Spices/seasonings
Brown sugar	Skimmed milk	Aniseed
Sugar	Whole milk	Cardamom
Dark chocolate	Fruits	Cinnamon
Milk chocolate	Apple	Clove
White chocolate	Cherry	Cumin
Egg	Coconut	Garlic
Cereals	Plum	Ginger
All-purpose flour	Oilseeds	Mustard
Basi ragi	Lupin	Nutmeg
Barley	Peanut	Parsley
Buckwheat	Sesame seed	Salt
Bulgur wheat	Soybean	Turmeric
Cornflake	Sunflower seed	Vanilla
Millet	Tree nuts	Synthetic colorant
Oat	Brazil nut	Blue No. 1
Publix 7 grain cereal	Cashew	Blue No. 2
Quinoa	Hazelnut	Green No. 3
Rice	Macadamia	Red No. 3
Rye	Pecan	Red No. 40
Semolina	Pine nut	Yellow No. 5
Sorghum	Pistachio	Yellow No. 6

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE was performed according to Fling and Gregerson (1986) using 4% stacking gel and 8% to 25% separating gel. Sample proteins were mixed with

suitable volumes of sample buffer (50 mM Tris-HCl, 1% w/v SDS, 0.01% w/v bromophenol blue, 30% v/v glycerol, with and without 2% v/v β -mercaptoethanol, pH 6.8) to make a final concentration of 0.50 mg/mL of each sample and heated for 10 min in a boiling water bath. Protein samples (20 μ g/lane) were loaded on the gels along with a molecular weight standard. The gels were run in running buffer (50 mM Tris-HCl, 190 mM glycine, 0.1% w/v SDS, pH 8.8) at a constant current (8 to 12 mA/gel) until the tracking dye reached the gel edge.

Immunoblottings. Immunoblottings were performed according to Venkatachalam and others (2002). For Western blot, proteins were transferred onto a 0.22 μ m nitrocellulose membrane following SDS-PAGE. For dot blot, samples containing 1 μ g protein were dotted on the nitrocellulose membrane and allowed to air dry. The unbound sites were blocked with 5% (w/v) NFDM in TBS-T for 1 h at RT. Following 3 washes with TBS-T (5 min each), the membranes were incubated with mAbs 4C10 (72.53 ng/mL), 4F10 (44.12 ng/mL) or 2A3 (14.34 ng/mL) overnight at 4 °C on a rocker (60 Hz, Rocker II model 260350, Boekel Scientific, Feasterville, Pa., U.S.A.). The membranes were washed 3 times with TBS-T (15 min each) and then incubated with horseradish peroxidase (HRP) labeled goat anti-mouse IgG pAb (80 ng/mL) for 1 h at RT on a rocker. The membranes were washed again as described above and incubated with a freshly prepared mixture of luminol, *p*-coumaric acid, and hydrogen peroxide for 5 min at RT. The membranes were then dried and covered with a transparent plastic wrap, and exposed to an X-ray film

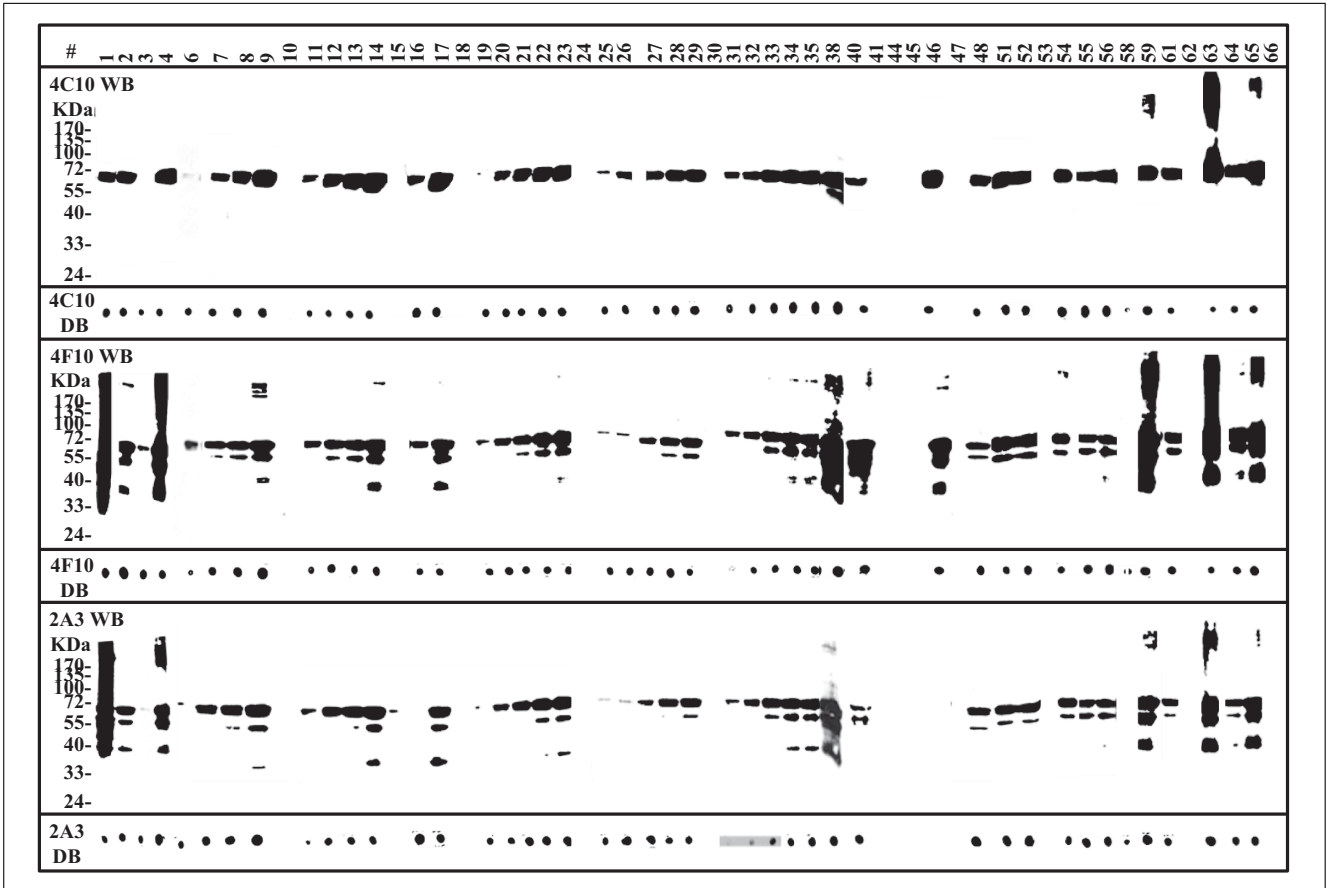


Figure 2–Western blot (WB) and dot blot (DB) in absence of β -mercaptoethanol using murine anti-amandin monoclonal antibodies 4C10, 4F10, and 2A3 as detection antibodies (20 μ g protein/lane). Sample numberings are the same as in Table 2.

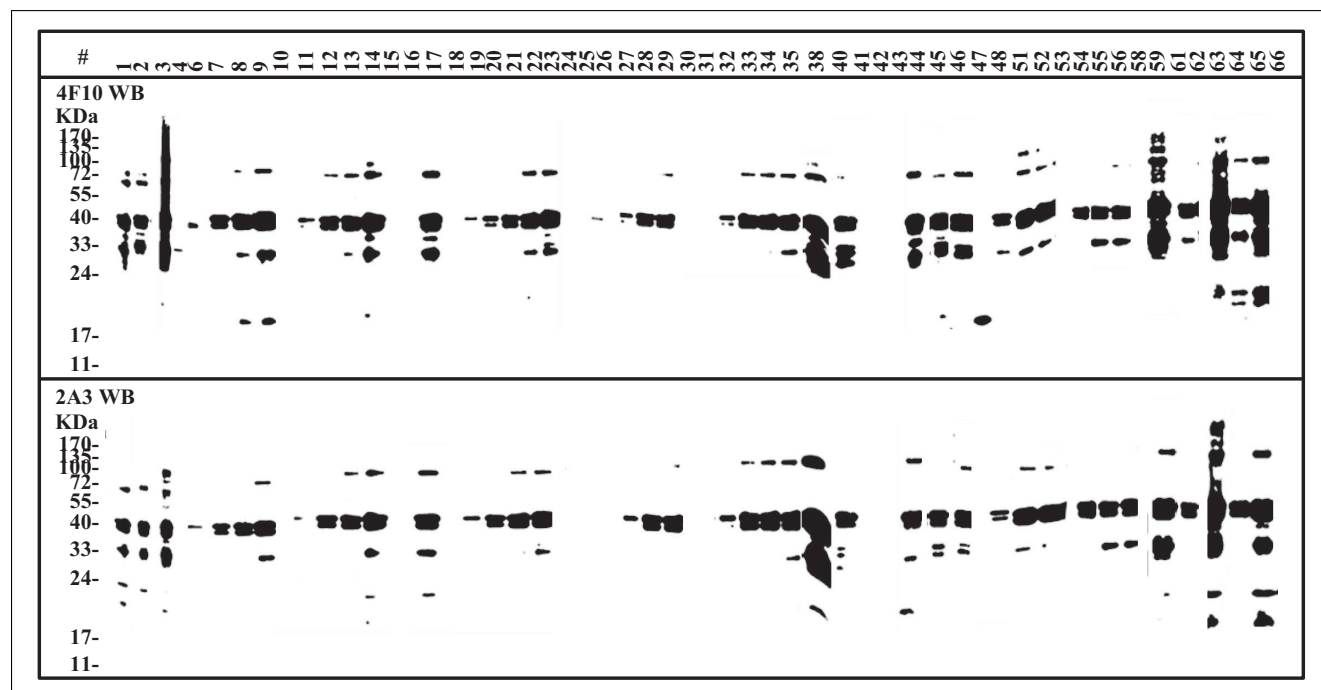


Figure 3—Western blot (WB) in presence of β -mercaptoethanol using murine anti-amandin monoclonal antibodies (mAb) 4F10 and 2A3 as detection antibodies (20 μ g protein/lane). The mAb 4C10 did not register any positive signal in Western blot under reducing condition. Sample numbering as in Table 2.

(Kodak X-OMAT AR Film, Eastman Kodak Co., Rochester, N.Y., U.S.A.) for autoradiographic visualization. The developed films were analyzed on ChemidocTM XRS+ system. The relevant band values, V , was used to assess the immunoreactivity in Western blots.

$$V = S/R$$

S = concentration of reactive almond protein in sample

R = concentration of reactive almond protein in reference (un-processed almond)

Concentration of reactive almond protein was calculated using standard curves based on almond protein concentration and band intensity (pixel) $\times$ area (mm²) as illustrated in Figure 1. A larger V value indicates a stronger immunoreactivity.

Statistical analysis. All experiments were carried out at least in duplicate and data are reported as the mean $\pm$ standard error of mean (SEM). One-way ANOVA was performed with SPSS software (21.0 for Windows, SPSS Inc., Chicago, Ill., U.S.A.). Fisher's least significant difference test was used to determine statistical significance at $P \leq 0.05$.

Results and Discussion

Sample water activity and color

The Maillard reaction depends strongly on water activity (A_w) and takes place at A_w as low as 0.3 and as high as 0.8 with maximum rate of reaction between 0.5 and 0.6 (Labuza and Baiser 1992). The A_w for the food matrices listed in Table 1 ranged from 0.37 to 0.80 ($n = 3$) which suggests that Maillard reaction possibly occurred prior to soluble protein extraction from the assessed samples. The Hunter color scale values for the food matrices listed in Table 1 are, $L^* = 31.75$ to 85.28 , $a^* = -2.29$ to 12.17 , and $b^* = 11.02$ to 33.05 . The L^* values for the tested food matrix flours

($n = 2$) were compared to the roast color value scale, Light = 53 ± 1 , Medium = 48.5 ± 1 , Dark = 43 ± 1 , to determine the lightness and darkness of the product (McDaniel and others 2012). The L^* values for the tested samples were consistent with Maillard browning or the natural product color (e.g. white chocolate). Therefore, browning of the selected food matrices can be attributed to the Maillard reaction.

Assay sensitivity and specificity

The immunoassays are sensitive and specific for almond detection. The mAb 4C10 recognizes a conformational epitope on amandin that overlaps with almond-allergic patients' IgE-binding epitopes. The mAbs 4F10 and 2A3 recognize sequential (linear) epitopes on amandin (Willison and others 2013). All 3 mAbs can reliably detect 10 to 200 ng/mL amandin in ELISAs (Su and others 2013) and did not register any false negative results in the tested almond-containing foods (Table 2). The assay signal corresponded well with the amount of almond in the tested foods. Using the almond-incurred sugar cookies as an example (samples 6 to 9 in Table 2), the R value dropped significantly ($P \leq 0.05$) with increased almond incurring levels. The decreased R value in samples containing more almond indicates a stronger immunoreactivity as less protein is required to register 50% of the maximum signal. The specificity of the mAb 4C10 has been described previously (Su and others 2013). Results of the current study are consistent with our previous findings. ELISA, Western blot, and dot blot results (Table 3, Figure 2 and 3) did not register any false positives. ELISA results suggest that the specificity of mAbs 4C10, 4F10, and 2A3 are comparable.

Assay robustness

The immunoassays were robust for almond detection. The ranges of immunoreactivity (R) values (Table 2) for tested food matrices containing almonds as determined by mAb-based

Table 4—List of food matrices with a significant decrease in the immunoreactivity of amandin compared to whole almonds.

4C10	4F10	2A3
- Roasted almonds (375 °F, 12 min) - Almond cookies (0.5%) - Almond sponge cake (0.5, 1%) - Almond dark and milk chocolate (0.5, 1, 2, 5, 10%) - Almond white chocolate (0.5, 1, 2%) - Cornflakes with almond (1%) - Trail mix with raisin, almond, and cashew	- Roasted almonds (325 °F, 20 min, 375 °F, 12 min) - Almond cookies (0.5, 1, 2, 5% and dark chocolate) - Almond sponge cake (0.5, 1%) and white cake (1%) - Almond dark, milk and white chocolate (0.5, 1, 2, 5, 10%, Hershey, and Dove) - Almond bar and brittle (30%) - Granola bars with roasted almond (2.8%) and peanut - Cornflakes with almond (1%) - Mix badam kheer and all trail mixes with almond	- Roasted almonds (325 °F, 20 min, 375 °F, 12 min) - Almond cookies (0.5, 1, 2, 5% and dark chocolate) - Almond sponge cake (0.5, 1%) and white cake (1%) - Almond dark chocolate (0.5, 1, 2, 5, 10%, Hershey, and Dove) - Almond bar and brittle (30%) - Granola bars with roasted almond (2.8%) and peanut - Cornflakes with almond (1%) - Mix badam kheer and all trail mixes with almond

indirect sandwich ELISAs were 0.67 to 15.19 (LSD = 1.90, $P \leq 0.05$) for 4C10, 1.00 to 11.83 (LSD = 0.86, $P \leq 0.05$) for 4F10, and 0.77 to 23.30 (LSD = 1.34, $P \leq 0.05$) for 2A3. To quantify the polypeptide bands on the developed Western blot films, whole almond protein extract (5 to 30 $\mu\text{g}/\text{lane}$) standard curves were prepared (Figure 1). All films were analyzed using the ChemidocTMXRS+ system. The relevant band values, V , of the tested foods (20 $\mu\text{g}/\text{lane}$) were used to calculate the degree of immunoreactivity relative to the unprocessed control ($V = 1$) (Table 2). The Western blot results ranged 0.07 to 1.79, 0.18 to 2.94, and 0.32 to 4.65 for 4C10, 4F10, and 2A3, respectively. The mAb 4C10 recognizes a conformational epitope that is substantially dependent on the intramolecular disulfide bond within the large subunit of amandin (Willison and others 2013). Therefore, 4C10 failed to register any positive signal in Western blot under reducing condition. As expected, pure amandin registered higher immunoreactivity compared to the whole almond protein extract. Thermal processing, particularly in presence of reducing sugar, significantly reduced amandin immunoreactivity in several food matrices such as cookies, sponge cakes, almond bars, and almond brittles (Table 4). Decreased amandin immunoreactivity was also found in almond-incurred chocolate and cornflakes, which was in agreement with our previous observations (Su and others 2013; Liu and others 2015). The reduction in amandin immunoreactivity may be attributed to epitope masking or degradation due to presence of sugars in the system, loss of disulfide bonds in amandin, and/or loss of protein solubility due to thermal aggregation or matrix effect (for example, protein–polyphenol insoluble complex formation). Similar findings have been reported suggesting Maillard reaction-induced reduction in immunoreactivity of soybean glycinin, β -conglycinin (van de Lagemaat and others 2007), and Gly m Bd30K (Babiker and others 1998), cherry Pru av 1 (Gruber and others 2004), squid tropomyosin (Nakamura and others 2006), buckwheat Fag e 1 (Nakamura and others 2008), and Fag t 3 (Yang and others 2013), milk α -lactalbumin (Bu and others 2009), hazelnut Cor a 11 (Iwan and others 2011), and silver carp parvalbumin (Zhao and others 2017). Zero signal from non-almond containing matched pairs of the food matrices illustrated that non-almond ingredients did not interfere with amandin detection (Table 2, Figure 2 and 3). Almond-incurred chocolate exhibited significantly lower immunoreactivity as compared to the control (Table 2 and 3). This is consistent with our previous findings (Su and others 2013). The low amandin recovery in chocolate matrices may be possibly due to chocolate polyphenols decreasing almond protein solubility (Sharma and others 2008; Tiwari and others 2010).

Conclusions

The observed decrease in immunoreactivity of amandin may be due to either one or a combination of (i) Maillard reaction, (ii) epitope degradation due to loss of amandin disulfide bond(s), and/or (iii) loss of protein solubility due to thermal aggregation. The immunoassays using the murine mAbs are specific, sensitive and robust for amandin detection in the tested food matrices and amandin incurred samples.

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