

A Sandwich Enzyme-Linked Immunosorbent Assay for the Detection of Almonds in Foods†

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ABSTRACT

An enzyme-linked immunosorbent assay was developed to detect almonds as potential allergenic contaminants in food. Polyclonal antibodies directed against roasted almonds were partially purified from immunized sheep and rabbits and used as capture and secondary antibodies, respectively, in a sandwich-type, 96-well plate format. Food samples and almond-spiked samples were extracted 1:10 in phosphate-buffered saline at 60°C for 2 h, centrifuged, and applied to wells coated with sheep anti-almond antibody. After incubation, washing, and the addition of rabbit anti-almond antibody, the amount of almond present was detected with the subsequent addition of goat anti-rabbit immunoglobulin G–alkaline phosphatase conjugate and *p*-nitrophenyl phosphate substrate. Plate absorbances were read at 410 nm, and standard curves were developed in all matrices to quantify unknowns. Antibodies developed were specific for almond; however, some cross-reactivity was observed with extracts of some tree nuts and sesame seeds. Sodium dodecylsulfate–polyacrylamide gel electrophoresis and Western immunoblotting indicated that sheep anti-almond antibody recognized proteins extracted from black walnuts, Brazil nuts, cashews, hazelnuts, macadamia nuts, pistachios, and sesame seeds in addition to those from almond. The assay was optimized to detect less than 1 ppm of almond and was used successfully to determine almond residues in cereal and chocolate without cross-reacting interferences. A retail survey of 20 brands of cereal demonstrated that the assay produced statistically consistent results. This assay provides a useful quality control tool for the food industry for the protection of consumers allergic to almonds.

Tree nuts are common allergenic foods, and consumers allergic to tree nuts often experience adverse reactions (8, 15, 23, 27). The estimated overall prevalence of food allergies is less than 2% in the general adult population (21) and approximately 6% in children (4), with at least 90% of all reactions resulting from the consumption of cow's milk, crustacea, eggs, fish, peanuts, soybeans, tree nuts, or wheat (9). Although at least 160 different food items have been documented to produce an allergic response (11), these eight foods and food groups have been identified as the major allergenic foods on the basis of the prevalence and severity of reactions (9).

Almonds (*Prunus amygdalus* L.), although technically a fruit seed, are most often classified as a tree nut (2, 12). Trace amounts of almond in food may present severe health consequences, including anaphylactic shock, when consumed by individuals allergic to almond (8, 15, 23, 27). These individuals must be vigilant in maintaining strict avoidance diets because there are no preventive medical treatments for food allergies. Despite this practice, the inadvertent ingestion of almonds does occur and could be the

result of nonlabeling or mislabeling of the food product, cross-contact with almonds during food production or preparation, or inadequate cleanup procedures after production of almond-containing foods, leading to carryover of almond residues into subsequently produced foods. These scenarios are not unique to almonds and can be described for any of the major allergenic foods (28).

Increased awareness of food allergies by consumers, industry, and government has been reflected in a greater number of food recalls in recent years (28). There are industry-directed approaches to minimizing food allergen issues during manufacturing, with the goal being an accurately labeled product that is informative to all consumers (6, 14, 28). Ensuring that a food product is free of allergenic residues is difficult, however, without an analytical testing method that is not only specific for that allergenic food but also able to detect minute quantities in a complex matrix such as food. The enzyme-linked immunosorbent assay (ELISA) is a sensitive and specific analytical method that has been successfully applied in the determination of residues from other commonly allergenic foods, such as peanut (10, 16, 31). The objective of this study was to develop an ELISA capable of detecting undeclared, potentially hazardous almond residues in foods.

MATERIALS AND METHODS

Immunogen and antibody semipurification. Polyclonal antibodies were produced at Covance Research Products, Inc. (Den-

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ver, Pa.). New Zealand white rabbits and sheep were immunized with a roasted almond immunogen using a hyperimmunization protocol described by Cordle et al. (5). The immunogen consisted of equal portions of almond meal and nonpareil select, Carmel select, Mission select, and Valencia almonds (Blue Diamond, Sacramento, Calif.; General Mills, Minneapolis, Minn.; Paramount Farms, Lost Hills, Calif.; Treehouse Farms, Earlimart, Calif.; and Zaragoza Alendras St., Madrid, Spain, respectively), which were ground and blended to a fine, uniform consistency.

Polyclonal antibodies were partially purified from sera using successive precipitation in 50 and 35% ammonium sulfate (7), followed by redissolution and extensive dialysis (12,000 to 14,000 molecular weight cut-off, Spectrum, Gardena, Calif.) against 0.01 M phosphate-buffered saline (PBS; 0.002 M NaH_2PO_4 , 0.008 M Na_2HPO_4 , 0.85% NaCl, and 0.02% NaN_3 , pH 7.4). To confirm and monitor antibody production, titers were routinely determined on collected sera using a noncompetitive ELISA in which log dilutions of serum were applied to plates coated with the immunogen (protocol below). Semipurified antibody lots with titers of 10,000 or more were pooled according to species and stored at -20°C . Titers were defined as the log reciprocal of the midlinear portion of dilution curves using plates coated with 0.1 μg of almond protein per well. The protein content of the pooled sera was determined by the Lowry et al. assay (18).

Food samples. Retail food samples were purchased locally in Lincoln, Nebraska. In addition, 40 samples were examined for cross-reactivity in the ELISA and consisted of other tree nuts, legumes, related *Rosaceae* fruits, and a variety of individual food ingredients. Specifically evaluated were allspice, almond extract, apricots (canned and dried), Bing cherries (canned), black walnuts, Brazil nuts, carob, cashews, cinnamon, cloves, cocoa, coconut, corn meal, eggs (raw and whole), hazelnuts (filberts), honey, macadamia nuts (two samples), nutmeg, peaches (canned and dried), peanuts, pecans, pine nuts, pistachios, plums (canned), poppy seeds, prunes, raisins, rice cereal, roasted soybeans, rolled oats, safflower oil, sesame seeds, soy lecithin, sunflower seeds, vanilla extract, walnuts, and whole-wheat flour.

To establish the statistical consistency of results, a retail survey of 20 breakfast cereals was conducted. The survey was designed to sample two lots of each brand of cereal, with each lot extracted in duplicate and each extract analyzed in duplicate. The resultant data were analyzed using the PROC MIXED procedure of SAS software (SAS Institute, Inc., Cary, N.C.).

Standards and food extractions. Food samples and almond-spiked samples were ground to uniform consistency (Sunbeam Corp., Delray Beach, Fla.) and extracted 1:10 (wt/vol) in 0.01 M PBS at 60°C for 2 h at 140 rpm (Cole-Palmer Instrument Co., Chicago, Ill.). The almond mixture used for standards and spiking consisted of the components of the immunogen described earlier. Extracts were transferred to screw-top polypropylene tubes (Becton Dickinson and Co., Franklin Lakes, N.J.) and centrifuged at $3,200 \times g$ and 10°C (International Equipment Co., Needham Heights, Mass.). The supernatant solution was stored at -20°C in polypropylene tubes until analysis. The Lowry et al. assay (18) was used to determine the protein content of extracts.

Almond-containing milk chocolate standards of 4.5 kg, containing 0, 0.5, 1, 2, 5, 10, and 25 ppm of almond (wt/wt) each, were prepared from separate 31.8-kg batches of milk chocolate formulated with 0- or 100-ppm almond flour (21.3% protein, Hughson Nut, Inc., Hughson, Calif.). The milk chocolate was prepared using individual ingredients of butter oil, chocolate liquor, cocoa butter, soy lecithin, skim milk powder, sugar, vanillin, and whole-milk powder (Barry-Callebaut, Ste.-Hyacinthe, PQ, Cana-

da). Tempered chocolate standards were thoroughly mixed (Hobart Corp., Troy, Ohio) before molding. For comparison, laboratory standards were prepared by extracting and serially diluting 0-ppm milk chocolate spiked with 100-ppm almond flour (wt/wt).

ELISA. The assay developed was modified from the sandwich ELISA described by Hefle et al. (10) and was as follows: 100 μl of coating buffer (0.015 M Na_2CO_3 , 0.035 M NaHCO_3 , and 0.02% NaN_3 , pH 9.6) containing 0.1 μg of sheep anti-almond antibody was applied to each well of a 96-well microtiter plate (MaxiSorp, Nalgel Nunc International, Roskilde, Denmark). Plates were incubated for 16 h at 4°C and then washed four times in buffer (0.05% Tween-20 in 0.01 M PBS) using a programmable automatic plate washer (Dynatech Laboratories, Inc., Chantilly, Va.). Remaining binding sites within wells were blocked with 350 μl of 0.1% gelatin (300 bloom porcine, Sigma Chemical Co., St. Louis, Mo.) per well in 0.01 M PBS, pH 7.4, and plates were incubated for 1 h at 37°C . Plates were washed with buffer, 100 μl of sample was applied to each well, and the plates were incubated for 1 h at 37°C . Samples were diluted in the appropriate matrix, either PBS or control extract, and loaded in triplicate on each plate. After washing, bound almond was captured during a 1-h, 37°C incubation with 0.2 μg of rabbit anti-almond antibody per well. Plates were washed in buffer, and the amount of almond present was detected by subsequent incubations of 1 h and 30 min with 100 μl of goat anti-rabbit immunoglobulin G-alkaline phosphatase conjugate (Pierce Chemical, Rockford, Ill.), diluted 1:5,000 in 0.1% bovine serum albumin (radioimmunoassay grade, Sigma) in 0.01 M PBS, and *p*-nitrophenyl phosphate substrate (SigmaFast, Sigma), respectively, interspersed with washing. Color development was stopped by the addition of 100 μl of 1 N NaOH. Plate absorbances were read at 410 nm on an MR600 or MR5000 plate reader (Dynatech). A series of standards were included on every plate, and standard curves were developed using either InPlot (DOS-based) or Prism (Windows-based) software (GraphPad Software, Inc., San Diego, Calif.).

RESULTS AND DISCUSSION

Immunized sheep and rabbits were responsive to the almond immunogen, with high titers (1.4×10^5 and 4.1×10^5 , respectively) 4 weeks into the protocol, and this responsiveness continued for the duration of the bleed schedule (46 and 22 weeks for rabbits and sheep, respectively). Titers performed on preimmunized sera samples indicated that the animals did not possess existing antibodies to almond. The protein content of the pooled titers were 13 and 19 mg/ml for rabbits and sheep, respectively. Preliminary assay trials indicated that an ELISA response optimal for the intended application was achieved using sheep anti-almond antibody, at a concentration of 1 $\mu\text{g}/\text{ml}$, as the primary coating or capture antibody and rabbit anti-almond antibody, at a concentration of 2 $\mu\text{g}/\text{ml}$, as the secondary or chase antibody.

Antibodies produced were specific for almond; however, some cross-reactivity was observed when the extracts of some other tree nuts and sesame seeds were analyzed in the ELISA. Of the more than 40 food items evaluated in the ELISA for cross-reactivity, a positive response ($>30\%$ maximum absorbance) occurred with black walnut, Brazil nut, cashew, hazelnut, macadamia, pistachio, and sesame seed extracts (Table 1). Peanuts, walnuts, pecans, pine nuts, sunflower seeds, wheat flour, oats, rice cereal, related *Ro-*

TABLE 1. Protein content of food extracts producing negative (<30% maximum absorbance) and positive ($\geq 30\%$ maximum absorbance) responses in the almond ELISA

<30% Maximum absorbance		$\geq 30\%$ Maximum absorbance	
Foodstuff	Protein ^a (mg/ml)	Foodstuff	Protein ^a (mg/ml)
Allspice	17.2	Black walnuts	3.8
Almond extract	ND	Brazil nuts	11.2
Apricots (canned)	0.4	Cashews	4.1
Apricots (dried)	3.5	Hazelnuts	9.9
Bing cherries	1.0	Macadamia nuts	3.3
Carob	2.6	Pistachios	8.5
Cinnamon	20.8	Sesame seeds	1.8
Cloves	41.8		
Cocoa	9.6		
Coconut	1.5		
Corn meal	0.7		
Eggs	102.0		
Honey	0.7		
Nutmeg	1.5		
Peaches (canned)	0.2		
Peaches (dried)	3.6		
Peanuts	11.8		
Pecans	1.6		
Pine nuts	3.8		
Plums (canned)	0.6		
Poppy seeds	5.6		
Prunes	7.6		
Raisins	2.2		
Rice cereal	0.9		
Rolled oats	0.8		
Safflower oil	ND		
Soybeans (roasted)	12.2		
Soy lecithin	0.2		
Vanilla extract	1.0		
Walnuts	1.6		
Whole-wheat flour	2.2		

^a Protein content of 1:10 extract as described in Materials and Methods and determined by the Lowry et al. assay (18). ND, Not detected in sample extract.

saceae fruits, and a variety of spices and other ingredients did not cross-react in the ELISA (Table 1). The ELISA responses of the 1:10 and diluted extracts of the positive cross-reactants are shown in Figure 1. This figure depicts the response relative to dilution of the extract and is not standardized relative to the protein content of the extracts. Table 2 summarizes the ELISA response of the cross-reacting extracts relative to the almond standard, based on soluble protein content. The minimum protein content necessary to elicit a positive response in the ELISA was 2,000 to 95,000 times greater for these seven food items than for almond. Thus, a positive ELISA response would not occur with these extracts when loaded at protein concentrations equivalent to those of the almond standard curve. In the application of this format of ELISA, these cross-reacting foods would present a foreseeable problem in the successful detection of almond only in instances in which these foods

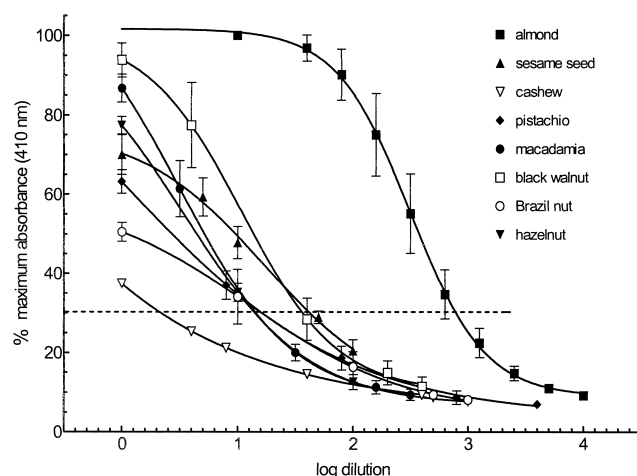


FIGURE 1. ELISA responses of positive cross-reacting food extracts as compared with 1,000-ppm (wt/vol) almond-spiked 0.01-M PBS. Data points represent the mean and standard deviation of three plates, except for the almond standard curve, which represents 20 plates. The dashed horizontal line indicates the departure from linearity at 30% maximum absorbance.

themselves were analyzed for the presence of residual almond (e.g., almond present in a macadamia nut sample).

Sodium dodecylsulfate–polyacrylamide gel electrophoreses (17) and Western immunoblotting with sheep anti-almond antibody demonstrated that the ELISA response was based on the recognition of proteins (data not shown). Acosta et al. (1) reported the recognition of purified almond major protein at 20 to 22 and 38 to 42 kDa by polyclonal rabbit antibodies directed against this water-soluble almond storage protein (26). Various proteins in the extracts of foods that produced a positive ELISA response in this study (Table 1) were also recognized by the sheep anti-almond antibody through immunoblotting. However, the number and intensity of the bands were less than those observed with almond, and none matched the molecular weight of the almond proteins. No effort was made to identify any proteins recognized by the sheep anti-almond antibody in these extracts. Using purified almond protein as an immunogen, Acosta et al. (1) reported polyclonal rabbit antibody

TABLE 2. Minimum concentration of protein resulting in a signal of 30% maximum absorbance of foods demonstrating a positive response in the almond ELISA, as estimated from curves generated in Figure 1, and protein content of these foods relative to that of almond at 30% absorbance

Foodstuff	Protein ($\mu\text{g/ml}$)	Protein content relative to almond at 30% absorbance (times greater)
Sesame seed	40	2,000
Cashew	1,900	95,000
Pistachio	530	26,500
Macadamia	230	11,500
Black walnut	100	5,000
Brazil nut	700	35,000
Hazelnut	700	35,000

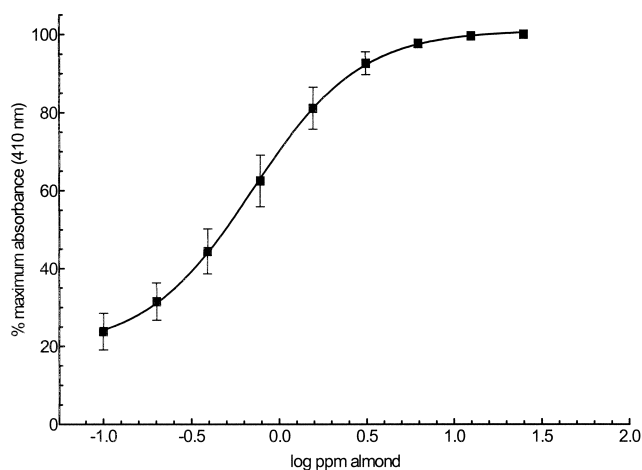


FIGURE 2. Standard curve of 1,000-ppm (wt/wt) almond-spiked corn, wheat, and oat-based breakfast cereal mixture extracted 1:10 in PBS and diluted in nonspiked cereal extracted 1:10 in PBS. Data points represent the mean and standard deviation of 42 plates.

recognition of walnut globulin, cashew globulin (13S), basmati rice globulin, and tepary bean phaseolin (7S) proteins at a concentration of 0.01 mg/ml in a noncompetitive ELISA.

The cross-reactivity observed in this ELISA was not unexpected considering the use of partially purified polyclonal antibodies, from two different species, directed against a crude extract of almond proteins. This cross-reactivity was not detrimental to the performance of the ELISA in its intended application in food. The ELISA was developed through the use of a composite almond immunogen to detect almond protein, as compared with allergenic almond proteins, as contaminants in food. In this manner, multiple almond proteins were recognized by the polyclonal antibodies, and detection of almond was maximized toward the ultimate goal of consumer protection. Sathe (26) described a dominant, water-soluble storage protein in almond with a molecular weight of more than 440 kDa (almond major protein) that was subsequently used as an immunogen for the production of antibodies in rabbits (1). The use of antibodies directed against a purified protein may result in better specificity; however, greater sensitivity may not result when applied in a complex matrix such as food (22, 30).

Individuals with food allergies produce immunoglobulin E, which recognizes specific food proteins (25). The symptoms of food allergy are associated with the release of physiological mediators from immunoglobulin E-bound mast cells or basophils after the ingestion and absorption of these proteins in allergic individuals (25). Immunoblotting of separated food proteins using sera from allergic patients illustrates the proteins recognized by immunoglobulin E and may allow the identification of specific allergenic proteins (20). Bargman et al. (3) used crude almond extracts and identified proteins with molecular weights of 45 to 50 and 70 kDa that were commonly bound by immunoglobulin E from the sera of individuals allergic to almonds. Using purified almond major protein as described by Sathe (26),

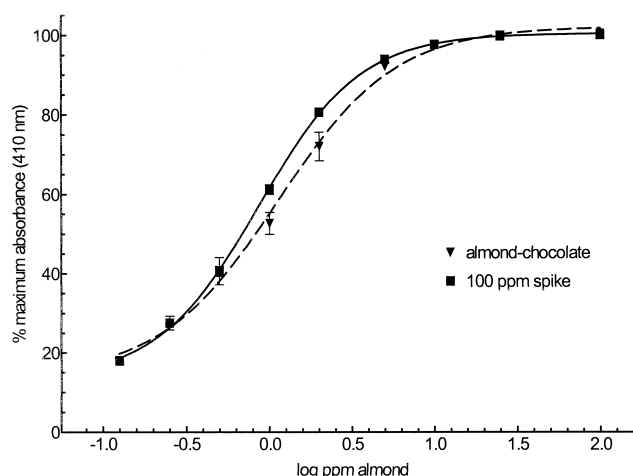


FIGURE 3. Comparison of ELISA responses of 100-ppm (wt/wt) almond flour, laboratory-spiked milk chocolate, and manufactured almond flour-containing milk chocolate standards, all extracted 1:10 in PBS and diluted in milk chocolate extracted 1:10 in PBS. Data points represent the mean and standard error of duplicate extractions, each of which was assayed in duplicate.

Roux et al. (24) demonstrated the recognition of this protein by immunoglobulin E in the sera of individuals allergic to almonds. In addition, Roux et al. (24) identified immunoglobulin E binding at molecular weights of 50 and 66 kDa, which is consistent with those reported by Bargman et al. (3). Although molecular characterization of the allergenic proteins is important for understanding, diagnosing, and ultimately treating a food allergy, it is not imperative for developing an analytical tool.

This ELISA is capable of detecting residues of less than 1 ppm of almond (limit of quantitation of 1 ppm of almond) and has been applied in foods such as cereal (Fig. 2), chocolate (Fig. 3), dairy foods, and other confectionery items. A retail survey of 20 brands of cereal (Table 3) verified the consistency of the assay results, indicating no significant differences in the variability of extracts ($P < 0.005$). This survey did not reveal almond cross-contact in cereals labeled without almonds; however, some samples contained no detectable almond (<1 ppm) even though almonds were listed as a minor ingredient (Table 3). This may represent the manufacturer's decision to include almonds in the ingredient statement as a precautionary measure to alert consumers allergic to almonds as compared with use of the "may contain" statement. Although almonds may not be present in a food when listed as a minor or last ingredient, it remains important for allergic consumers to continually avoid such products because of the chance that almonds are present and the potentially dire health consequences associated with consumption.

To validate the extraction protocol and ELISA response for almond-spiked food samples, manufactured almond-containing milk chocolate standards and laboratory-spiked almond-free milk chocolate were compared (Fig. 3). Comparable standard curves demonstrated that the almond was effectively extracted (86 to 100%) from chocolate with use of the extraction protocol. In analyses of retail samples, almond cross-contact residues were detected in two of four

TABLE 3. Results of retail survey of 20 brands of dry breakfast cereal for the presence of almond using the almond ELISA

Cereal sample	Almonds declared	Almond (ppm) ^a	
		Lot 1	Lot 2
A	Yes	1,200 ± 94	5,200 ± 250
B	Yes	3,400 ± 88	8,000 ± 760
C	Yes	4,500 ± 18	7,000 ± 180
D	Yes	34,000 ± 180	34 000 ± 3,500
E	Yes	11,000 ± 18	9,800 ± 280
F	Yes	11,000 ± 190	9,200 ± 880
G	Yes	11,000 ± 370	4,700 ± 320
H	Yes ^b	<1	<1
I	Yes ^b	<1	<1
J	Yes ^b	2.2 ± 0.2	<1
K	No	<1	<1
L	No	<1	<1
M	No	<1	<1
N	No	<1	<1
O	No	<1	<1
P	No	<1	<1
Q	No	<1	<1
R	No	<1	<1
S	No	<1	<1
T	No	<1	<1

^a Mean ± SE.

^b Almonds were declared on ingredient list as one of the last ingredients.

lots of a chocolate-covered peanut confectionery item (Table 4). These items were not labeled as containing almond. A comparison of the whole ground product and the chocolate coating indicated that almond was present in the chocolate coating. Peanuts alone did not produce a response in the ELISA.

Consistent with the objective of this research, this ELISA successfully detected residues of almond at concentrations pertinent to allergenic concern in foods. The minimum clinically provoking dose of a food allergen is undefined and depends on the allergen in question and the sensitivity of the allergic individual. Hourihane et al. (13) reported minor, subjective allergic symptoms in patients allergic to peanuts who were orally challenged with 100 µg of peanut protein. A casein concentration as low as 0.06% was associated with fatal anaphylaxis after consumption of 100 g of a contaminated sausage (19). Concentrations of 0.14 to 0.16% of undeclared egg ovalbumin in meatballs caused allergic reactions in individuals allergic to eggs, and undeclared concentrations of 0.003 to 0.013% were found in pasta (19). Other published studies of allergen dose-response relationships are summarized by Taylor and Lehrer (29). The ELISA outlined in this study was capable of detecting almond residues at concentrations of at least 1 ppm in food, which is the equivalent of 0.0001%. This ELISA allows the food manufacturer to evaluate finished product and/or components of the product for potentially hazardous almond residues and to assess the adequacy of cleanup processes to remove these residues.

TABLE 4. Almond cross-contact residues in four lots of a retail sample of chocolate-coated peanuts^a

Lot	Almond (ppm)	
	Whole ground sample	Chocolate coating
1	4	5
2	57	93
3	<1	<1
4	<1	<1

^a Data represent the mean of duplicate analyses.

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