Peanut Allergen (Ara h 1) Detection in Foods Containing Chocolate†

A. POMÉS,* R. VINTON, AND M. D. CHAPMAN

INDOOR Biotechnologies, Inc., 1216 Harris Street, Charlottesville, Virginia 22903, USA

ABSTRACT

Inadvertent exposure to peanut in foods poses health risks for peanut-allergic individuals that can be reduced by improving detection systems for allergen contaminants in food products and manufacturing processes. Detection of peanut in chocolate has been especially difficult. We report the optimization of conditions for measuring a major peanut allergen, Ara h 1, in chocolate with the use of a two-site monoclonal antibody sandwich enzyme-linked immunosorbent assay. Ara h 1 was extracted from peanut in the presence or absence of chocolate with phosphate buffer, salt, and three dried milks (goat, soy, or nonfat) (0 to 25% wt/vol) for 15 min at 60°C or for 2.5 h at room temperature. The best conditions for Ara h 1 extraction in the presence of chocolate were 5% nonfat dry milk for 2.5 h at room temperature. Spiking experiments of chocolate with peanut confirmed improvement of the extraction: Ara h 1 was detected in extractions of 0.16 to 0.33% peanut in chocolate. Interestingly, the best conditions for Ara h 1 extraction were different for peanut alone than with chocolate, regarding time, temperature, and percentage of nonfat dry milk in the extraction buffer. In chocolate with peanut foods, the total Ara h 1 values were 10-fold higher than when products were extracted with phosphate buffer alone and could be up to 400-fold higher for individual foods. The dramatic improvement of Ara h 1 extraction should allow specific allergen monitoring in chocolate-containing food products and assessment of Ara h 1 exposure.

Peanut, a common ingredient of food products in the United States, is also one of the main foods associated with allergic reactions that can cause life-threatening anaphylactic responses *(2, 8, 29, 30, 35)*. Peanut allergens are heat resistant and conserved during food production and processing *(1, 5, 20)*. Consumers can be inadvertently exposed to peanut allergens when foods become contaminated from processing lines shared with peanut products. Detection systems for peanut allergens in food products should reduce the risk of undesired exposure. Useful peanut allergen assays should assess threshold levels for sensitization and induction of allergic reactions *(31)*.

Several tests for peanut have been developed that use polyclonal antibodies raised either against peanut extracts or against the peanut protein conarachin A *(9, 13, 16, 24, 34)*. Similar assays are used to measure other allergens in foods, such as hazelnut, almonds, and eggs, with the use of either polyclonal antibodies or human IgE antibodies *(10–14, 17, 21)*. These assays measure "total" peanut components or proteins, or antigens such as conarachin A, but do not measure specific peanut allergens. Peanut components measured by commercial assays are expressed in parts per million (ppm) relative to an arbitrary peanut standard. This makes the results difficult to standardize, and the tests do not provide quantitative measurements of actual allergen exposure.

Seven peanut (*Arachis hypogaea*) allergens have been identified, cloned, and designated by the World Health Organization and International Union of Immunological Societies subcommittee of Allergen Nomenclature: Ara h 1 (vicilin), Ara h 2 (conglutin), Ara h 3 (glycinin), Ara h 4 (glycinin), Ara h 5 (profilin), Ara h 6 (conglutin), and Ara h 7 (conglutin) *(3, 4, 6, 7, 19, 27, 32)*. We recently developed a monoclonal antibody–based enzyme immunoassay (ELISA) to monitor the major peanut allergen Ara h 1 in food products *(26)*. Unlike the other peanut assays, this assay is specific for a major allergen, Ara h 1, and is fully quantitative: results are expressed in absolute units (ng or µg Ara h 1).

The presence of chocolate impairs the detection of proteins in food extracts *(12–14, 16, 18, 21, 25, 28)*. Detection of peanut protein in the presence of chocolate can be improved by purifying the extract with immunoaffinity chromatography *(25)*. This process is cumbersome and not useful for routine screening of peanut products. Some commercial tests for peanut protein recommend using heat (Neogen Corporation, Lansing, Mich.), gelatin (Tepnel, Fluitshire, UK, and r-Biopharm, Inc., Marshall, Mich. *(18)*) or skimmed milk powder (Neogen, Tepnel, and r-Biopharm) to improve extraction efficiency, especially in powders (e.g., cocoa and corn flour) and in high-cocoa chocolate samples. However, the effects of some of these additives on assays for peanut detection have not been published.

Chocolate also impaired detection of Ara h 1 when foods were extracted without additives *(26)*. In the present study, different extraction conditions and additives for extraction of Ara h 1 from peanut, with or without chocolate,

* Author for correspondence. Tel: 434-984-2304; Fax: 434-984-2709; E-mail: apomes@inbio.com.

were compared. The effects of extraction conditions on Ara h 1 were also investigated by spiking chocolate with known amounts of peanut and by comparing Ara h 1 extracted from chocolate food products containing peanut.

MATERIALS AND METHODS

Materials. Oil-roasted Virginia peanuts (Planters Company, East Hanover, N.J.) were used in the study with chocolate. Four different peanut market types were analyzed alone: Runner (Mr. Mac's Peanuts, Eufaula, Ala.), Spanish and Virginia (J.J. Hull's Incredible Edibles, Toledo, Ohio), and Valencia (Sunland, Inc., Portales, N.Mex.). The milk powders for extraction were Carnation Instant Nonfat Dry Milk (Nestlé USA, Inc., Solon, Ohio), Myenberg Instant Powdered Goat Milk (Jackson-Mitchell, Inc., Turlock, Calif.), and Better Than Milk Soy Beverage Mix (Division of Fuller Life, Inc., Maryville, Tenn.). Finished foods containing peanut, chocolate, or both were purchased from local supermarkets or kindly provided by Dr. W. Jeffrey Hurst from Hershey Foods Technical Center, Hershey, Pa. These foods included Hershey's products (Reese's Pieces [peanut butter candy in a crunchy shell], NutRageous [milk chocolate, peanuts, caramel], Reese Sticks [crispy wafers, peanut butter, milk chocolate], Reese's milk chocolate Peanut Butter Cups), M&M's, Snickers, Nestlé Butterfingers, Nestlé Baby Ruth, Brach's Clusters, Brach's Maple Goodies, and Keebler Fudge Sticks. Only Brach's Maple Goodies contain toffee. The five negative controls were the Hershey's products Kit Kat (crisp wafers in chocolate), Krackel (crisped rice in milk chocolate), Nuggets with almonds, Hershey's Milk Chocolate, and Hershey's Kisses.

Ara h 1 extraction from peanut in presence of chocolate. Oil-roasted Virginia peanut (0.5 g) and chocolate (2.5 g of chocolate chips) were ground separately with a food processor followed by mortar and pestle. Ground peanut and chocolate were weighed, combined, and extracted in 50-ml conical tubes with 30 ml of extraction buffer. Although different kinds of chocolate are available in the market, the experiments were performed with semisweet chocolate baking chips containing sugar, chocolate liquor, cocoa butter, butterfat, soy lecithin (as emulsifier), and vanilla (Food Lion, Salisbury, N.C.). Control samples of peanut alone (0.5 g) were extracted under the same conditions. Peanut-chocolate samples were extracted in phosphate-buffered saline, pH 7.5 (PBS), containing 1 M NaCl and 0 to 25% of either nonfat dry milk (NFDM), dry goat milk, or dry soy milk. The nonfat milk had 0 g total fat, the goat milk had 7 g total fat with 4 g saturated fat (per 340 g total milk), and the soy milk contained 2.5 g total fat with 0.2 g polyunsaturated and 1.9 g monounsaturated fat (per 736 g of dry milk). The dry soy milk contained tofu (water, soy solids, calcium sulfate), maltodextrin (from corn), modified food starch, sunflower oil, dicalcium phosphate, natural flavors (no MSG, no dairy), sea salt, anatase titanium dioxide, vegetable mono and diglycerides, vitamin B12, and vitamin E (mixed tocopherols). The extractions were carried out for 15 min at 60°C with continuous mixing or by rocking for 2.5 h at room temperature with mixing every 15 min. The four peanut market types were extracted for 15 min at 60°C (3 g in 30 ml of extraction buffer with 1% NFDM). All samples were allowed to stand for 10 min, then centrifuged at 16,000 × g. The supernatants were collected and stored at −20°C.

Spiking experiments. Spiking experiments with decreasing amounts of peanut in chocolate were performed in order to assess the detection limit of the extraction procedure. Extraction was performed under the best extraction conditions found for peanut

with chocolate, i.e., 2.5 h at room temperature with PBS, 1 M NaCl, and 5% NFDM. Peanut and chocolate chips were ground and weighed separately and combined to a total of 3 g before adding 30 ml of the extraction buffer. Known amounts of peanut (1, 5, 10, 25, and 50 mg) were extracted in the presence of chocolate and assayed for Ara h 1 by ELISA.

Ara h 1 extraction from commercial products containing peanut. Eleven candy samples (foods with chocolate and peanut, including peanut butter fudge sticks, bars, and sweets) were extracted and tested (see product description in "Materials" section). The drier foods were ground easily with only a food processor, but oily products containing more peanut needed the additional use of a mortar and pestle, and this was the case for most of the samples. Five chocolate products that were not supposed to contain peanut from the ingredient declaration and that had nondetectable levels of Ara h 1 in the ELISA were used as negative controls.

The best extraction conditions for Ara h 1 established in the presence of chocolate were applied to the extraction of foods containing chocolate and compared to the best conditions for extraction of peanut alone. The samples (3 g) were extracted with the use of 30 ml PBS with 1 M NaCl containing 1% NFDM at 60°C for 15 min or 5% NFDM at room temperature for 2.5 h. Seven of these samples were also analyzed for comparison by extracting with phosphate buffer only for 2.5 h at room temperature.

Two-site monoclonal antibody ELISA. The assay was performed as previously described (26). Monoclonal anti–Ara h 1 antibodies 2C12 and biotinylated 2F7 were used as capture and detection antibodies, respectively, followed by streptavidin-peroxidase and ABTS [2,2′-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid)] substrate for color development. Plates were read after 10 min at 405 nm with a Microplate scanning spectrophotometer (Power Wave 200, Bio-Tek Instruments, Winooski, Vt.). The assay was quantitated with the use of a standard curve of doubling dilutions of purified Ara h 1, from 0.008 to 4 μg/ml. Values for food samples were interpolated from the linear part of the curve and expressed as ng/ml, μg/ml, ng Ara h 1 per gram of food, or μg Ara h 1 per gram of food. Because the samples were obtained by extraction of 3 g of food in 30 ml of buffer, values can be transformed between units (i.e., ng/ml × 10 = ng/g of food product).

RESULTS

Optimization of extraction conditions for peanut with and without chocolate. Peanut was extracted alone or mixed with ground chocolate chips. Table 1 shows the Ara h 1 levels in peanut samples extracted with and without chocolate and with different milk concentrations (0, 1, 2.5, and 5%) at room temperature for 2.5 h or at 60°C for 15 min. The best extraction conditions for peanut alone were 1% NFDM at 60°C for 15 min. Because increasing concentrations of NFDM or goat milk (10, 15, 20, and 25%) did not improve Ara h 1 extraction (data not shown), we continued the study using 0 to 5% milk. Ara h 1 recovery from peanut extraction at room temperature increased from 124 to 334 μg/ml by adding 1% NFDM. At 60°C, the yield of Ara h 1 was significantly higher in PBS with 1 M NaCl alone (381 μg/ml) and was further increased by addition of 1 to 5% NFDM (Table 1).

Ara h 1 was not detectable when peanut was extracted with chocolate in PBS with 1 M NaCl at either room tem-

TABLE 1. *Effect of temperature and milk protein on extraction of Ara h 1 from peanut or peanut with chocolate[a]*

| | Ara h 1 (µg/ml) | | | | | |
| | Room temperature, 2.5 h | | | 60°C, 15 min | | |
% dry milk	Nonfat dry milk	Goat milk	Soy milk	Nonfat dry milk	Goat milk	Soy milk
Peanut samples						
0	124	126	102	381	168	260
1	334	246	125	662	403	165
2.5	293	191	109	421	227	145
5	261	178	168	424	298	253
Peanut with chocolate samples						
0	<0.03	0.1	<0.03	<0.03	<0.03	<0.03
1	0.7	1.0	<0.03	0.04	0.3	<0.03
2.5	3.8	1.9	<0.03	0.9	1.0	<0.03
5	9.7	4.1	<0.03	4.5	2.6	<0.03

[a] Ara h 1 extracted from 0.5 g of peanut alone or from 0.5 g of peanut mixed with 2.5 g of chocolate in PBS with 1 M NaCl containing NFDM, goat milk, or soy milk. The inter- and intra-assay variations are 11.8 and 3.1%, respectively *(26)*.

perature or 60°C. The maximal recovery of Ara h 1 from peanut with chocolate (9.7 µg/ml) was obtained with 5% NFDM at room temperature for 2.5 h (Table 1). This represents a 3.7% recovery for chocolate and peanut versus peanut alone. However, because the extraction efficiency can be influenced by the size of the sample (and there was 0.5 g of peanut alone and 3 g of peanut plus chocolate),

Extraction of peanut at 60°C for 15 minutes

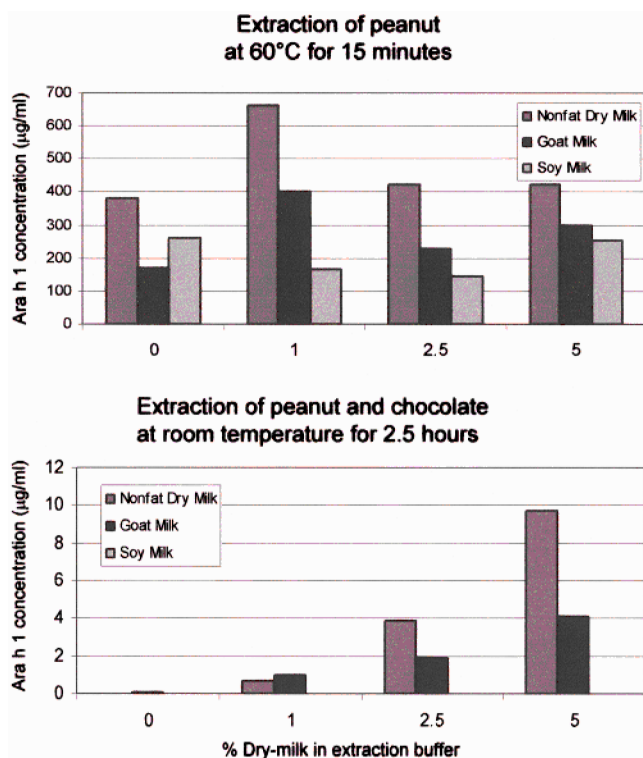**Extraction of peanut and chocolate at room temperature for 2.5 hours**

FIGURE 1. *(Top panel) Ara h 1 levels in peanut (0.5 g) extracted at 60°C for 15 min using phosphate buffer with 1 M NaCl and three kinds of dry milk at different concentrations. (Bottom panel) Ara h 1 levels in peanut (0.5 g) extracted in the presence of chocolate (2.5 g) at room temperature for 2.5 h using phosphate buffer with 1 M NaCl and three kinds of dry milk at different concentrations.*

this comparison might not be the ideal way to give a percentage of recovery. At this milk percentage, the Ara h 1 levels obtained when using NFDM were higher than when using goat milk. Ara h 1 was not detected when extracted with soy milk, which is not a real milk but was chosen for testing as a different source of a mixture of proteins, lipids, and carbohydrates. Addition of 2% Tween-20 to PBS with 1 M NaCl increased Ara h 1 recovery, but no advantage was found in adding 2% Tween-20 to the NFDM-containing buffer (data not shown) *(26)*. Increasing concentrations of NFDM or goat milk (10, 15, 20, and 25%) did not significantly improve Ara h 1 extraction. Considering that the use of lower amounts of milk is preferable and that optimal conditions for extraction of peanut alone included the use of 1% NFDM, we focused the study of extraction of peanut with chocolate using 0 to 5% milk. The results show that use of 5% NFDM greatly improved the recovery of Ara h 1 from peanut plus chocolate, although the levels of Ara h 1 recovered were much lower than when the same amount of peanut was extracted alone, without chocolate (Table 1). Figure 1 represents the Ara h 1 yield under the best conditions tested for peanut alone (top panel) or for peanut with chocolate (bottom panel).

In order to assess the sensitivity of the Ara h 1 ELISA for detection of peanut in the presence of chocolate, spiking experiments were performed. Decreasing amounts of peanut (50, 25, 10, 5, 1 mg) were extracted with chocolate in a total sample weight of 3 g with 5% NFDM at room temperature for 15 min. Ara h 1 was detected in samples spiked with 5 to 10 mg of peanut (Table 2), which indicates a detection limit between 0.16 and 0.33% of peanut in the presence of chocolate. This is a significant improvement considering that Ara h 1 had been undetectable when peanut was extracted in the presence of chocolate with PBS for 2.5 h at room temperature (2 g of peanut with 1 g of chocolate: 66% peanut) or in PBS with 1 M NaCl (0.5 g of peanut with 2.5 g of chocolate: 17% peanut).

Ara h 1 in chocolate food products. Ara h 1 levels were compared in chocolate products containing peanut,

TABLE 2. *Spiking experiment of peanut in chocolate*

Peanut (mg)[a]	O.D. 405 nm (extract diluted 1:2)
0 (background)	0.060 ± 0.003
1	0.067
5	0.094
10	0.152
25	0.241
50	0.450

[a] Peanut in chocolate with a total sample weight of 3 g.

FIGURE 2. *Ara h 1 levels in chocolate products extracted under two different conditions: 1% NFDM (60°C) or 5% NFDM (room temperature). Each symbol represents one of the samples extracted and analyzed for Ara h 1 levels. The limit of detection of the ELISA is 30 ng/ml (indicated by a dashed horizontal line). Eleven samples of chocolate plus peanut are above the detection limit, whereas the five negative controls (chocolate products without peanut) are below it. The number of samples studied under each condition is indicated in parentheses under the x-axis. Geometric mean (thicker horizontal line) values and 95% confidence intervals are shown for each column in black.*

and Ara h 1 was detected in all cases with the use of either 5% NFDM at room temperature or 1% NFDM at 60°C (Fig. 2). There was no significant difference between these conditions: total Ara h 1 extracted with 5% NFDM at room temperature and with 1% NFDM at 60°C was 1,361 µg/ml (goat milk, 38 µg/ml) and 1,617 µg/ml (goat milk, 36 µg/ml), respectively (Fig. 2). However, 5% NFDM at room temperature would be recommended for logistical purposes. The total Ara h 1 detected in seven samples extracted with the use of 1 or 5% NFDM was 10-fold higher than when products were extracted with PBS alone. Ara h 1 values were up to 400-fold higher than when extracted with PBS alone for individual foods (data not shown). Ara h 1 was not detected in the five chocolate products used as negative controls.

DISCUSSION

The conditions for extracting Ara h 1 from peanut in the presence of chocolate were optimized, and Ara h 1 was detected in all the chocolate foods containing peanut that were tested. The results suggest that the optimal extraction procedure for Ara h 1 in chocolate is 5% NFDM for 2.5 h at room temperature. Spiking experiments show that under these conditions, Ara h 1 could be detected in chocolate with 0.16 to 0.33% peanut. This percentage represents a significant improvement compared with extraction with phosphate buffer alone or with 1 M NaCl, although future studies will need to elucidate whether this detection limit is enough to measure the smallest Ara h 1 levels that can produce allergic reactions. No significant differences were found between the amounts of Ara h 1 extracted from chocolate products under the best two conditions, and small differences could be explained by (i) a nonhomogeneous distribution of peanut pieces in the food, (ii) variability in mixing of samples during extraction, or (iii) variability in composition of the product. Overall, the logistics of incubating a large number of samples at 60°C might not be feasible for most laboratories; thus, the recommended extraction conditions for Ara h 1 from chocolate products would be to use 5% NFDM for 2.5 h at room temperature. If time is an issue, the 2.5 h can be shortened to 15 min by extracting at 60°C.

Given the wide variety of types of peanut used in the food industry, the Ara h 1 content was analyzed in four different peanut market types: Runner, Spanish, Valencia, and Virginia. Ara h 1 was measured in the range 98 to 265
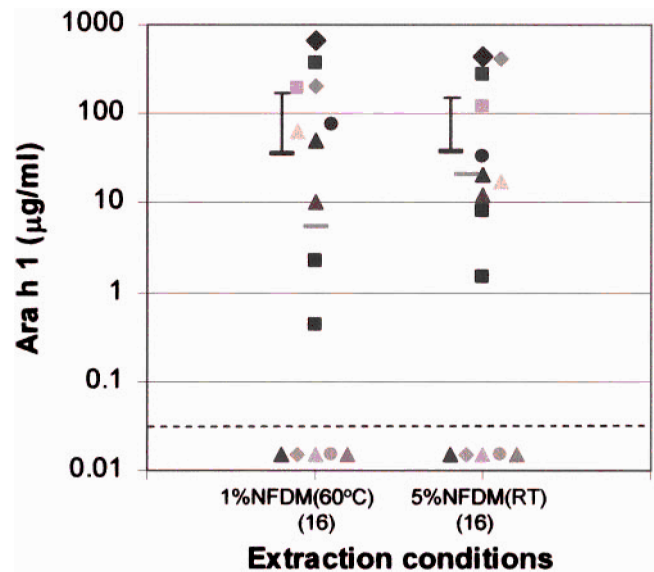
µg/ml for raw peanuts (data not shown). Therefore, differences in Ara h 1 content greater than threefold are not expected with the use of different market types. The Ara h 1 levels in extracts from raw and roasted peanuts from these four market types were also compared. ELISA curves were parallel, indicating equivalent antibody binding between native and heat-processed Ara h 1. Interestingly, preliminary experiments showed that the content of Ara h 1 from roasted peanut extracts was fivefold greater on average than the content from raw peanut extracts (data not shown). This result suggests that the epitopes recognized by the monoclonal antibodies used in the ELISA became more accessible in the roasted than in the raw allergen or that a difference in Ara h 1 extraction efficiency exists. A similar increase has been reported for binding of IgE to Ara h 1 from roasted versus raw peanuts *(23)*.

The yield of the Ara h 1 extraction depends on the allergen binding properties of the components of the matrix combined with peanut. It is well known that chocolate is a difficult matrix for the extraction of proteins because of its high content of tannins and other phenolic compounds that have a high binding affinity to proteins *(18)*. Tannins can bind proteins during extraction and can also interfere with the ELISA test by binding to the antibody. Addition of fish gelatin to the extraction buffer has been used to bind tannins *(18)*. Similarly, the effect of NFDM could be to bind the tannins in chocolate that sequester Ara h 1. Other matrices, such as cookie or pancake mix, bind Ara h 1 in different degrees *(26)*. Therefore, accurate allergen quanti-

fication will depend on standards prepared with the use of similar ingredients to those present in the food product, although the high variability in food components makes this an impracticable effort.

The Ara h 1 monoclonal antibody assay combined with other assays for major peanut allergens should provide complementary information for risk assessment of allergen exposure. Monoclonal antibodies against Ara h 2 have been produced and are being used to develop a new peanut allergen test that quantifies allergen in absolute units. These specific allergen assays will be useful for (i) investigating threshold doses of specific allergen exposure in allergic patients and (ii) risk assessment of inadvertent exposure of allergic patients to foods, either as a result of allergen contamination of foods or mislabeling of food products. The threshold doses for allergenic foods are difficult to investigate, and assays with good detection limits should be used to assess the threshold levels *(31)*. Hourihane et al. *(15)* reported limits of 100 μg and 2 mg of peanut protein for the first subjective and first objective allergic reactions, respectively. However, the limits of protein content for safe foods detected with the use of polyclonal antibody immunoassays cannot be directly compared with the limits of specific peanut allergens measured with the use of monoclonal antibody assays. The amount of a specific allergen in foods is expected to be smaller than the amount of peanut components, because Ara h 1 and Ara h 2 constitute 12 to 16% and 6 to 9% of total peanut protein, respectively *(22)*. The existence of complementary assays that measure peanut allergens such as Ara h 1 and Ara h 2, in combination with assays that measure peanut components, will allow studies on threshold levels of allergen exposure, as well as improvement in food labeling if the right detection limit of allergen level for allergic reactions is achieved. Warning labels should be explicit enough to prevent exposure of peanut-allergic people to undesired allergens *(33)*. The Ara h 1 test, alone or combined with a test for Ara h 2, will be a useful tool in the study of thresholds of allergen exposure for peanut hypersensitivity.

ACKNOWLEDGMENTS

REFERENCES

1. Beyer, K., E. Morrow, X. M. Li, L. Bardina, G. A. Bannon, A. W. Burks, and H. A. Sampson. 2001. Effects of cooking methods on peanut allergenicity. *J. Allergy Clin. Immunol.* 107:1077–1081.

2. Bock, S. A., A. Munoz-Furlong, and H. A. Sampson. 2001. Fatalities due to anaphylactic reactions to foods. *J. Allergy Clin. Immunol.* 107:191–193.

3. Burks, A. W., L. W. Williams, C. Connaughton, G. Cockrell, T. J. O'Brien, and R. M. Helm. 1992. Identification and characterization of a second major peanut allergen, Ara h II, with use of the sera of patients with atopic dermatitis and positive peanut challenge. *J. Allergy Clin. Immunol.* 90:962–969.

4. Burks, A. W., L. W. Williams, R. M. Helm, C. Connaughton, G. Cockrell, and T. O'Brien. 1991. Identification of a major peanut al-

lergen, Ara h I, in patients with atopic dermatitis and positive peanut challenges. *J. Allergy Clin. Immunol.* 88:172–179.

5. Burks, A. W., L. W. Williams, W. Thresher, C. Connaughton, G. Cockrell, and R. M. Helm. 1992. Allergenicity of peanut and soybean extracts altered by chemical or thermal denaturation in patients with atopic dermatitis and positive food challenges. *J. Allergy Clin. Immunol.* 90:889–897.

6. Clarke, M. C., S. A. Kilburn, J. O. Hourihane, K. R. Dean, J. O. Warner, and T. P. Dean. 1998. Serological characteristics of peanut allergy. *Clin. Exp. Allergy* 28:1251–1257.

7. de Jong, E. C., M. Van Zijverden, S. Spanhaak, S. J. Koppelman, H. Pellegrom, and A. H. Penninks. 1998. Identification and partial characterization of multiple major allergens in peanut proteins. *Clin. Exp. Allergy* 28:743–751.

8. Emmett, S. E. F., J. Angus, J. S. Fry, and P. N. Lee. 1999. Perceived prevalence of peanut allergy in Great Britain and its association with other atopic conditions and with peanut allergy in other household members. *Allergy* 54:380–385.

9. Hefle, S. L., R. K. Bush, J. W. Yunginger, and F. S. Chu. 1994. A sandwich enzyme-linked-immunosorbent-assay (ELISA) for the quantitation of selected peanut proteins in foods. *J. Food Prot.* 57:419–423.

10. Hefle, S. L., E. Jeanniton, and S. L. Taylor. 2001. Development of a sandwich enzyme-linked immunosorbent assay for the detection of egg residues in processed foods. *J. Food Prot.* 64:1812–1816.

11. Hlywka, J. J., S. L. Hefle, and S. L. Taylor. 2000. A sandwich enzyme-linked immunosorbent assay for the detection of almonds in foods. *J. Food Prot.* 63:252–257.

12. Holzhauser, T., S. Oliver, and S. Vieths. 2002. Detection of potentially allergenic hazelnut (*Corylus avellana*) residues in food: a comparative study with DNA PCR-ELISA and protein sandwich-ELISA. *J. Agric. Food Chem.* 50:5808–5815.

13. Holzhauser, T., and S. Vieths. 1999. Indirect competitive ELISA for determination of traces of peanut (*Arachis hypogaea* L.) protein in complex food matrices. *J. Agric. Food Chem.* 47:603–611.

14. Holzhauser, T., and S. Vieths. 1999. Quantitative sandwich ELISA for determination of traces of hazelnut (*Corylus avellana*) protein in complex food matrixes. *J. Agric. Food Chem.* 47:4209–4218.

15. Hourihane, J., S. A. Kilburn, J. A. Nordlee, S. L. Hefle, S. L. Taylor, and J. O. Warner. 1997. An evaluation of the sensitivity of subjects with peanut allergy to very low doses of peanut protein: a randomized, double blind, placebo-controlled food challenge study. *J. Allergy Clin. Immunol.* 100:596–600.

16. Hurst, W. J., E. R. Krout, and W. R. Burks. 2002. A comparison of commercially available peanut Elisa test kits on the analysis of samples of dark and milk chocolate. *J. Immunoassay Immunochem.* 23:451–459.

17. Keating, M. U., R. T. Jones, N. J. Worley, C. A. Shively, and J. W. Yunginger. 1990. Immunoassay of peanut allergens in food-processing materials and finished foods. *J. Allergy Clin. Immunol.* 86:41–44.

18. Keck-Gassenmeier, B., S. Bénet, C. Rosa, and C. Hischenhuber. 1999. Determination of peanut traces in food by a commercially-available ELISA test. *Food Agric. Immunol.* 11:243–250.

19. Kleber-Janke, T., R. Crameri, U. Appenzeller, M. Schlaak, and W. M. Becker. 1999. Selective cloning of peanut allergens, including profilin and 2S albumins, by phage display technology. *Int. Arch. Allergy Immunol.* 119:265–274.

20. Koppelman, S. J., C. A. Bruijnzeel-Koomen, M. Hessing, and H. H. de Jongh. 1999. Heat-induced conformational changes of Ara h 1, a major peanut allergen, do not affect its allergenic properties. *J. Biol. Chem.* 274:4770–4777.

21. Koppelman, S. J., A. C. Knulst, W. J. Koers, A. H. Penninks, H. Peppelman, R. Vlooswijk, I. Pigmans, G. van Duijn, and M. Hessing. 1999. Comparison of different immunochemical methods for the detection and quantification of hazelnut proteins in food products. *J. Immunol. Methods* 229:107–120.

22. Koppelman, S. J., R. A. Vlooswijk, L. M. Knippels, M. Hessing, E. F. Knol, F. C. van Reijsen, and C. A. Bruijnzeel-Koomen. 2001. Quantification of major peanut allergens Ara h 1 and Ara h 2 in the

peanut varieties Runner, Spanish, Virginia, and Valencia, bred in different parts of the world. *Allergy* 56:132–137.

23. Maleki, S. J., S. Y. Chung, E. T. Champagne, and J. P. Raufman. 2000. The effects of roasting on the allergenic properties of peanut proteins. *J. Allergy Clin. Immunol.* 106:763–768.

24. Mills, E. N. C., A. Potts, G. W. Plumb, N. Lambert, and M. R. A. Morgan. 1997. Development of a rapid dipstick immunoassay for the detection of peanut contamination of food. *Food Agric. Immunol.* 9:37–50.

25. Newsome, W. H., and M. Abbott. 1999. An immunoaffinity column for the determination of peanut protein in chocolate. *J. AOAC Int.* 82:666–668.

26. Pomés, A., R. M. Helm, G. A. Bannon, A. W. Burks, A. Tsay, and M. D. Chapman. 2003. Monitoring peanut allergen in food products by measuring Ara h 1. *J. Allergy Clin. Immunol.* 111:640–645.

27. Rabjohn, P., E. M. Helm, J. S. Stanley, C. M. West, H. A. Sampson, A. W. Burks, and G. A. Bannon. 1999. Molecular cloning and epitope analysis of the peanut allergen Ara h 3. *J. Clin. Invest.* 103: 535–542.

28. Roux, K. H., S. S. Teuber, J. M. Robotham, and S. K. Sathe. 2001. Detection and stability of the major almond allergen in foods. *J. Agric. Food Chem.* 49:2131–2136.

29. Sicherer, S. H., A. Munoz-Furlong, A. W. Burks, and H. A. Sampson. 1999. Prevalence of peanut and tree nut allergy in the US determined by a random digit dial telephone survey. *J. Allergy Clin. Immunol.* 103:559–562.

30. Skolnick, H. S., M. K. Conover-Walker, C. B. Koerner, H. A. Sampson, W. Burks, and R. A. Wood. 2001. The natural history of peanut allergy. *J. Allergy Clin. Immunol.* 107:367–374.

31. Taylor, S. L., S. L. Hefle, C. Bindslev-Jensen, S. A. Bock, A. W. Burks, Jr., L. Christie, D. J. Hill, A. Host, J. O. Hourihane, G. Lack, D. D. Metcalfe, D. A. Moneret-Vautrin, P. A. Vadas, F. Rance, D. J. Skrypec, T. A. Trautman, I. M. Yman, and R. S. Zeiger. 2002. Factors affecting the determination of threshold doses for allergenic foods: how much is too much? *J. Allergy Clin. Immunol.* 109:24–30.

32. Viquez, O. M., C. G. Summer, and H. W. Dodo. 2001. Isolation and molecular characterization of the first genomic clone of a major peanut allergen, Ara h 2. *J. Allergy Clin. Immunol.* 107:713–717.

33. Wood, R. A. 2002. Food manufacturing and the allergic consumer: accidents waiting to happen. *J. Allergy Clin. Immunol.* 109:920–922.

34. Yeung, J. M., and P. G. Collins. 1996. Enzyme immunoassay for determination of peanut proteins in food products. *JAOAC Int.* 79: 1411–1416.

35. Yunginger, J. W., K. G. Sweeney, W. Q. Sturner, L. A. Giannandrea, J. D. Teigland, M. Bray, P. A. Benson, J. A. York, L. Biedrzycki, and D. L. Squillace. 1988. Fatal food-induced anaphylaxis. *JAMA* 260:1450–1452.