

Can Commercial Peanut Assay Kits detect Peanut Allergens?

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Mara C.L. Nogueira¹, Regina McDonald¹, Carmen Westphal^{1,2}, Soheila J. Maleki³ and Jupiter M. Yeung^{1*},

¹ National Food Processors Association, 1350 I Street, N.W.

Washington, DC 20005, U.S.A.

² Current address: Food and Drug Administration, Center for Food Safety and Applied Nutrition,

8301 Muirkirk Rd., Laurel, MD 20708, U.S.A.

³ United States Department of Agriculture, Agricultural Research Service,

1100 Robert E. Lee Blvd., New Orleans, LA 70124, U.S.A.

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***Corresponding Address:**

Jupiter Yeung, Ph.D.

National Food Processors Association

1350 I Street, N.W., Suite 300
Washington, DC 20005-3305
Tel: (202) 639-5961
Fax: (202) 639-5991
Email: jyeung@nfpa-food.org

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Abstract:

Peanut is the food group mostly associated with severe and fatal allergic reactions. In the United States, more than 90% of peanut allergic individuals' serum IgE recognized peanut proteins Ara *h* 1 and Ara *h* 2, therefore establishing these proteins as major peanut allergens. The amount of Ara *h* 1 and Ara *h* 2 in three varieties of peanut cultivars that are commonly processed in the industrialized countries was determined to be 12-16% and 6-9 % respectively. Current commercial peanut test kits use polyclonal peanut-specific antibodies to detect soluble or buffer extractable peanut proteins. Since the two major peanut allergens Ara *h* 1 and Ara *h* 2 are isolated from soluble peanut proteins, it is generally assumed these commercial kits can detect peanut allergens, although none of these kits claim to detect peanut allergen. This study showed for the first time that the peanut test kits could in fact detect

major peanut allergens Ara *h* 1 and Ara *h* 2, in both native or heat denatured structures, therefore these kits qualified to be classified as peanut allergen ELISA.

INTRODUCTION

Peanut is the food most associated with severe and fatal allergic reactions, although the reasons for this dominance are still not clear (1). In recent years, the protein allergens in peanuts have been identified, isolated and cloned (2, 3, 4, 5). In the United States, more than 90% of peanut allergic individuals' serum IgE recognized and reacted to peanut proteins Ara *h* 1 and Ara *h* 2, therefore establishing Ara *h* 1 and Ara *h* 2 as major peanut allergens (2, 3). However, in three different European populations, Ara *h* 1 was recognized by lower percentages of patients (35%, 65% and 70%), as described by Kleber-Janke and colleagues (4) Clarke et al., (6) and de Jong et al. (7).

The allergenic nature, protein composition and protein contents of Ara *h* 1 and Ara *h* 2 in three varieties of peanut cultivars (Virginia, Valencia and Spanish) that are commonly processed in the western industrialized countries were studied by Koppelman and colleagues (8). Although in the United States Runner peanut is a common variety in the market place, it is a hybrid of Virginia and Spanish cultivars, and therefore, it was not included in the study. This study showed total protein contents to be between 24 and 29%, and the extractable proteins between 18 and 26% by the Kjeldahl method. The amount of Ara *h* 1 was determined to be 12-16%, while Ara *h* 2 varied between 6 and 9 %. These investigators also reported that the levels of Ara *h* 1 and Ara *h* 2 were not significantly different between the tested varieties, and therefore the serological differences among peanut-allergic patients from

different parts of the world could not be explained by the allergen composition of different peanuts.

Ara *h* 1 is a glycoprotein, and belongs to the vicilin seed storage protein family (9). Purified Ara *h* 1 is a 63.5 kD glycoprotein, that is assembled in di- and trimeric complexes with an isoelectric point (pI) of 4.55 (2, 9). It has distinct heat stable IgE-binding sites (10), both on the protein and carbohydrate moieties (1,9,11). Ara *h* 2 is also a glycoprotein, but belongs to the conglutin family (6). Purified Ara *h* 2 migrates as a doublet at 17 kD on a reducing gel during sodium dodecyl sulfate- polyacrylamide gel electrophoresis (SDS-PAGE) with pI of 5.2. Ara *h* 2 contains ten IgE epitopes recognized by sera from a peanut sensitive population (3,7).

In the mid-1990s, sensitive commercial food allergen test kits emerged in the marketplace offering laboratories the opportunity to analyze peanut proteins in processed food products or in samples taken from various locations in a processing facility. Currently, there are four commercial enzyme-linked immunosorbent assays (ELISA) for rapid and highly specific detection of peanut proteins. In May 2003, the AOAC International completed the multi-laboratory performance validation of the commercially available peanut protein test kits. This performance validation provides the regulatory agencies an interim analytical method that could be useful for regulatory enforcement and to monitor for the presence of peanut proteins (allergens) in multiple food matrices.

These commercial peanut test kits use polyclonal peanut-specific antibodies to detect soluble or buffer extractable peanut proteins. An allergen is a protein, such as Ara *h* 1 or Ara *h* 2, that can induce an allergic reaction in sensitive individuals. None of these kits claim to detect peanut allergen per sé. Since the two major peanut allergens Ara *h* 1 and Ara *h* 2 are isolated from soluble peanut proteins, it is generally assumed these commercial kits can detect peanut allergens. The aim of our study was to determine if the peanut test kits can in fact detect major peanut allergens Ara *h* 1 and Ara *h* 2, and whether the kits detect Ara *h* 1 and Ara *h* 2 derived from raw, light and dark roasted peanuts equally well.

EXPERIMENTAL

Materials and Reagents

Commercial defatted raw, light roasted, and dark roasted peanut flours were used for the purification of Ara *h* 1 and Ara *h* 2 proteins according to Burks et al. (2,3). Purities of Ara *h* 1 and Ara *h* 2 were estimated to be 85-95% based on the SDS PAGE using the manufacturer's procedure for NuPAGE[®] MES SDS in a 12% Bis-Tris gel (Invitrogen, Carlsbad, CA). Results were visualized by colloidal Coomassie brilliant blue staining. Batches of purified proteins were pooled and protein concentrations were expressed as an

average of results obtained by photometric methods of O.D. at 280 nm and Beer's Law, Waddel's method of O.D. at 215 and 225 nm, the Bradford method (Bio-Rad, CA) and Pierce BCA (bicinchoninic acid) protein assay (Pierce, Rockford, IL). Each sample was diluted fresh to 0.1 µg/µL using the specific kit extraction buffer, and serial dilutions were made so that the final concentrations were 0.1, 0.01, 0.001, 0.0001 and 0.00001 µg/µL for analysis.

Commercial Peanut Test Kits

Four peanut protein kits were used for this study: the Veratox[®] Peanut #8430, (Neogen Corporation, Lansing, MI, <http://www.neogen.com/>), Ridascreen[®] Peanut #R6201 (R-Biopharm AG, Darmstadtand, Germany, <http://www.r-biopharm.com/>), ELISA-Tek[™] #510831 (ELISA Technologies, Gainesville, FL, <http://www.elisa-tek.com>, same as BioKits peanut test kits, Tepnel Life Sciences, Manchester, England, <http://www.tepnel.com/>), and Prolisa[™] #PLF.9400 (Pro-Lab Diagnostics, Richmond Hill, Ontario, Canada, <http://www.pro-lab.com/>). All kits are sandwich ELISA systems that use polyclonal antibodies specific for soluble peanut proteins.

The limits of detection (LOD) for each kit ranged from as low as 0.4 ppm to 2.5 ppm. Each assay was performed according to the manufacturer's instructions at the time of this study. Sample dilutions were incubated in the peanut antibody-coated wells in either

singles or duplicates according to the sample layout protocol. Samples were washed to remove any unbound material. After the addition of an antibody-enzyme conjugate and further incubation, any excess unbound complexes were removed through washing. With the addition of a substrate, the bound enzyme-conjugate yielded a visual color change that was proportional to the amount of Ara *h* 1 and Ara *h* 2 present in the extracts.

The Veratox[®] and Ridascreen[®] test kits come with their own software to calculate the peanut concentrations in sample extracts. The ELISA-Tek[™] and Prolisa[™] test kits do not provide any software, so an alternative curve-fitting program (GraphPad Prism Software, San Diego, CA) was used to calculate the peanut concentrations in the samples analyzed by these kits.

Sample Dilutions

The VERATOX[®] test kit is the only one that did not require any further dilutions of the samples before addition to the microtiter wells. The ELISA-Tek[™] required a 1:20 dilution (100 μ L + 1.9 ml of sample diluent), the ELISA-Tek[™] required a 1:5 dilution (100 μ L + 400 μ L of extraction buffer) and the Prolisa[™] required a 1:4 dilution (125 μ L + 375 μ L of sample diluent). The dilution factors were already included in the Veratox[®] and Ridascreen[®] software. For the quantification of peanut proteins in the

samples analyzed by the Prolisa™ test kit, the dilution factor was taken into consideration when running the GraphPad Prism software. Results were expressed in ppm of peanuts for ELISA-Tek™, Ridascreen® and Veratox® kits, and ppm peanut proteins for Prolisa™, according to manufacturer's instruction.

Instrumentation

The optical densities (OD) of the microtiter wells were measured using the Dynex MRX Revelation plate reader (Dynex Technologies, Inc., Chantilly, VA, with Revelation software) with the sample filter set at 450 nm for the ELISA-Tek™, Ridascreen® and Prolisa™ kits and 650 nm for the Veratox® test kit. The instrument was checked monthly for calibration to ensure the reader continued to meet specifications.

RESULTS AND DISCUSSION

The yield of proteins extracted from raw peanuts was always higher than from the roasted peanuts. Concentrations of purified Ara *h* 1 and Ara *h* 2 ranged from 2.55-0.30 µg/µl, depending on the batch and degree of roasting. The darker the roasting, the less

protein could be extracted. The difference in yields was not surprising, since it is well known that most proteins, when subjected to thermal treatment, undergo conformational changes at all levels of its structure. Hydrophobic groups that are originally oriented inward could turn outward, become exposed to unfavorable interactions with an aqueous solvent, and consequently making the protein less soluble in water-based extraction buffers (12). Koppelman et al. (10) showed that Ara *h* 1 from peanuts that were heat-treated prior to purification exhibited a denatured state and a decrease in solubility. Although Ara *h* 1 adopted a different conformation upon heat denaturation, it was heat-stable, and its IgE-binding affinity was not affected. These findings are consistent with those reported by Nordlee et al. (12) and Beyer et al. (13).

Even though protein concentrations were determined according to Beer's law using O.D. 280 and the extinctions coefficient, BioRad protein determination assay, BCA , and Waddell method using O.D. 215 and 225, accurate protein concentrations were not obtained for the roasted peanut samples. We believe this is due to the fact that the biochemical modifications that occur during roasting alter UV and visible absorbance values of the roasted proteins. The only method to determine the concentration of the roasted proteins accurately is to visually adjust their concentrations using SDS-PAGE followed by Coomassie blue dye (Fig. 1) or Sypro (Molecular Probes, Eugene, OR) staining which is protein content independent. The roasted Ara *h* 2 proteins are more soluble than the roasted Ara *h* 1 proteins. Since the Ara *h* 1 purified from dark roast peanuts can only be concentrated to a certain degree

before it began to fall out of solution, what was loaded and observed on the gel was the most concentrated sample obtained and utilized in our study. Here we are comparing the recognition of the different proteins utilizing different kits and our primary concern was to utilize the same amount of each protein (i.e. dark roasted Ara h 1) in each assay.

It is always debatable whether peanut protein or allergen test kits can in fact detect peanut allergens. It is originally assumed that if peanut proteins are detected, peanut allergens should be there. Although this assumption is obvious, since Ara h 1 and Ara h 2 are purified from peanut extracts, hard data was lacking. For the first time, we showed data that all the four commercial peanut test kits could detect Ara h 1 and Ara h 2, as shown in Table 1. Among the four test kits examined in this study, the ELISA-Tek™ and Prolisa™ were more sensitive in detecting Ara h 1 derived from raw peanuts, followed by the Ridascreen® and Veratox® test kits (ELISA-Tek™ = Prolisa™ > Ridascreen® > Veratox®). However, the Prolisa™ test kit was more sensitive to detect Ara h 2 derived from raw peanuts, followed by ELISA-Tek™, Veratox® and Ridascreen® (Prolisa™ > ELISA-Tek™ = Veratox® > Ridascreen®). The levels of Ara h 1 and Ara h 2 detected and reported for each kit in Table 1 were not critically important or even comparable, since the reporting units used by different manufacturers were different, e.g. ppm of peanuts or ppm of peanut proteins. To further complicate the issue, the peanut standards or calibrators used in these kits were all different, so data obtained could not be correlated. This is consistent with the analytical uncertainties observed by Pomés et al (14).

Although the same amounts of Ara *h* 1 and Ara *h* 2 derived from raw, light roasted and dark roasted peanuts were used to evaluate the sensitivity or binding efficiency of the test kits, it was anticipated the reactivity of the IgG antibodies in the kit might not respond the same to the allergens, since Maleki et al. reported roasting increased IgE binding to both Ara *h* 1 (15) and Ara *h* 2 (16). In Table 2, ELISA-Tek™ showed equal sensitivity to Ara *h* 1 derived from light and dark roasted peanut, but was more sensitive to Ara *h* 1 from raw peanuts (Table 1). The Veratox® test kit was equally sensitive to Ara *h* 1 derived from raw, light roasted and dark roasted peanuts, while the Ridascreen® was more sensitive to Ara *h* 1 from light roasted peanuts, followed by raw Ara *h* 1 from raw and dark roasted Ara *h* 1. Although Prolisa™ test kit appeared to be most sensitive to Ara *h* 1 and Ara *h* 2 from raw peanuts, Table 1, no further comparison on sensitivity to roasted peanut allergens were done, since Pro-Lab Diagnostics was not included in the 2003 AOAC International performance certification study.

The ELISA-Tek™ and Veratox® were equally sensitive to Ara *h* 2 derived from raw (Table 1), light roasted and dark roasted peanuts (Table 3). The Ridascreen® test kit was equally sensitive to Ara *h* 2 from light and dark peanuts (Table 3), and more sensitive to these than to Ara *h* 2 derived from raw peanuts (Table 1).

In parallel studies, Nordlee et al. (13) and Maleki et al. (15) observed that roasted peanut proteins bind to IgE at higher levels than raw peanuts, and that the IgE-recognition sites in roasted peanuts were different from those in raw peanuts. While Nordlee et al. (13) speculated that heat treatment increased allergenicity of peanut proteins by increasing the availability of allergenic binding sites, Maleki et al. (15,16) demonstrated covalent modification of the proteins during the roasting process created novel IgE epitopes. Both Maleki and Beyer observed increased IgE binding, that does not necessarily mean that there should be enhanced rabbit IgG binding, which is what the test kits antibodies were made of. Our findings demonstrated that the polyclonal anti-peanut antibodies used in the kits do not follow the same affinity pattern of human IgE.

Conclusion

This study demonstrated that all four peanut assay kits were able to recognize a wide variety of soluble peanut proteins, including Ara *h* 1 and Ara *h* 2 from raw peanuts, irrespective of their native, or heat altered structures as observed by three of four test kits evaluated in this study. Hence, the peanut-ELISA kits belong to a class-specific immunoassay for soluble peanut proteins, analogous to a class-specific thiabendazole fungicides ELISA (17), and they also qualify to be classified as peanut allergen ELISA.

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Table 1: Detection of Ara h 1 and Ara h 2 proteins extracted from raw peanut using four different test kits.

Concentration (µg/µl)		0.1	0.01	0.001	0.0001	
0.00001						
Test kit						
Ara h 1	1	NA	NA	OVER	1.2	ND
	2	NA	OVER	10.2	ND	ND
	3	NA	7.1	ND	ND	ND
	4	NA	NA	20.0	4.3	ND
Ara h 2	1	NA	1.6	ND	ND	NA
	2	9.9	ND	ND	NA	NA
	3	NA	9.1	ND	NA	NA
	4	NA	21.8	8.8	ND	ND

All data expressed in ppm of peanuts, except for test kit 4, which data is expressed in ppm of peanut protein

ND = not detected, NA = not assayed, Over = data is over the highest calibrator

1 = BioKits, Tepnel BioSystems or Elisa Technologies

2 = Ridascreen, R-Biopharm

3 = Veratox, Neogen Corporation

4 = Prolisa

Table 2: Detection of Ara h 1 extracted from light and dark roasted peanuts at different concentrations using 3 different test kits

		Concentration (µg/µl)				
		0.1	0.01	0.001	0.0001	
Test kit						
Light Roasted	1	NA	9.3	3.9	ND	NA
	2	NA	OVER	OVER	56.8	4
	3	NA	10.8	ND	NA	NA
Dark Roasted	1	OVER	OVER	1.2	ND	NA
	2	NA	17.6	ND	NA	NA
	3	NA	7.8	ND	NA	NA

All data expressed in ppm of peanuts

ND = not detected, NA = not assayed, Over = data is over the highest calibrator

1 = BioKits, Tepnel BioSystems or Elisa Technologies

2 = Ridascreen, R-Biopharm

3 = Veratox, Neogen Corporation

Table 3: Detection of Ara *h* 2 extracted from light and dark roasted peanuts at different concentrations using 3 different test kits

Concentration ($\mu\text{g}/\mu\text{l}$)		0.1	0.01	0.001	0.0001
0.00001					
Test					
Light Roasted	1	1.9	1.5	ND	NA
	2	NA	OVER	4.6	NA
	3	NA	4.6	ND	NA
Dark Roasted	1	OVER	9.5	ND	NA
	2	NA	23.6	3.7	NA
	3	NA	3.7	ND	NA

All data expressed in ppm of peanuts

ND = not detected, NA = not assayed, Over = data is over the highest calibrator

1 = BioKits, Tepnel BioSystems or Elisa Technologies

2 = Ridascreen, R-Biopharm
3 = Veratox, Neogen Corporation

Fig. 1: SDS-PAGE analysis of 0.7 •g each of Ara h 1, Ara h 2 and defatted light roasted peanut extract stained with Coomassie blue. MW: molecular markers (188, 98, 62, 49, 38, 28, 17, 14, 6, 3 kDa); R1: Ara h 1 from raw peanut; L1: Ara h 1 from light roasted peanut; D1: Ara h 1 from dark roasted peanut; L7: defatted light roasted peanut extract; R2: Ara h 2 from raw peanut; L2: R2: Ara h 2 from light roasted peanut; D2: R2: Ara h 2 from dark roasted peanut

Ara h 1

