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Impact of Thermal Processing on ELISA Detection of Peanut Allergens

Tong-Jen Fu*,† and Nicole Maks‡

†Division of Food Processing Science and Technology, Institute for Food Safety and Health, U.S. Food and Drug Administration, 6502 South Archer Road, Bedford Park, Illinois 60501, United States

‡Institute for Food Safety and Health, Illinois Institute of Technology, 6502 South Archer Road, Bedford Park, Illinois 60501, United States

ABSTRACT: This study examined the effect of heat treatment on the solubility of peanut proteins and compared the performances of two commercial ELISA kits (Veratox Quantitative Peanut Allergen Test and BioKits Peanut Assay Kit) for quantitation of peanut residues as affected by different heat treatments (moist and dry heat) and detection targets (mixture of proteins vs specific protein). Both laboratory-prepared and commercial peanut flour preparations were used for the evaluation. The two ELISA kits tended to underestimate the levels of protein in samples that were subjected to elevated heat, respectively, by more than 60- or 400-fold lower for the autoclaved samples and by as much as 70- or 2000-fold lower for the dark-roast commercial flour samples. The BioKits test, which employs antibodies specific to a heat labile protein (Ara h 1), in general exhibited a greater degree of underestimation. These results suggest that commercial ELISA kits may not be able to accurately determine the amount of proteins present in thermally processed foods due to changes in the solubility and immunoreactivity of the target proteins. Users need to be aware of such limitations before applying ELISA kits for evaluation of food allergen control programs.

KEYWORDS: *food allergen, peanut flours, ELISA test kits, thermal processing*

INTRODUCTION

Peanuts are an important staple of the human diet. They are consumed either alone as a snack or used as an ingredient in desserts, confections, and various main dishes. Peanut flours with different roasting colors and fat contents are used to add flavor, aroma, and protein to a wide variety of food products. Unfortunately, peanuts are also one of the common causes of food hypersensitivity in humans. Peanut allergy affects 0.6% of the U.S. population[1] and accounts for the majority of fatalities caused by anaphylactic reactions.[2,3] Because of the severity of allergic reactions and the frequent use of peanuts as ingredients in foods, inadvertent exposure to peanut allergens is a significant public health concern. Consumers rely on food labels to disclose the presence of allergenic ingredients. In the United States, the Food Allergen Labeling and Consumer Protection Act (FALCPA) came into effect in 2006, requiring manufacturers to clearly list ingredients derived from eight allergenic foods (milk, egg, fish, crustacean shellfish, tree nuts, wheat, peanuts, and soybeans). FALCPA defines the term "major food allergens" as one of these eight food groups or any ingredient that contains proteins derived from these foods.[4]

Current labeling regulations deal only with allergens that are knowingly added as ingredients but do not address allergens that can be inadvertently introduced into food as a result of production errors or cross contact during manufacturing. To prevent the incidence of undeclared allergens, food manufacturers have increasingly developed and implemented allergen control programs.[5] Elements of effective allergen controls have been discussed.[5,6] An assessment of allergen control measures practiced in targeted food industries indicated that >94% of the surveyed companies implemented certain allergen control programs and >71% conducted analytical testing of allergens.[7] In 2011, the United States passed the Food Safety Modernization Act, which requires each food production facility to put in place preventive control plans. Implementation of a food allergen control program has been recommended.[8]

Reliable allergen detection methods play a key role in an effective allergen control program.[5,6] Commercial ELISA test kits remain the most frequently used methods. Food manufacturers use these tests to verify or validate the effectiveness of various components of allergen control programs, including ingredient analysis, validation of cleaning and sanitation procedures, and routine finished product analysis.[5−7] ELISA test kits are also used by public health authorities to investigate consumer complaints and to monitor industry compliance with food allergen labeling regulations.[9−11] The number of ELISA test kits that are commercially available has increased significantly in recent years.[12,13] Test kits for detection of peanut allergens are among the most readily available.

Existing ELISA test kits can be divided into two groups on the basis of the detection targets. Some test kits detect total soluble proteins or a mixture of proteins; others use antibodies that are reactive toward specific allergenic or marker proteins. Many test kits are designed to allow quantitative analysis

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involving a two-step process, extraction of the target proteins and detection of the extracted proteins via binding with specific antibodies. The concentrations are interpolated from standard curves generated with reference standards typically composed of native forms of the target proteins. In some kits, the final concentrations in the sample are expressed as parts per million (ppm) peanut material using an internal conversion factor that converts protein concentration to amount of peanut material.[14] Because detection is achieved via antigen−antibody binding reactions, any changes in the target protein, and thus its recognition by the kit antibodies, can greatly influence assay results. Proteins undergo unfolding and thus lose their tertiary and secondary structures after heat treatments. Formation of aggregates may follow and, at higher temperatures, chemical modification can occur.[15,16] These modifications often lead to changes in the solubility and immunoreactivity of target proteins and, thus, can affect ELISA detection. As thermal treatments affect individual proteins differently, the performance of different ELISA test kits may vary depending on the nature of the target proteins.[17]

The performance of commercial ELISA kits for detection of peanut residues in thermally processed foods has been evaluated. Koch et al.[18] examined three ELISA test kits for detection of raw or oil-roasted ground peanut spiked in cookies and showed that raw peanuts exhibited 3−4 times higher responses when compared with oil-roasted peanuts. These researchers also observed a significant difference in peanut contents measured by different test kits. For example, the level of proteins in cookies spiked with raw peanuts detected by the Prolisa peanut PAK test was about 3 times higher than that determined by the Ridascreen Peanut test. This between-kit variation was also observed by Westphal et al.,[14] who compared three commercial ELISA kits for quantitation of peanut content in buffers containing 10 ppm of proteins extracted from light- and dark-roast peanut flours. The results obtained from one kit were found to be consistently lower than those obtained from the other two kits, and only one kit could detect the presence of peanut proteins in PBS extracts of the dark-roast flours. Recently, Khuda et al.[19] evaluated the accuracy and precision of five commercial ELISA kits for the detection of peanut, egg, and milk allergens spiked in sugar cookies and reported that the measured concentrations of peanut proteins in incurred cookie dough varied among the different test kits, ranging from 11.0 to 101.8% of the incurred levels. Food processing negatively affected the detection of allergen residues by all test kits. For example, the test kits manufactured by Neogen and R-Biopharm both gave relatively accurate measurements of peanut contents in the unheated dough, but both greatly underestimated the levels of peanut proteins in cookies baked at 190 °C for 30 min (17.4 and 17.1% of the incurred levels, respectively).

Although previous studies have demonstrated that thermal processing can significantly affect protein quantitation by ELISA test kits, few have examined the underlying causes for such an effect. As the number of commercial test kits with different formats and detection targets continues to increase, food manufacturers are faced with the constant challenge of choosing the right test kits for their applications. A detailed understanding of the factors that may affect ELISA detection of proteins in thermally processed foods will allow an informed choice and a better recognition of the limitations of the selected test kits. Toward that end, we evaluated the performance of two commercial ELISA test kits, in comparison with a total protein assay, for quantitation of proteins in peanut flours that have been subjected to both moist- and dry-heat treatments. How different thermal treatments affect the quantitation of ELISA test kits that target different proteins was examined, and whether the decrease in detectability was due to the inability to extract peanut proteins or to the changes in the immunoreactivity of the extracted proteins was determined. The two commercial kits evaluated were the Veratox Peanut Allergen Test, which is reactive toward total peanut proteins, and the BioKits Peanut Assay Kit, which employs antibodies specific to a marker protein, Ara h 1. Laboratory-prepared peanut flours as well as commercial peanut flour samples with different fat contents and various degrees of roasting were used as the model food systems.

MATERIALS AND METHODS

Laboratory-Prepared Peanut Flours. One hundred and fifty grams of raw peanuts (Runner variety) purchased from Mr. Mac's Peanuts (Eufaula, AL, USA) were deskinned and ground using the Osterizer blender (Sunbeam Co., Delray Beach, FL, USA) for 1 min. The ground peanuts were defatted by mixing with 1.5 L of hexane in an orbital shaker at 250 rpm for 1 h at room temperature before filtration through a Whatman no. 2 filter paper. The extraction was repeated two more times. The flours were air-dried in a fume hood and homogenized in a mortar with a pestle and were stored at −20 °C until use. The same protocol was used to prepare defatted flours from shelled raw peanuts (Runner variety) purchased from Golden Peanut Co. (Alpharetta, GA, USA).

Commercial Peanut Flours. Partially defatted flours of the Runner variety with different degrees of roasting (light, medium, dark, medium strong, and strong) and fat contents (12, 12/14, and 28%) were obtained from Golden Peanut Co. and Nutrin Co. (Washington, DC, USA). A dark-roast peanut flour sample used in cereals and bakery products was donated from company A. A portion of each commercial peanut flour sample was defatted by mixing with 10× (w/v) of hexane as described above. One gram of each of the original or defatted flour samples was mixed with 4 mL of DI water before division into 1 mL aliquots for further extraction and analysis.

Heat Treatments. Laboratory-prepared peanut flours were subjected to moist- or dry-heat treatments. For the moist-heat treatment, 4 mL of DI water was added to each of the polyethylene tubes containing 1 g of peanut flour, and the mixtures were heated in a water bath at 60 or 100 °C for 10 min or were autoclaved in a Sterimatic autoclave (Market Forge Industries Inc., Everett, MA, USA) at 121 °C for 10 min. The unheated and heat-treated samples were divided into 1 mL aliquots for further extraction and analysis. For the dry-heat treatment, glass vials containing 1 g of peanut flour were heated in a muffle furnace (Barnstead International, Dubuque, IA, USA) at 60, 100, 120, 176, 204, 232, 260, or 400 °C for 10 min. Each of the unheated and heat-treated samples was mixed with 4 mL of DI water before division into 1 mL aliquots for further extraction and analysis.

Protein Quantification by the BCA Assay. The 1 mL flour−water mixtures of the unheated and heat-treated samples were further extracted in 9 mL of phosphate-buffered saline (PBS) for 15 min at 60 °C in a shaking water bath at 150 rpm. The samples were centrifuged at 10000*g* for 10 min in an Eppendorf model 5810R centrifuge (Hamburg, Germany). The amount of extractable proteins was determined by the bicinchoninic acid total protein assay (BCA; Pierce, Rockford, IL, USA) per the manufacturer's instructions. Bovine serum albumin was used as the protein standard. For samples dry-heated at 260 and 400 °C, prior to the BCA analysis, the PBS extracts were passed through P6 spin columns (Pierce) to remove interfering brown pigments that resulted from heat treatments.

Protein Quantification by ELISA Test Kits. The Veratox Quantitative Peanut Allergen Test manufactured by Neogen Co. (Lansing, MI, USA) uses a sandwich format. It employs antibodies targeting total soluble peanut protein. The result is reported as parts

Figure 1. (A) Laboratory-prepared peanut flours subjected to dry-heat treatment for 10 min at different temperatures; (B) commercial peanut flour samples with different fat contents and different degrees of roasting. GRaw, Golden, raw; G12L, Golden 12% fat, light roast; G28L, Golden 28% fat, light roast; G12D, Golden 12% fat, dark roast; G28D, Golden 28% fat, dark roast; N28 M, Nutrin 28% fat, medium roast; N28MS, Nutrin 28% fat, medium-strong roast; N12S, Nutrin 12/14% fat, strong roast; A, company A.

per million peanut, and the range of quantitation is 2.5−25 ppm. The protocol recommended by the kit manufacturer was followed except that the amount of extraction buffer used was adjusted to accommodate the 1 mL sample size. Briefly, the 1 mL peanut flour−water mixtures were extracted with 25 mL of prewarmed extraction buffer (containing PBS plus one-fifth of a scoop of extraction additive) at 60 °C for 15 min in a shaking water bath (150 rpm). After centrifugation at 10000*g* for 10 min, the supernatant was serially diluted to appropriate concentrations for ELISA analysis per the kit instructions. The absorbance of the standards and samples was measured at 650 nm using a microtiter plate reader (Biotek Instruments, Winooski, VT, USA). The peanut concentrations in the samples were interpolated from the standard curve using Neogen's log/logit software.

The BioKits Peanut Assay kit (Neogen) is a sandwich-type enzyme immunoassay utilizing biotin−avidin enhancement. The assay uses polycolonal antibodies specific to Ara h 1. The results are reported as parts per million peanut with a range of detection of 1−20 ppm peanut. For our analysis, the 1 mL flour−water mixtures were extracted with 10 mL kit extraction buffer (containing Tris buffer, 0.3 M NaCl, and fish gelatin) that was preheated to 60 °C in a water bath. The samples were mixed in a stomacher (Seward Laboratory Systems Inc., Port Saint Lucie, FL, USA) for 2 min at medium speed and extracted for 15 min at room temperature. One milliliter of the sample extract was removed and centrifuged at 10000*g* for 10 min. The supernatant was collected and diluted 10-fold in the working diluent solution, which was further diluted in the working diluent to appropriate concentrations for ELISA analysis per kit instructions. The absorbance of the standards and samples was measured at 450 nm using a microtiter plate reader. The level of peanut residues in the samples was interpolated from the standard curve generated with the Ara h 1 standard solutions using a sigmoidal curve fit.

SDS-PAGE Analysis. PBS extracts of the flour−water mixtures were mixed with equal volumes of 2× Laemmli buffer (Sigma, St. Louis, MO, USA) and boiled for 10 min. Fifteen microliters of the samples was loaded in a 10−20% Tris−tricine mini-gel (Invitrogen, Carlsbad, CA, USA) and run in an XCell SureLock Mini-Cell (Invitrogen) at 250 V. Proteins were visualized by Coomassie Brilliant Blue staining. SeeBlue and Mark 12 (Invitrogen) were used as molecular weight (MW) markers.

Comparison of the Efficacy of Extraction Protocols for Recovery of Peanut Proteins. The 1 mL flour−water mixtures of the unheated and heat-treated samples were subjected to extraction using either of the two protocols: (1) extraction with 9 mL of PBS (i.e., Veratox extraction buffer without extraction additive) for 15 min in a shaking water bath at 60 °C and 150 rpm or (2) extraction with 9 mL of Tris buffer with 0.3 M NaCl (i.e., BioKits extraction buffer without fish gelatin) preheated to 60 °C, stomached for 2 min, and extracted for 15 min at room temperature. The protein concentration in each of the extracts was determined using the BCA assay as described above.

Statistical Analysis and Experimental Design. The heat treatments were repeated six and two times for the laboratory-prepared and commercial flour samples, respectively. For each trial, duplicate peanut flour samples were treated at each temperature. For the test kit analyses, all samples from each trial were assayed with one or two separate ELISA runs and each with duplicate wells. Differences in the normalized concentrations between samples treated at different temperatures were analyzed by analysis of variance using OriginPro software (Origin Lab, Northampton, MA, USA). Fisher's LSD test was used for comparison of the means. In all cases, the level of significance was set at $p < 0.05$.

RESULTS

Laboratory-Prepared Peanut Flour Samples. The defatted flours prepared from Mr. Mac's peanuts were subjected to various degrees of heat treatments for 10 min. Although there was not much color change in flour samples that were moist-heated or dry-heated at mild temperatures, browning was evident in samples dry-heated at temperatures ≥204 °C (Figure 1A). The laboratory-prepared flour sample heated at 204 °C was lighter in color than all of the commercial flour samples (Figure 1B), whereas the samples heated at 232 and 260 °C exhibited roasting colors similar to those of the light-roast and dark-roast commercial flour samples, respectively. Peanut flour heated at 400 °C appeared burnt.

Protein Quantification by BCA. The levels of protein extracted by PBS from the unheated and heat-treated peanut flour samples were determined by the BCA total protein assay.

Table 1. Amount of Protein or Peanut Residues in Unheated and Moist-Heated Peanut Flour Samples As Determined by the BCA Assay, the Veratox Peanut Allergen Test, and the BioKits Peanut Assay Kit in a Single Run

	BCA	Veratox	Biokits
moist-heat treatment	ppm peanut protein	ppm peanut	ppm peanut
unheated	50,905 ± 799	727,500 ± 231,138	5,117,500 ± 2,083,753
60 °C, 10 min	49,750 ± 1018	702,500 ± 138,414	5,222,500 ± 1,503,515
boiled, 10 min	26,300 ± 2432	250,000 ± 64,807	489,750 ± 52,690
autoclaved, 10 min	16,745 ± 615	5100 ± 1849	1792 ± 1686

Table 2. Normalized Concentrations of Protein or Peanut Residues in the Moist- or Dry-Heated Peanut Flour Samples As Determined by the BCA Total Protein Assay, the Veratox Peanut Allergen Test, and the BioKits Peanut Assay Kit[a]

heat treatment		normalized concentration in samples (%)		
		BCA	Veratox	BioKits
moist heat	unheated	100.0 $^{A, 1}$	100.0 $^{A, 1}$	100.0 $^{A, 1}$
	60 °C, 10 min	93.4 ± 3.8 $^{A, 1}$	98.9 ± 15.7 $^{A, 1}$	96.4 ± 18.0 $^{A, 1}$
	100 °C, 10 min	53.6 ± 4.7 $^{B, 1}$	45.1 ± 12.2 $^{B, 1}$	30.7 ± 16.7 $^{B, 2}$
	autoclaved, 10 min	44.7 ± 14.6 $^{B, 1}$	0.7 ± 0.3 $^{C, 2}$	0.1 ± 0.1 $^{C, 2}$
dry heat	unheated	100.0 $^{A, 1}$	100.0 $^{A, 1}$	100.0 $^{A, 1}$
	60 °C, 10 min	93.8 ± 12.6 $^{A, 1}$	112.4 ± 9.6 $^{A, 2}$	107.8 ± 15.6 $^{A, 2}$
	100 °C, 10 min	93.2 ± 12.6 $^{A, 1}$	99.6 ± 13.4 $^{A, 1}$	81.1 ± 10.5 $^{A, 2}$
	120 °C, 10 min	94.1 ± 9.5 $^{A, 1}$	90.9 ± 14.9 $^{A, 1}$	93.0 ± 20.5 $^{A, 1}$
	176 °C, 10 min	83.5 ± 10.0 $^{A, 1}$	89.3 ± 9.1 $^{A, 1}$	89.2 ± 17.2 $^{A, 1}$
	204 °C, 10 min	76.9 ± 9.3 $^{B, 1}$	85.7 ± 12.9 $^{A, 1}$	77.2 ± 14.7 $^{A, 1}$
	232 °C, 10 min	50.1 ± 9.2 $^{C, 1}$	37.3 ± 13.8 $^{B, 1}$	25.0 ± 19.1 $^{B, 2}$
	260 °C, 10 min	14.3 ± 6.2 $^{D, 1}$	1.5 ± 1.4 $^{C, 2}$	0.6 ± 0.7 $^{C, 2}$
	400 °C, 10 min	2.7 ± 1.8 $^{E, 1}$	0.05 ± 0.06 $^{C, 2}$	0.01 ± 0.01 $^{C, 2}$

[a]Each value represents the average ± standard deviation of results obtained from six separate runs. Values in the same column followed by the same letter are not significantly different ($p > 0.05$). Values in the same row followed by the same number are not significantly different ($p > 0.05$).

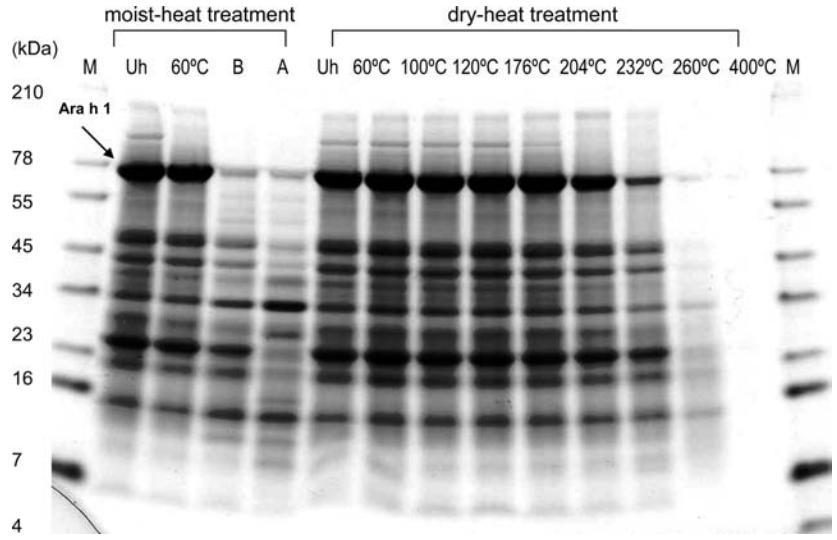

Figure 2. SDS-PAGE analysis of PBS extracts of peanut flours that have been moist- and dry-heated at different temperatures. The Ara h 1 band is indicated by the arrow. M, molecular weight marker (SeeBlue); Uh, unheated; B, boiled; A, autoclaved.

Table 1 shows the typical results obtained for the moist-heated samples. To facilitate the comparison of the impact of heat treatment on the performances of different methods, the protein concentrations determined in the heat-treated samples were normalized against those of the unheated samples. Table 2 lists the average of the normalized concentrations obtained from six separate heat treatment runs.

Boiling and autoclaving caused an approximately 50% decrease in the amount of protein extracted. On the other hand, dry heating at 100 or 120 °C did not significantly ($p > 0.05$) affect the extractability of peanut proteins. Significant decreases ($p < 0.05$) in the level of extracted protein occurred when the flour samples were dry-heated at temperatures ≥204 °C. At 232 and 260 °C, the amounts of protein recovered were about 50 and 14%, respectively, of the unheated samples. Very little protein was recovered from samples heated at 400 °C.

Quantification by ELISA Test Kits. The amounts of peanut residues in the moist-heated samples as determined by the

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Veratox and BioKits tests for one experiment are listed in Table 1. The two test kits gave very different results, even for the same unheated samples. To facilitate the comparison, for each test, the concentrations obtained for the heat-treated samples were normalized against those of the unheated samples. The average values of the normalized concentrations obtained from six separate heat treatment runs are listed in Table 2.

In general, heat treatment resulted in a lower level of peanut detected by the two ELISA test kits, but the extent of decrease differed. For the boiled samples, the normalized concentration determined by the Veratox kit, which is reactive to multiple peanut proteins, was similar to that determined by BCA. On the other hand, the level determined by BioKits, which employs antibodies specific to Ara h 1, was lower. For the autoclaved samples, the normalized concentrations determined by either test kit were significantly lower than those determined by BCA. The BioKits registered a normalized concentration of 0.1%, which was >400-fold lower than the 44.7% detected by BCA. The Veratox test also underestimated the level of protein in the autoclaved samples (~60-fold lower than that detected by BCA), although the extent of underestimation was not as pronounced as that exhibited by the BioKits test.

For samples dry-heated at temperatures up to 204 °C, both test kits performed well, giving normalized concentrations similar to those obtained by BCA. However, for samples dry-heated at higher temperatures, quantitation of peanut residues by the two ELISA kits was significantly affected. At 232 °C, the levels of protein measured by BioKits were significantly lower than those obtained by BCA or the Veratox kit. Both the Veratox and BioKits underestimated the amount of peanut protein in samples heated at 260 °C, giving a normalized concentration of 1.5 and 0.6%, respectively, compared with the 14.3% detected by BCA.

SDS-PAGE Analysis. The protein profiles of the unheated and heat-treated flour samples are shown in Figure 2. The levels of the major peanut allergen Ara h 1 in the boiled and autoclaved samples were significantly lower than in the unheated sample. The intensity of a number of other bands also decreased in the boiled samples and continued to fade in the autoclaved samples. When compared with those observed in the boiled samples, there seemed to be a general decrease in band intensity in the autoclaved samples, especially those representing proteins with higher MW, although some protein bands became more intense (e.g., the band with an MW of ~34 kDa).

For the dry-heat-treated samples, the banding patterns remained largely unchanged in samples heated at temperatures up to 176 °C. At 204 °C, the intensity of some bands began to decrease, and at 232 °C, the intensity of additional bands, including that of Ara h 1, decreased significantly. For samples heated at 260 °C, very limited numbers of bands were visible. No protein or peptide fragments were visible on the gel for samples heated at 400 °C.

Commercial Peanut Flour Samples. As shown in Figure 1B, the extent of browning in the commercial flour samples can be ranked from the darkest to the lightest as follows: company A ≅ Golden 28% fat, dark roast > Golden 28% fat, light roast ≅ Golden 12% fat, dark roast ≅ Nutrin 12% fat, strong roast ≅ Nutrin 28% fat, medium-strong roast > Nutrin 28% fat, medium roast > Golden 12% fat, light roast > Golden, raw.

Quantification by BCA and ELISA Test Kits. Initial BCA and ELISA analyses for samples that were defatted versus samples that were not defatted yielded similar results. Therefore, all of the later analyses were done on the original flour samples without defatting. The protein or peanut concentration in each of the commercial flour preparations as determined by BCA and the two ELISA test kits was normalized against the concentrations determined for the Golden raw peanut flour samples. The average values of the normalized concentrations obtained from two separate experiments are summarized in Table 3.

Table 3. Normalized Concentration of Protein or Peanut Residues in Commercial Peanut Flour Preparations with Various Fat Contents and Roast Colors As Determined by the BCA Assay, the Veratox Peanut Allergen Test, and the BioKits Peanut Assay Kit[a]

	normalized concentration in samples (%)		
flour preparation	BCA	Veratox	BioKits
Golden, raw	100.0 ^{A,1}	100.0 ^{A,1}	100.0 ^{A,1}
Golden 12%, light	25.2 ± 1.6 ^{B,1}	9.3 ± 1.4 ^{B,2}	2.8 ± 0.2 ^{B,3}
Golden 28%, light	18.0 ± 0.5 ^{C,1}	2.3 ± 0.2 ^{C,2}	0.7 ± 0.5 ^{C,3}
Golden 12%, dark	20.7 ± 2.8 ^{C,1}	2.3 ± 0.9 ^{C,2}	0.8 ± 0.03 ^{C,2}
Golden 28%, dark	20.0 ± 2.5 ^{C,1}	0.5 ± 0.1 ^{D,2}	0.01 ± 0.00 ^{D,2}
Nutrin 28%, medium	22.3 ± 0.3 ^{C,1}	6.0 ± 1.5 ^{E,2}	2.8 ± 0.3 ^{B,3}
Nutrin 28%, medium strong	21.0 ± 0.9 ^{C,1}	3.8 ± 0.1 ^{C,2}	1.3 ± 0.2 ^{E,3}
Nutrin 12/14%, strong	20.5 ± 1.8 ^{C,1}	1.3 ± 0.2 ^{D,2}	0.7 ± 0.1 ^{C,2}
company A	18.9 ± 1.3 ^{C,1}	0.3 ± 0.04 ^{D,2}	0.01 ± 0.01 ^{D,2}

[a]Each value represents the average ± standard deviation of results obtained from two separate runs. Values in the same column followed by the same letter are not significantly different ($p > 0.05$). Values in the same row followed by the same number are not significantly different ($p > 0.05$).

Regardless of the degree of roasting or fat content, the amount of protein in the PBS extracts of the commercial peanut flour samples as determined by BCA was about 18−25% of that of the raw peanut flour. However, the relative amount of protein in these samples as indicated by the two ELISA kits was lower and the degree of underestimation differed depending on the extent of roasting and the test kit used. For example, for the Golden light-roast peanut flour with 12% fat, the normalized concentration determined by the Veratox was 9.3%, compared with the 25.2% measured by BCA. For the dark-roast flour with 28% fat, although the normalized concentration determined by BCA was slightly lower than that obtained for the light-roast flour with 12% fat (20.0% vs 25.2%), the normalized concentration measured by the Veratox test was only 0.5%. The degree of underestimation was much greater when using the BioKits test, which registered a normalized concentration of only 0.01% (approximately 2000-fold lower than the level determined by BCA). A similar trend was observed when testing the flour samples obtained from Nutrin Co. The normalized protein concentrations for these samples as measured by the two ELISA kits were lower than those determined by BCA, and a greater degree of underestimation was observed when the samples were tested using the BioKits test. For the flour sample from company A, the BioKits test

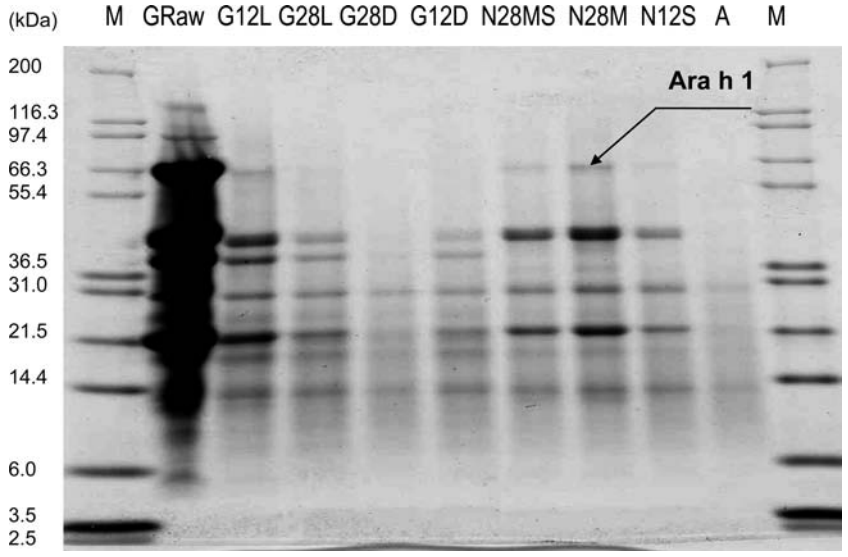

Figure 3. SDS-PAGE analysis of PBS extracts of commercial peanut flour samples with different fat contents and different degrees of roasting. The Ara h 1 band is indicated by the arrow. GRaw, Golden, raw; G12L, Golden 12% fat, light roast; G28L, Golden 28% fat, light roast; G28D, Golden 28% fat, dark roast; G12D, Golden 12% fat, dark roast; N28MS, Nutrin 28% fat, medium-strong roast; N28 M, Nutrin 28% fat, medium roast; N12S, Nutrin 12/14% fat, strong roast; A, company A; M, molecular weight marker (Mark12).

Table 4. Comparison of the Efficiency of the Extraction Protocols Used by the BCA Assay (PBS/60 °C) and Veratox Kit without Skim Milk (PBS/60°C) and the BioKits Assay without Fish Gelatin (Tris/0.3 M NaCl/60 °C) for the Moist- or Dry-Heat-Treated Flour Samples[a]

		actual (mg/mL) or normalized (%) protein concentrations in samples			
		PBS/60 °C		Tris/0.3 M NaCl/60 °C	
heat treatment		mg/mL	%	mg/mL	%
moist heat	unheated	49.5 ± 22.2	100.0 $^{A, 1}$	37.7 ± 6.0	100.0 ^{A,1}
	60 °C, 10 min	47.7 ± 22.9	95.2 ± 4.7 $^{A, 1}$	36.0 ± 7.0	95.2 ± 3.2 $^{A, 1}$
	100 °C, 10 min	25.5 ± 8.8	53.6 ±7.1 $^{B, 1}$	20.8 ± 1.8	56.1 ± 9.7 $^{B, 1}$
	autoclaved, 10 min	16.2 ± 0.5	38.2 ± 18.9 $^{B, 1}$	14.6 ± 0.3	39.4 ± 6.6 $^{C, 1}$
dry heat	unheated	51.2 ± 21.8	100.0 $^{A, 1}$	39.3 ± 8.2	100.0 $^{A, 1}$
	60 °C, 10 min	46.8 ± 14.1	96.4 ± 19.3 $^{A, 1}$	38.7 ± 8.4	98.2 ± 2.67 $^{A, 1}$
	100 °C, 10 min	45.2 ± 12.9	93.5 ± 19.1 $^{A, 1}$	38.0 ± 8.6	96.6 ± 4.5 $^{A, 1}$
	120 °C, 10 min	46.9 ± 15.0	95.5 ± 14.3 $^{A, 1}$	36.7 ± 8.7	93.0 ± 2.4 $^{A, 1}$
	176 °C, 10 min	44.2 ± 15.5	89.4 ± 12.0 $^{A, 1}$	35.7 ± 10.3	90.0 ± 8.0 $^{A, 1}$
	204 °C, 10 min	42.1 ± 16.0	83.9 ± 6.4 $^{A, 1}$	37.2 ± 9.9	94.1 ± 11.2 $^{A, 1}$
	232 °C, 10 min	24.1 ± 6.5	50.3 ± 12.7 $^{B, 1}$	22.2 ± 1.0	58.0 ± 10.7 $^{B, 1}$
	260 °C, 10 min	6.6 ± 1.6	15.6 ± 9.4 $^{C, 1}$	5.3 ± 0.5	13.9 ± 3.6 $^{C, 1}$
	400 °C, 10 min	1.2 ± 0.9	2.8 ± 1.9 $^{C, 1}$	1.1 ± 0.6	3.0 ± 1.8 $^{D, 1}$

[a]Protein levels in extracts were determined with the BCA assay. Each value represents the average ± standard deviation of the actual or normalized concentrations obtained from three separate runs. Values in the same column followed by the same letter are not significantly different ($p > 0.05$). Values in the same row followed by the same number are not significantly different ($p > 0.05$).

registered a level of peanut protein that was about 1900-fold lower than the level measured by BCA (0.01 vs 18.9%).

Whether fat content affects protein quantification by ELISA test kits is less clear. The levels of peanut residues measured by the two test kits were lower for the Golden peanut flour samples with 28% fat than for the flours with 12% fat. The difference in protein level determined may not be due to the difference in fat content, but rather the different degrees of roasting between these samples. As evident in Figure 1B, the light-roast and dark-roast samples with 28% fat had a darker brown color than those with 12% fat.

SDS-PAGE Analysis. The amount of protein, specifically that of Ara h 1, extracted from the commercial flour samples was significantly lower than that obtained from the raw peanut flour (Figure 3). The intensity of the protein bands generally correlated with the degree of roasting, with the bands for the Golden 12% fat, light-roast flour being the most intense, and those of the Golden 28% fat, dark-roast flour and the flour sample from company A fading. Among the Golden peanut flour samples, the protein bands for samples with higher fat content appeared less intense than those for samples with lower fat content. For example, in the Golden light-roast flours, the protein bands for the sample with 28% fat were lighter than those for the sample with 12% fat. Similarly, in the dark-roast flours, the protein bands for the sample with 28% fat were less intense than those for the sample with 12% fat. As discussed above, these differences could be due to the greater degree of roasting that the flours with higher fat content received (Figure

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Table 5. Comparison of the Efficiency of the Extraction Protocols Used by the BCA Assay and Veratox kit without Skim Milk (PBS/60 °C) and the BioKits Assay without Fish Gelatin (Tris/0.3 M NaCl/60 °C) for the Commercial Flours of Various Fat Contents and Degrees of Roasting[a]

	actual (mg/mL) or normalized (%) protein concentrations in samples			
	PBS/60 °C		Tris/0.3 M NaCl/60 °C	
sample	mg/mL	%	mg/mL	%
Golden, raw	63.4 ± 0.6	100.0 ^{A}	54.8 ± 4.0	100.0 ^{A}
Golden 12%, light	16.4 ± 1.2	25.9 ± 1.6 $^{B, 1}$	13.8 ± 1.5	25.3 ± 4.6 $^{B, 1}$
Golden 28%, light	12.0 ± 1.3	19.0 ± 1.9 $^{C, 1}$	9.6 ± 0.1	17.6 ± 3.0 $^{C, 1}$
Golden 12%, dark	13.8 ± 1.9	21.8 ± 2.7 $^{C, 1}$	11.0 ± 0.04	20.1 ± 2.0 $^{C, 1}$
Golden 28%, dark	13.2 ± 1.6	20.9 ± 2.4 $^{C, 1}$	11.4 ± 0.5	20.8 ± 1.5 $^{C, 1}$
Nutrin 28%, medium	13.8 ± 0.5	21.8 ± 0.9 $^{C, 1}$	11.2 ± 1.2	20.6 ± 3.7 $^{C, 1}$
Nutrin 28%, medium strong	13.2 ± 0.5	20.8 ± 0.7 $^{C, 1}$	10.8 ± 1.1	19.7 ± 3.0 $^{C, 1}$
Nutrin 12/14%, strong	12.7 ± 1.0	20.0 ± 1.5 $^{C, 1}$	10.0 ± 0.5	18.4 ± 2.2 $^{C, 1}$
company A	12.0 ± 0.7	19.0 ± 0.9 $^{C, 1}$	9.4 ± 0.7	17.2 ± 2.5 $^{C, 1}$

[a]Protein levels in extracts were determined with the BCA assay. Each value represents the average ± standard deviation of the actual or normalized concentrations obtained from three separate runs. Values in the same column followed by the same letter are not significantly different ($p > 0.05$). Values in the same row followed by the same number are not significantly different ($p > 0.05$).

1B). The protein bands for the Golden 28% fat, light-roast flour exhibited intensity similar to those of the Golden 12% fat, dark-roast flour. This similarity correlated with the similar roasting colors for the two samples (Figure 1B), despite what the product names may have otherwise indicated.

Comparison of the Efficacy of Extraction Protocols. To determine whether the variation in protein quantitation among the three methods was due to differences in the extraction conditions used, we compared the amount of proteins that can be extracted from the unheated and thermally treated samples following the protocol used in the BCA and Veratox tests (PBS/60 °C without skim milk) and the BioKits assay (Tris/0.3 M NaCl/60 °C without fish gelatin).

Table 4 summarizes the actual and normalized concentrations of protein recovered from both the moist- and dry-heated peanut flour samples using the two different extraction protocols. For each of the unheated or heat-treated samples, although the actual amount of protein extracted using the PBS protocol was about 20% greater than that recovered using the Tris/NaCl protocol, both extraction protocols resulted in a similar normalized protein concentration for each of the heat-treated samples. For example, both extraction protocols yielded normalized concentration values, approximately 50 and 40% for the boiled and autoclaved samples, respectively. For samples dry-heated at 232 or 260 °C, both protocols were able to recover about 50 or 15% of protein relative to the unheated samples.

Similar results were observed for the commercial flour samples. There was no difference in the level of normalized concentrations determined using either extraction protocol for each of the commercial flour samples, even though the actual amount of protein extracted in PBS was consistently about 20% greater than that recovered using the Tris/NaCl buffer (Table 5).

DISCUSSION

This study examined the effect of heat treatment on the solubility of peanut proteins and compared the performances of two commercial ELISA kits for quantitation of peanut residues as affected by different heat treatments (moist and dry heat) and detection targets (mixture of proteins vs specific protein). Both laboratory-prepared and commercial peanut flour preparations were used for the evaluation. Peanut flours contain most of the components found in a typical food, including proteins, carbohydrates, lipids, minerals, and other nutrients, and thus can serve as a good model food system. The laboratory-prepared peanut flour provided a simple system to evaluate the performance of ELISA test kits for detection of peanut proteins under different heat treatment conditions. The commercial peanut flour provided a "real-world" sample for the evaluation.

It was observed that different methods registered different readings (in ppm values) even for samples aliquoted from the same preparation. Variations in protein quantitation among different commercial ELISA test kits have been reported.[18−21] Factors attributed to these variations include the use of different antibodies, different extraction conditions, different standards or calibrators used to construct the standard curve in relating antibody−antigen binding reactions to protein concentrations, and different conversion factors used for converting protein concentrations to specified reporting units, for example, parts per million peanuts or parts per milliion peanut proteins.[14,19,20] Normalization of the measured concentration was done in this study so that the impact of heat treatments on the quantitative analysis of different methods could be compared. The same approach was used in previous studies to evaluate different ELISA test kits for detection of egg residues in thermally processed samples.[17,22] Calculating results as a "percent of unheated control" was also discussed by Downs and Taylor[23] as a way to indicate the relative effect of heating on the detection capacities of milk test kits.

Boiling of the laboratory-prepared peanut flours caused a 50% decrease in the amount of protein extracted as determined by the BCA method. The higher temperature and pressure applied during autoclaving resulted in a similar decrease in protein yield and changes in the intensities of certain protein/peptide bands as revealed by the SDS-PAGE analysis. Dry-heat treatments also resulted in a decrease in protein solubility, although the decrease occurred at a much higher temperature (>176 °C), suggesting that peanut proteins are more resistant to thermal denaturation under dry-heat conditions. Dry heating at 232 and 260 °C for 10 min produced flours with roasting colors similar to those of the commercial flour samples and significantly lowered the extractability of proteins. This agreed

with the low levels of protein that were extracted from the various commercial peanut flour samples. Samples with a darker roasting color had a lower level of extractable protein, although the total protein levels among the various commercial flour samples were not very different from each other, ranging from 18 to 25% of that of the raw peanut flour sample (Table 3). The issues regarding the extractability of proteins in roasted peanuts have been discussed in the literature.[24−26] Poms et al.[24] showed that, although dry blanching at 120 °C for 20 min did not affect protein solubility in Tris buffer saline (pH 8.2), dry roasting at 190 °C for 20 min lowered the protein yield by 80%. Pomés et al.[25] found that the total protein level in peanut extracts decreased with increasing roasting time and was lowered by 78% in peanuts roasted at 177 °C for 30 min.

SDS-PAGE analysis indicated that one of the proteins that was greatly affected by heat treatment was Ara h 1. The protein is a 63.5 kDa glycoprotein belonging to the vicilin seed storage protein family and accounts for 12−16% of the total protein in peanut extracts.[21,27] The level of soluble Ara h 1 was greatly reduced in the boiled/autoclaved samples but remained unaffected in samples dry-heated at temperatures up to 176 °C. Koppelman et al.[28] showed that heating Ara h 1 in solution resulted in an irreversible transition between 80 and 90 °C, leading to an increase in the β-structure and a concomitant aggregation of the protein. Blanc et al.[29] showed that boiling of Ara h 1 for 15 min resulted in the formation of rod-like branched aggregates with reduced IgE-binding capacity.

The intensity of the Ara h 1 band in the laboratory-prepared flours dry-heated at temperatures ≥232 °C or the commercial flour samples was much lower than that of the raw peanut flour sample and decreased significantly in samples with increasing degrees of roasting (Figures 2 and 3). The decrease in the extractability of Ara h 1 in roasted peanuts has been observed by a number of researchers.[21,28−30] Koppelman et al.[28] showed the extraction yields of Ara h 1 from ground peanuts heat-treated at 110 and 140 °C for 15 min were, respectively, 75 and 32% of the yield obtained from the unheated peanuts. At 155−200 °C, at which the ground peanuts appeared brown or dark brown, no Ara h 1 was extracted into the buffer. Blanc et al.[29] showed that Ara h 1 extracted from roasted peanuts appeared to be highly denatured and composed of compact, globular aggregates, but retained the IgE-binding capacity of the native protein.

The Veratox and BioKits tests evaluated in this study, although able to correctly indicate the amounts of proteins present in samples heated under mild conditions, tended to underestimate the levels of protein in samples that were subjected to elevated heat, respectively, by more than 60- or 400-fold lower for the autoclaved samples and by as much as 70- or 2000-fold lower for the dark roast commercial flour samples. The degree of underestimation was greater for peanut flour samples with a darker roasting color. The BioKits test, which employs antibodies specific to a heat labile protein (Ara h 1), in general exhibited a greater degree of underestimation.

The differences in quantitation of thermally treated proteins between the ELISA kits and BCA could be due to a number of factors. They could be due to the difference in the amount of protein extracted. We compared the protein yield obtained using the extraction buffers employed in the different methods and found that there was no significant difference in the normalized concentration of protein extracted by the PBS or the Tris/NaCl buffer. The skim milk and fish gelatin were omitted in this comparison to avoid interference of these proteins in the analysis. It has been reported that the addition of these additives may improve the extraction efficacy of proteins,[31] suggesting that the actual amount of proteins extracted by the Veratox or BioKits could be greater than the values indicated in Table 4. However, this possibility still would not explain the underestimation of residual protein in heat-treated samples by these two test kits.

The discrepancy in protein quantitation between the ELISA kits and BCA could also be due to the heat-induced changes in the immunoreactivity of extracted proteins, thus affecting the proper recognition by the antibodies used in the test kits. Westphal et al.[14] showed that the antibodies provided in ELISA kits they evaluated exhibited a reduced binding to proteins extracted from dark-roast peanut flour relative to proteins extracted from light-roast peanut flour. Pomés et al.[25] reported that roasting at 177 °C for 15 min resulted in a decrease in the binding affinity between Ara h 1 and certain monoclonal antibodies by 100-fold. Autoclaving caused a significant decrease in the antigenicity of Ana o 1, a vicilin in cashew nut, and rendered the protein undetectable on a Western blot when probed with certain antibodies.[32] In a sandwich ELISA, a protein with decreased antigenicity or a weaker binding to the kit antibody will result in a lower OD reading. In interpolation from a standard curve that is constructed on the basis of native proteins, this can lead to a lower level of quantitation and, thus, an underestimation of the target protein.

The greater degree of underestimation by the BioKits can be explained by the kit's targeting of a relatively heat labile protein, Ara h 1, for detection. The lower extractability of Ara h 1 in comparison with other proteins in highly processed samples would result in a lower level of protein registered by the test kit. With the use of a conversion factor that was typically designed on the basis of unheated peanut, this would translate to a lower reading in parts per million peanut material. The greater reduction in the antigenicity of Ara h 1 in comparison with other proteins under elevated heat could also contribute to the greater degree of underestimation by this kit.

The current study demonstrated that protein quantitation by immunoassay may not be accurate when dealing with thermally processed samples due to the heat-induced changes in protein solubility and antigenicity. Different proteins may be affected by thermal processing in different manners, which can lead to differences in quantitation between different ELISA tests. Designing ELISA tests that target marker proteins that are structurally and immunochemically stable throughout food manufacturing may help to alleviate the problems associated with ELISA detection in processed foods. Westphal et al.[14] suggested that Ara h 2 could be a good candidate for this purpose as it retains most of its immunoreactivity in both light and dark peanut flours. However, Schmitt et al.[30] showed that the solubility of Ara h 2 and the ability to bind with anti-Ara h 2 antibody decreased in peanut samples boiled or roasted for extended periods of time, suggesting that underestimation may be unavoidable when ELISA tests are used for quantitation of peanut residues in highly processed foods.

Food manufacturers are in need of validated methods. Guidance on the validation procedures for quantitative food allergen ELISA has recently been published.[33] The performance of commercially available ELISA test kits for the detection of peanut protein in different food matrices has been evaluated in multilaboratory studies.[34,35] Many of these studies used samples spiked with target allergens but have not gone through actual food processing conditions, casting doubt on the performance

of these tests for detection of allergens in processed foods. In recent years, as the potential effects of thermal treatments on ELISA quantitation of protein became recognized, the use of incurred samples (i.e., samples with a known amount of target allergens processed in a manner mimicking industrial manufacturing processes) in validation studies has increased.[19,23] Considerations for the preparation of naturally incurred standards in different food matrices have been discussed.[10] It is not possible to produce incurred standards for all manufacturing conditions, but a better understanding of the effect of different heating conditions on ELISA quantitation, as presented in the current study, will aid in the design of more relevant incurred standards for future validation studies.

Food manufacturers assess risk based on allergen content, and many rely on the use of ELISA test kits to determine the levels of allergens in food and thus the associated risk. Food manufacturers need to recognize that ELISA test kits could indicate low levels of protein in highly processed food when proteins are actually present in high levels. These considerations are also essential when ELISA kits are used to validate allergen control programs, such as allergen cleaning programs.[6] A low reading registered by ELISA test from swabs of food contact surfaces soiled with highly processed materials may not necessarily indicate a low level of allergen residues present.

Allergen risk assessment based on ELISA test results can be further complicated by the fact that the impact of processing on ELISA analysis may not reflect the true effect on human IgE reactions. For example, Koch et al.[18] compared commercial peanut ELISA kits with human serum-based immunoassays and showed that whereas the response of ELISA test kits to roasted peanuts was dramatically reduced, the binding by human sera was not affected. Mondoulet et al.[36] also showed that no significant difference in IgE immunoreactivity was observed between whole protein extracts from raw and roasted peanuts, and the IgE binding capacity of purified Ara h 1 and Ara h 2 actually increased as a result of roasting. Nogueira et al.[21] reported that the polyclonal antipeanut antibodies used in the test kits they evaluated did not follow the same affinity pattern exhibited by human IgE toward roasted peanuts.

In conclusion, this study showed that commercial ELISA kits may not be able to accurately determine the amount of protein present in thermally processed foods due to heat-induced changes in the solubility and immunoreactivity of target proteins. Users need to be aware of such limitations and take into account the nature of the test, the intended target, and the heat treatment conditions before applying ELISA test kits for evaluation of food allergen control programs or utilizing the test results for allergen risk assessment.

AUTHOR INFORMATION

Corresponding Author

*Phone: (708) 728-4149. Fax: (708) 728-4177. E-mail: tongjen.fu@fda.hhs.gov.

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