

***Performance Tested Method*SM Multiple Laboratory Validation Study of ELISA-Based Assays for the Detection of Peanuts in Food**

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***Performance Tested Method*SM multiple laboratory validations for the detection of peanut protein in 4 different food matrixes were conducted under the auspices of the AOAC Research Institute. In this blind study, 3 commercially available ELISA test kits were validated: Neogen Veratox[®] for Peanut, R-Biopharm RIDASCREEN[®] FAST Peanut, and Tepnel BioKits for Peanut Assay. The food matrixes used were breakfast cereal, cookies, ice cream, and milk chocolate spiked at 0 and 5 ppm peanut. Analyses of the samples were conducted by laboratories representing industry and international and U.S governmental agencies. All 3 commercial test kits successfully identified spiked and peanut-free samples. The validation study required 60 analyses on test samples at the target level 5 µg peanut/g food and 60 analyses at a peanut-free level, which was designed to ensure that the lower 95% confidence limit for the sensitivity and specificity would not be <90%. The probability that a test sample contains an allergen given a prevalence rate of 5% and a positive test result using a single test kit analysis with 95% sensitivity and 95% specificity, which was demonstrated for these test kits, would be 50%. When 2 test kits are run simultaneously on all samples, the probability becomes 95%. It is**

therefore recommended that all field samples be analyzed with at least 2 of the validated kits.

Peanuts contain large amounts of oil and storage proteins, many of which are allergenic (1–4). Although peanuts are a common source of protein, even minute amounts can cause allergic reactions. Peanut allergens can trigger an immune response in allergic individuals who ingest them, causing a number of reactions that range from mild hives and itching to anaphylaxis (5, 6). Anaphylaxis is a systemic severe allergic reaction, involving vomiting, diarrhea, difficulty breathing, swelling of the mouth and tongue, and a rapid drop in blood pressure. Recently, news about people with peanut allergies being in danger has made headlines (7). It has been estimated that more than 100 people die each year as a result of food allergy related anaphylaxis and thousands more require medical treatment (8).

Despite the growth in the population allergic to foods during the past 20 years, no validated methods for the detection of any food allergens at levels that posed a health concern were available. To address this issue, the U.S. Food and Drug Administration (FDA) in collaboration with AOAC and the manufacturers of commercial assays for the detection of peanut protein (9–11) undertook an effort to validate commercial ELISA-based assays at the *Performance Tested Methods*SM level. The validation process was conducted with 3 laboratories: the FDA Northeast Regional Laboratory (Jamaica, New York); the Allergen Method Development Group, Food Research Division, Bureau of Chemical Safety,

Health Canada (Ottawa, Ontario, Canada); and the National Food Processors Association Laboratory (Washington, DC).

Although earlier versions of these test kits could be used for quantitative purposes, the focus was placed on the qualitative analysis of samples containing 0 and 5 ppm peanut. Each diagnostic kit successfully detected the presence of peanut in each of the spiked samples of the 4 commodities (0 false negatives). The false-positive rate was 0.4, 0.8, and 0.0% for the Veratox[®] for Peanut Allergen Assay manufactured by Neogen, the RIDASCREEN[®] FAST Peanut Assay manufactured by R-Biopharm, and the BioKits Peanut Assay Kit manufactured by Tepnel BioSystems, respectively. AOAC certifications for these test kits have been issued (12–14).

Materials and Methods

Food Matrixes

Breakfast cereals (General Mills Original Cheerios), cookies (Nabisco Honey Teddy Grahams), vanilla ice cream (Höagen-Dazs), and milk chocolate (Hershey) were purchased from a local supplier.

Standard Reference Material

The peanut standard used was peanut butter obtained from the National Institute for Standards and Technology (NIST; Gaithersburg, MD), Standard Reference Material (SRM) No. 2387.

Immuno-Diagnostic Kits

The Veratox for Peanut Allergen Assay manufactured by Neogen Corporation (Lansing, MI), the RIDASCREEN FAST Peanut Assay manufactured by R-Biopharm (Darmstadt, Germany), and the BioKits Peanut Assay Kit manufactured by Tepnel BioSystems and distributed by ELISA Technologies (Gainesville, FL) were obtained from their respective suppliers.

Sample Preparation

Peanut butter spiking solution.—A 50 mg portion of peanut butter was thoroughly mixed into a 2% carboxy methyl cellulose (CMC) solution using a polytron for 2 min. To this suspension, 0.05% thimerosal and 0.25% bovine serum albumin (BSA) were added using a polytron for 2 min (final mass 70 g). A 4.87 g portion of standard peanut butter suspension was further diluted to 25 mL with phosphate-buffered saline (PBS) immediately prior to use for spiking. Homogeneity of the suspension was confirmed by analyzing 4 portions of the solution using the Veratox for Peanut Allergen kit.

Control spiking solution.—The 2% CMC solution contained 0.05% thimerosal and 0.25% BSA in PBS (pH 7.4).

Spiking.—Samples containing 0 and 5 ppm peanut were prepared by adding 200 μ L of either the peanut butter spiking solution or the control spiking solution in a zigzag motion to each food sample.

Cereal.—Cereal was ground in an explosion-proof Waring blender 1/3 full at full speed. Ground products were pooled and mixed by tumbling in a V-shaped blender for 3 h. Two hundred 5 g aliquots were weighed into 250 mL screw-capped polypropylene containers. Half of the containers (100) were spiked with peanut butter, and the other half were spiked with the negative control spiking solution. The samples were stored at 4°C prior to shipping for this validation study.

Cookies.—Cookie samples were prepared as described for the cereal samples.

Ice cream.—Ice cream samples were prepared as described for the cereal samples except for the omission of the grinding and blending steps; the samples were stored at –20°C prior to shipping for this validation study.

Milk chocolate.—Milk chocolate was shaved into thin pieces, stored at –20°C, and blended at high speed into a fine powder in a Waring blender. The two hundred 5 g aliquots were prepared in 250 mL screw-capped polypropylene containers. Half of the containers (100) were spiked with peanut butter spiking solution and the other half with the negative control spiking solution. The samples were stored at 4°C prior to shipping for this validation study.

Coding.—Samples were assigned alpha-numeric codes designed to identify the collaborative laboratory, the test kit being used, and the peanut content without revealing to the analyst any of the information regarding the nature of the samples in this blind study.

Analysis.—Analyses of the samples by the collaborating laboratories were conducted within 7 days of sample preparation.

Immuno-Diagnostic Assays

Veratox.—The Veratox for Peanut Allergen Assay was used as prescribed by the manufacturer. This entailed mixing the entire 5 g sample with 1 scoop of blocking reagent and 125 mL prewarmed extraction buffer at 60°C for 15 min in a water bath shaker at 150 rpm. The extract was then centrifuged, and 150 μ L of the aqueous layer along with 150 μ L of each standard (supplied by Neogen) was placed in the red marked strips. A 100 μ L volume of each extract and standard was then transferred into the strip of antibody-coated microwells. After 10 min of incubation at room temperature, the strips were washed 6 times with wash buffer. A 100 μ L volume of the detector antibody conjugate was then pipetted into each of the wells and incubated for 10 min at room temperature. The strip was washed again 6 times with wash buffer, and 100 μ L K-Blue[™] substrate was added to each well. After 10 min of incubation at room temperature, 100 μ L of the acid stop solution was added to each well and the strip was read at 650 nm using the 303EIA Reader (Awareness Technology, Inc., Palm City, FL) with the Peanut Allergen Program. A minimum correlation coefficient (r) >0.98 for the calibration curve was required, or else the analysis had to be repeated.

RIDASCREEN.—The RIDASCREEN FAST Peanut Assay was used as prescribed by the manufacturer. This entailed mixing the entire 5 g sample with 100 mL prewarmed

extraction buffer for 20 min at 60°C in a water bath shaker. The extract was then centrifuged, and the aqueous layer (nonfat) was removed for analysis. A 100 µL volume of each sample and each standard supplied by R-Biopharm was placed in the antibody-coated microwells. After 30 min of incubation at room temperature, the strips were washed 3 times with wash buffer; 100 µL enzyme conjugate was then pipetted into each of the wells and incubated for 30 min at room temperature. The strip was washed again 3 times with wash buffer and 50 µL substrate and 50 µL chromogen were added to each well. After 30 min of incubation at room temperature, 100 µL acid stop solution was added to each well, and the absorbance was read at 450 nm against an air blank.

BioKits.—The *BioKits* Peanut Assay Kit was used as prescribed by the manufacturer. This entailed mixing the entire 5 g samples with 50 mL prewarmed extraction buffer to the cereal, cookie, and chocolate samples or 50 mL extraction buffer at room temperature to the ice cream samples and shaking for 15 min at room temperature. The samples were centrifuged at 10 000 × g for 10 min, and the aqueous layer was removed for further analysis. This involved diluting the aqueous fraction 1:10 and pipetting 100 µL into the antibody-coated microwells. Standards supplied by the manufacturer were used without further dilution. After 30 min of incubation at room temperature, the strips were washed 5 times with wash buffer. A 50 µL volume of the anti-conarachin-A Biotin was then pipetted into each of the wells and incubated for 15 min at room temperature. The strip was washed again 5 times with wash buffer, and 50 µL Avidin peroxidase conjugate was then pipetted into each of the wells and incubated for 15 min at room temperature. The wells were washed again 5 times, and 100 µL tetramethylbenzidine substrate was added to each well. After 15 min of incubation at room temperature, 50 µL stop solution was added to each well, and the absorbance was read at 450 nm against an air blank.

Specificity of Immuno-Diagnostic Assays

The specificity of all 3 commercial test kits was examined with 32 challenge commodity extracts (*see below*) and conducted by the manufacturers. The 32 challenge commodities were barley, buckwheat, wheat, wheat gluten, rice, rye, oats, corn, soybeans, green peas, lima beans, chick peas, sunflower seeds, pumpkin seeds, sesame seeds, poppy seeds, almond, Brazil nut, cashew, chestnut, coconut, hazelnut, macadamia, pistachio, pecan nut, pine kernel, walnut, milk proteins as skim milk powder, cocoa, lecithin, bovine gelatin, and porcine gelatin.

Veratox.—A 5 g sample of each challenge commodity was extracted with 125 mL PBS and 1 scoop extraction additive by shaking at 60°C for 15 min in a water bath. The extract was filtered and the Veratox for Peanut test was conducted according to manufacturer's guidelines. Samples were analyzed in duplicate and repeated twice on 2 different days.

RIDASCREEN.—One-gram samples of each challenge commodity were extracted with 20 mL extraction buffer, and

the RIDASCREEN FAST Peanut test was performed according to manufacturer's specifications. If a sample displayed cross-reactivity, it was further diluted to 100 ppm and re-analyzed with the RIDASCREEN assay.

BioKits.—Four 5 g portions of each challenge commodity were prepared. Three of the 5 g portions were spiked to a final concentration of 5 ppm peanut butter. The fourth 5 g portion was used as a control and spiked with buffer. Each sample was analyzed according to the manufacturer's guidelines.

Ruggedness

Veratox.—According to the instructions supplied with the Veratox for Peanut Allergen Assay, the analysis of sample extracts generated at 60°C should be performed at room temperature. The ruggedness of the kit to variations in temperature was examined by performing the assay with the components warmed to 24°C and the extracts warmed to 24°, 30°, and 32°C. The experiment was conducted in quadruplicate on 2 different days.

RIDASCREEN.—The influence of temperature variation on the RIDASCREEN FAST Peanut Assay was investigated by analyzing a blank sample and 2 spiked samples (5 ppm) in triplicate at 18°, 24°, and 30°C as part of 2 separate studies conducted by 2 technicians. All reagents and samples were equilibrated to the specified temperature for 30 min prior to analysis.

BioKits.—The *BioKits* Peanut Assay Kit test of ruggedness was split into 2 parts covering the extraction and ELISA activities. Analyses were performed with variations in the extraction conditions, such as sample weight, extraction volume, extraction buffer components, extraction buffer temperature, extraction motion, extraction time, and extraction dilution. Also varied were the sample/reagent volume, laboratory temperature, incubation times, washing, mixing, addition drift, and timing after Acid Stop addition. Additional robustness and ruggedness tests performed by the manufacturers focused on the temperature of extraction and the volumes of the reagents and samples. The results were consistent with the specifications of each assay and are available from the manufacturer.

Data Analysis

Although earlier versions of all 3 commercial kits could be used for the quantitative detection of peanut protein, the focus of this validation study was the qualitative detection of 0 and 5 ppm peanut. As such, the results are presented as the fraction and the percent of samples correctly identified. The kits were performance-validated for use in detecting peanut, on a pass/fail basis, at a target level of 5 µg peanut/g food item. The validation study required 60 analyses on test commodities at the target level of 5 µg peanut/g food and 60 analyses at a peanut-free level, which was designed to ensure that the lower 95% confidence limit for the sensitivity and specificity would not be <90%.

Results and Discussion

Specificity

Veratox.—The Veratox for Peanut Allergen Assay did not display cross-reactivity with any of the 32 challenge commodities listed in Table 1. The maximum response observed with a challenge extract was an optical density (OD) of 0.161, indicative of 0.62 ppm peanut. This was considered within the background, inasmuch as it was less than the 2.5 ppm limit of detection specified by the manufacturer. Thus, the Veratox for Peanut Allergen Assay was ascribed as specific for peanuts with no cross-reactivity to any of the 32 challenge commodities.

RIDASCREEN.—The cross-reactivity of the RIDASCREEN FAST Peanut Assay with the 32 commodities is summarized in Table 1. Analyzed at 50 mg/mL, the chick peas, green peas, and lima beans generated a response comparable with that observed with standards containing 5–10 ppm peanut. Upon dilution to 100 ppm, cross-reactivity to green peas, chick peas, and lima beans did not occur. All other substances were comparable to background, without any measurable cross-reaction.

BioKits.—The BioKits Peanut Assay Kit was challenged with potentially cross-reactive proteins from the 32 challenge commodities listed in Table 1. The BioKits Peanut Assay Kit correctly identified as negative all 32 potentially cross-reactants.

Detection of 5 ppm Peanut

Veratox.—The 3 collaborative laboratories using the Veratox for Peanut Allergen Assay correctly identified 100% (240/240) of breakfast cereal, cookies, ice cream, and milk chocolate that were spiked with 5 ppm peanut and correctly identified >99% (239/240) of the samples of breakfast cereal, cookies, ice cream, and milk chocolate that did not contain any measurable amounts of peanut (Table 2).

RIDASCREEN.—The 3 laboratories correctly identified 100% (240/240) of the breakfast cereal, cookies, ice cream, and milk chocolate samples spiked with 5 ppm peanut using the RIDASCREEN FAST Peanut Assay. Analyses of the breakfast cereal, cookies, ice cream, and milk chocolate samples that did not contain any measurable amounts of peanut correctly identified 99% (238/240; Table 2).

BioKits.—Using the BioKits Peanut Assay Kit, all 3 laboratories correctly identified 100% (240/240) of the samples of breakfast cereal, cookies, ice cream, and milk chocolate samples spiked with 5 ppm peanut and correctly identified 100% (240/240) of the breakfast cereal, cookies, ice cream, and milk chocolate that did not contain any measurable amounts of peanut (Table 2).

In this validation, 3 ELISA-based diagnostic kits were examined. Although each kit method used procedures specific to itself, all 3 ELISAs used similar extraction procedures. These included mixing the sample with prewarmed extraction buffer and incubation in a shaker bath followed by either filtration or centrifugation.

Table 1. Cross-reactivity with extracts^a

Extract concentration	RIDASCREEN		
	Veratox assay 40 mg/mL	assay 50 mg/mL	BioKits assay 50 mg/mL
Grains			
Barley	—	—	—
Buckwheat	—	—	—
Wheat	—	—	—
Wheat gluten	—	—	—
Rice	—	—	—
Rye	—	—	—
Oat	—	—	—
Corn	—	—	—
Legumes and vegetables			
Soybean (dosoy soya flour)	—	—	—
Green pea	—	— ^b	—
Lima (butter) bean	—	— ^b	—
Chick pea	—	— ^b	—
Seeds			
Sunflower	—	—	—
Pumpkin	—	—	—
Sesame	—	—	—
Poppy	—	—	—
Nuts			
Almond	—	—	—
Brazil	—	—	—
Cashew	—	—	—
Chestnut	—	—	—
Coconut	—	—	—
Hazelnut	—	—	—
Macadamia	—	—	—
Pistachio	—	—	—
Pecan	—	—	—
Pine nut kernel	—	—	—
Walnut	—	—	—
Miscellaneous			
Skim milk powder	—	—	—
Cocoa	—	—	—
Lecithin	—	—	—
Bovine gelatin	—	—	—
Porcine gelatin	—	—	—

^a Extracts were analyzed using the Veratox[®] for Peanut Allergen Assay, the RIDASCREEN[®] FAST Peanut Assay, and the BioKits Peanut Assay Kit at 40 mg/g by Neogen (Lansing, MI).

^b Analysis at 50 mg/mL generated a response comparable to that observed with standards containing 5–10 ppm peanut. However, analysis at 0.1 mg/mL generated a response within background.

Table 2. Qualitative detection of peanut in spiked samples^a

Kit	0 µg peanut/g ^b				Total	5 µg peanut/g ^b				Total
	Cereal	Cookies	Milk chocolate	Ice cream		Cereal	Cookies	Milk chocolate	Ice cream	
Veratox [®] Assay	59/60	60/60	60/60	60/60	239/240	60/60	60/60	60/60	60/60	240/240
RIDASCREEN [®] Assay	59/60	59/60	60/60	60/60	238/240	60/60	60/60	60/60	60/60	240/240
BioKits Assay	60/60	60/60	60/60	60/60	240/240	60/60	60/60	60/60	60/60	240/240

^a All samples were analyzed by the participating laboratories within 7 days of receipt. Data were compiled by the AOAC Research Institute.

^b The fraction of correctly identified samples for each of the commodities along with the total for each level spiked.

In the examination of specificity, it should be noted that there is a connection between concentration and cross-reactivity. With the RIDASCREEN FAST Peanut, this connection became apparent when RIDASCREEN FAST Peanut was challenged with concentrated extracts. The method tested positive for peanut in concentrated extracts of green peas, chick peas, and lima beans; however, when the extracts were diluted to 100 ppm, the cross-reactivity was not observed.

In conclusion, the results from the study indicated that the test kits correctly identified the test samples ($\pm$). Though the previously mentioned validation criteria are useful performance indicators, it is important in a regulatory sense to know how the test kits will be expected to perform when actually applied in practice to screen lots of product that have varying prevalence rates with respect to the presence of an allergen. This performance is estimated as the probability that the test sample actually contains an allergen given a positive response by the test. For example, the probability that a test sample contains an allergen, given a prevalence rate of 5% and a positive test result using a single test kit analysis with 95% sensitivity and 95% specificity, which was demonstrated for these test kits, would be 50%; however, when 2 test kits are run simultaneously on all samples, the probability becomes 95%.

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