

FOOD COMPOSITION AND ADDITIVES

Preparation of Peanut Butter Suspension for Determination of Peanuts Using Enzyme-Linked Immunoassay Kits

MARY W. TRUCKSESS, VICKERY A. BREWER, KRISTINA M. WILLIAMS, and CARMEN D. WESTPHAL

U.S. Food and Drug Administration, Center for Food Safety and Applied Nutrition, 5100 Paint Branch Pkwy, College Park, MD 20740

JAMES T. HEERES

Joint Institute for Food Safety and Applied Nutrition, University of Maryland, 5100 Paint Branch Pkwy, College Park, MD 20740

Peanuts are one of the 8 most common allergenic foods and a large proportion of peanut-allergic individuals have severe reactions, some to minimal exposure. Specific protein constituents in the peanuts are the cause of the allergic reactions in sensitized individuals who ingest the peanuts. To avoid accidental ingestion of peanut-contaminated food, methods of analysis for the determination of the allergenic proteins in foods are important tools. Such methods could help identify foods inadvertently contaminated with peanuts, thereby reducing the incidence of allergic reactions to peanuts. Commercial immunoassay kits are available but need study for method performance, which requires reference materials for within- and between-laboratory validations. In this study, National Institute of Standards and Technology Standard Reference Material 2387 peanut butter was used. A polytron homogenizer was used to prepare a homogenous aqueous Peanut Butter suspension for the evaluation of method performance of some commercially available immunoassay kits such as Veratox for Peanut Allergen Test (Neogen Corp.), Ridascreeen Peanut (R-Biopharm GmbH), and Bio-Kit Peanut Protein Assay Kit (Tepnel). Each gram of the aqueous peanut butter suspension contained 20 mg carboxymethylcellulose sodium salt, 643 µg peanut, 0.5 mg thimerosal, and 2.5 mg bovine serum albumin. The suspension was homogenous, stable, reproducible, and applicable for adding to ice cream, cookies, breakfast cereals, and chocolate for recovery studies at spike levels ranging from 12 to 90 µg/g.

It is estimated that about 1–2% of adults and 5–8% of children have some type of food allergy, with allergy to peanuts, eggs, and milk being responsible for over 80% of food hypersensitivity reactions (1). In the last 10 years, there has been an increase in both the incidence and severity of allergic reactions to peanut proteins (2–4). The only option available for the prevention of peanut hypersensitivity reaction is strict avoidance. However, the potential for accidental ingestion remains high due to heavy consumption of peanut-containing foods; peanut proteins in foods as the result of mishandling or inaccurate labeling; and the legal use in the ingredient statement of the terms “artificial” or “natural flavor” and not “peanut flavor” (5).

In order to minimize the risk of serious or fatal outcomes due to accidental consumption of peanut allergens, and to provide science-based guidance to consumers and manufacturers, accurate methods for the detection of small amounts of peanut proteins are necessary. It has been determined that the consumption of as little as 100 µg peanut protein can cause allergic reactions in a peanut-allergic individual (6). Ideally, the detection limit of peanut or peanut proteins in an *in vitro* assay system should be at or below the level capable of inducing an allergic response in peanut-sensitive patients.

Recent research efforts have been centered on the development of methods for the detection of peanut proteins in food. In 2001, there were 4 commercial kits available for the detection of peanut protein, all based on some form of immunochemical detection. Because there is no uniform peanut protein standard known, kit manufacturers all use their own peanut protein extracts as standards and as immunogens. Three of the kits use conversion factors to translate data of peanut proteins to peanuts. Quantitative results of the identical samples differ when analyzed by different kits (7, 8). There is no established procedure for preparation of a peanut spiking solution for recovery studies. To solve these problems, a reference peanut butter was obtained from the National Institute of Standards and Technology (NIST). A peanut butter suspen-

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Corresponding author's e-mail: mary.trucksess@cfsan.fda.gov.

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sion was prepared. The suspension was evaluated for homogeneity, stability, and applicability for spiking into ice cream, cookies, breakfast cereals, and chocolate. These foods were selected because they are often produced with shared, dry clean, or product flush equipment that is susceptible to cross-contamination.

Experimental

Food Samples

Samples were purchased from local grocery stores and consisted of ice cream (Häagen-Dazs), cookies (Nabisco Teddy Grahams), breakfast cereals (General Mills Cheerios), milk chocolate (Nestlé), and dark chocolate (Hershey). Breakfast cereals and cookies were each ground in a Waring blender at high speed in small portions. The portions were combined and mixed by shaking in a polypropylene bag. Chocolate was cut into small pieces and frozen at -20°C , and then ground in a Waring blender in small portions with the addition of powdered dry ice. After the sublimation of the dry ice, all portions were combined and mixed.

Apparatus

(a) *Polytron homogenizer*.—Cat. No. 27 11 2005, Model PT10/35 (Brinkmann Instruments, Inc., Westbury, NY).

(b) *Generator*.—Cat. No. 27 11 3959, Model PTA 20TS (Brinkmann).

(c) *Centrifuge*.—Operating at $>23\,500\text{ g}$.

(d) *Shaker*.—IKA-Schuttler MTS 4 shaker (orbital shaker; Fisher Scientific, Pittsburgh, PA), or equivalent.

(e) *Pipet and pipet tips*.—20, 100, and 1000 μL ; 5 and 10 mL adjustable air displacement pipets with disposable tips.

(f) *Multichannel pipet*.—Labsystems 8- or 12-channel, 200 μL (Fisher Scientific), or equivalent.

(g) *Enzyme-linked immunosorbent assay (ELISA) plate reader*.—Microwell spectrophotometer with variable wavelength, Spectra Max 340 (Molecular Devices Corp., Sunnyvale, CA), or equivalent.

(h) *ELISA strip reader*.—Stat Fax 302 plus (Awareness Technology Inc., Palm City, FL).

(i) *Microwell holder*.—Dynatech 96 well holder (Fisher Scientific).

(j) *Reagent boat*.—Plastic, 30 mL, wedge-shaped troughs for use with multichannel pipet.

(k) *Software*.—SOFTmax PRO2.11 (Molecular Devices Corp.) and Log/logit (Awareness Technology Inc.).

Reagents

(a) *Bio-Kit Peanut Protein Assay Kit*.—ELISA Technologies (distributor; Gainesville, FL).

(b) *Veratox for Peanut Allergen Test*.—Neogen Corp. (Lansing, MI).

(c) *Ridascreen Peanut*.—R-Biopharm GmbH (Marshall, MI).

(d) *Standard Reference Materials (SRM) 2387 Peanut Butter*.—NIST (Gaithersburg, MD). The SRM 2387 Peanut Butter was prepared by a peanut butter manufacturer using its

usual recipe for creamy peanut butter: roasted peanuts, sugar, partially hydrogenated vegetable oils, and salt. Most of the peanuts were Florunner. The final product contained about 90% peanuts and included no other sources of protein. The reference value for total protein in SRM 2387 is $22.2 \pm 0.5\%$. Protein was calculated from nitrogen using a factor of 5.46 (9).

(e) *Carboxymethylcellulose sodium salt*.—Cat. No. C9481, Sigma-Aldrich Chemicals (St. Louis, MO).

(f) *Bovine serum albumin*.—Cat. No. B4287 (Sigma-Aldrich).

(g) *Thimerosal*.—Cat. No. T8784 (Sigma-Aldrich).

(h) *Phosphate buffered saline*.—Cat. No. P-3813 (Sigma-Aldrich).

Procedure

Preparation of 2% CMC solution.—The solution was prepared by using a polytron to homogenize 12 g CMC in 600 mL distilled water for 5 min; then 300 mg thimerosal and 1.5 g bovine serum albumin were added. The mixture was homogenized for an additional 1 min. The appearance of the solution was milky, and then turned clear upon standing at room temperature for 1 h.

Preparation of peanut butter suspension.—A 50 mg amount of peanut butter was weighed into a 100 mL beaker, and CMC solution was added into the same beaker to a total weight of 70 g. A small, flat, stainless steel spatula was used to disperse the peanut butter into the solution until there was no visible peanut butter adhering to the spatula. The mixture was homogenized with the polytron for 5 min.

Methods of Analyses

Three commercially available immunoassay kits were used: Veratox for Peanut Allergen Test, Ridascreen Peanut, and Bio-Kit Peanut Protein Assay Kit. The assay kits are double antibody sandwich format and are based on the specific interaction between antibody and antigen. The peanut proteins are sandwiched between 2 antibodies; one antibody captures the analytes, and the second antibody is conjugated to the enzyme horseradish peroxidase. The enzyme uses tetramethylbenzidine/hydrogen peroxide for color development. A stopping reagent is then added to stop the reaction. The intensity of the color is proportional to the peanut protein concentration and is measured with an ELISA reader. Veratox test kit was used for homogeneity and stability studies. All 3 kits were used for recovery studies of peanut in extraction buffer and for determination of peanut in control food. Veratox and Ridascreen test kits were used for recovery studies of peanut in control food samples. The procedure of the Veratox test kit was the most rapid of the 3 kits; therefore, the majority of our work was done using this kit. The Bio-Kit had the longest extraction procedure and incubation time, and only 2 standards which required dilution for calibration curve were provided. There was no extraction buffer included in the kit.

Testing

Homogeneity.—Three suspensions of similar composition were prepared on the same day. Four portions (ca 100 mg

Table 1. Stability of peanut butter suspension^a determined by the Veratox for Peanut Allergen kit

Temperature, °C	Recovery, % ^b			
	Day 2	Day 7	Day 10	Day 17
22	103	93	89	78
4	93	81	84	71
-20	96	105	105	83

^a With no bovine serum albumin added.^b $n = 4$, the repeatability relative standard deviation, RSD_r , ranged from 8.7 to 12.4% at a level of 12.8 $\mu\text{g/g}$.

each) of each suspension were weighed into 240 mL polypropylene containers and were extracted and analyzed in duplicates with the Veratox for Peanut Allergen Test kit following the manufacturer's recommended procedure. The test samples were extracted with buffers (included in the kits) at 60°C in a shaking water bath. The extracts containing the peanut proteins were centrifuged. The supernatants were separated, diluted with buffer (included in the kit), and analyzed.

Stability.—Test samples (100 mg peanut butter suspension) were stored at room temperature (about 22°), 4°, and -20°C. Test samples were removed at 1-, 2-, 3-, and 5-week intervals, and analyzed using the Veratox Peanut Allergen Test kit.

Control food samples.—Food samples prepared according to the *Experimental* section were analyzed by using the 3 immunoassay kits.

Recovery.—Recoveries of peanut in extraction buffer were determined using the 3 commercial immunoassay kits.

The Veratox for Peanut Allergen kit and the Ridascreen Peanut kit were used for recoveries of peanut added to foods. Four types of commercial blank food samples that were previously tested for the presence of peanut were used. No peanut was found in the blank food samples at the limit of detection of the methods (about 1 μg peanut/g food). The approximately 48, 96, or 192 mg of suspension containing 30, 60, and 120 μg peanut, respectively, was weighed into a polypropylene screw-capped container, and the blank sample (5 g for Veratox kit, 1 g for Ridascreen kit) was added to each and analyzed. The added peanut levels for the Veratox kit were 6, 12, and 24 $\mu\text{g/g}$; the added peanut levels for the Ridascreen kit were 30, 60, and 120 $\mu\text{g/g}$.

Results and Discussion

Effect of CMC on the Suspension

The effect of variation of CMC concentration on the peanut butter suspension was tested. CMC concentrations of 0.5, 1.0, 1.5, and 2.0% were used. CMC at concentration <1.5% showed precipitation upon standing at room temperature after 2 days. A suspension containing 20 mg CMC/g and about 720 μg peanut butter/g was prepared. The theoretical value of peanut in each suspension was calculated on the basis of the peanut concentration of SRM 2387 peanut butter of 90%. For

example, a 70 g suspension containing 50 mg Peanut Butter has a theoretical value of:

$$\text{Peanut} = 50 \text{ mg}/70 \text{ g} \times 90\%, \text{ or } 50\,000 \mu\text{g}/70 \text{ g} \times 0.9 = 643 \mu\text{g/g}$$

Effect of Thimerosal on the Suspension

In the preliminary study, the Veratox test kits were used for the homogeneity testing and stability study. Six portions (100 mg suspension) of the same suspension (50 mg peanut butter/70 g 2% CMC) were analyzed according to the procedure (extracted with 125 mL extraction buffer; this is the amount used for 5 g food). Results were compared with the theoretical value (12.8 $\mu\text{g/g}$ food). The average recovery was 111%, with a repeatability relative standard deviation (RSD_r , $n = 6$) of 8.2%. The suspension was kept at room temperature for 5 days. Six samples were analyzed again in the same manner. Average recovery of peanut was 79% with an RSD_r of 10.1%. From these preliminary results, it was concluded that the suspension was homogeneous but was unstable upon standing at room temperature. Subsequently, an antibacterial agent, thimerosal, was added as an ingredient.

Effect of Bovine Serum Albumin on the Suspension

The stability of the suspension was determined as affected by temperature over time of storage. Identical portions of suspensions were stored at 22° (room temperature), 4°, and -20°C, and analyzed after 2, 7, 10, and 17 days. The temperature effect was minimal. The storage time effects were shown by recoveries in Table 1. After 17 days, the recoveries of all suspension dropped to 71–83%. We concluded that the suspensions were stable at all 3 temperatures for at least 10 days. The low recoveries might be due to peanut protein degradation. Recoveries were improved by the addition of bovine serum albumin to the suspension. This was tested on identical samples. Subsequently, bovine serum albumin was added to the list of reagents in the suspension as a stabilizer. New suspensions were stored only at room temperature.

Homogeneity testing was repeated for the aqueous peanut butter suspensions containing CMC, peanut butter, thimerosal, and bovine serum albumin. Three suspensions were prepared on the same day. The following day, 4 por-

Table 2. Homogeneity and recovery of peanut^a in extraction buffer^b

Peanut suspension	Peanut added, $\mu\text{g/g}$	Peanut found, $\mu\text{g/g}$	Recovery ^c , %	RSD_r ^d , %
1	6.1	6.6	108	1.7
2	6.3	5.8	92	4.4
3	5.8	5.8	100	6.1

^a Using Veratox for Peanut Allergen kit.^b Use of 125 mL extraction buffer (same volume for 5 g test sample).^c $n = 8$.^d Within-laboratory repeatability relative standard deviation (RSD_r) = $SD/\text{mean (recovery)} \times 100$.

Table 3. Recovery (%) of peanut added to foods by the Veratox for Peanut Allergen kit

Peanut added, $\mu\text{g/g}^a$	Ice cream		Cookies		Breakfast cereal		Milk chocolate	
	Rec., %	RSD _r , %	Rec., %	RSD _r , %	Rec., %	RSD _r , %	Rec., %	RSD _r , %
6	89	4.7	85	8.0	102	5.8	72	5.5
12	101	5.4	94	22.2	111	6.0	72	5.7
24	101	4.0	89	14.3	98	2.9	102	16.8
Mean	97		89		104		82	

^a $n = 6$.

tions of each were extracted and analyzed in duplicate. Data of homogeneity testing are shown in Table 2. At levels of 5.8–6.3 $\mu\text{g/g}$, the average recoveries ($n = 8$) ranged from 92 to 108%, and RSD_r ranged from 1.7 to 6.1%. After 5 weeks' storage, recoveries of suspension at 12.8 $\mu\text{g/g}$ level in extraction buffer ranged from 95 to 109% ($n = 6$). Suspensions were stable for at least 5 weeks at room temperature (22°C).

Recovery Studies

Recoveries of suspension using 3 different commercial kits at various levels were determined. The Veratox for Peanut Allergen kit recoveries ranged from 94 to 107% at 6 $\mu\text{g/g}$ (4 test extracts, 4 analyses of each extract); the Ridascreen kit ranged from 55 to 60% at 24 $\mu\text{g/g}$; and the Bio-Kit ranged from 86 to 94% at levels of 3.2, 6.4, and 12.8 $\mu\text{g/g}$. All gave coefficients of variation <10%.

Four common types of foods, breakfast cereals, chocolate (milk chocolate and dark chocolate), ice cream, and cookies, were spiked and analyzed for recovery studies using the Veratox and Ridascreen kits. All commodities except ice cream were ground to powder and a composite of each food was made by mixing. Analyses of replicate test samples ($n = 4$) gave results similar to or below the negative control standard (0 $\mu\text{g/g}$) of the 3 test kits. Test samples were spiked at 6, 12, and 24 $\mu\text{g/g}$ for testing with the Veratox kit, and were spiked at 30, 60, and 120 $\mu\text{g/g}$ for the Ridascreen kit. Tables 3 and 4 give data of the recovery study of spiked foods. Recoveries of peanut in ice cream, cookies, and breakfast cereals ranged from 85 to 111% for the Veratox kit, and from 60 to

81% for the Ridascreen kit. Milk chocolate gave recovery data similar to that of the other foods using the Veratox kit. Recoveries were about 20% lower than the other foods using the Ridascreen kit. Recoveries for dark chocolate were lower than that for ice cream, cookies, and breakfast cereals. They were about 20–30% for both kits. In general, the Ridascreen kit gave lower recoveries than the Veratox kit. Quantitative results of identical samples differ when analyzed by different kits because the antibodies and the kit standards are different (7, 8).

Additions of various proteins such as nonfat dry milk, fish gel, caseins, and skim milk were investigated to improve the recovery of peanut protein from spiked dark chocolate. The Veratox kits were used to analyze spiked test samples at 12 $\mu\text{g/g}$ before extraction. Various amounts (1, 2, 3, 4, 5, 10, 15, 20, 25, and 30 g) dry milk was used. It was found that 5 g dry milk gave maximum recoveries of about 60% for the Veratox kit as shown in Table 2. Fresh-water fish gelatin (10%) did not improve recoveries. When skim milk was used as solvent to prepare the phosphate saline buffer in the Veratox kit, or was used as a diluting solvent in the Ridascreen kit, recoveries were similar to those of adding nonfat dry milk.

It is not possible to correlate the total protein content of the NIST reference material to the standards of the kits that were used. The 3 kits (Veratox for Peanut Allergen Test, Ridascreen Peanut Kit, Bio-Kit Peanut Protein Assay Kit) that were used contain standards that consist of water-soluble peanut proteins, and these are used to create a standard curve. However, these standards are calibrated to detect peanut material from a sample extract, not water-soluble peanut protein. In other words, a

Table 4. Recovery (%) of peanut added to foods by the Ridascreen Peanut Kit

Peanut added, $\mu\text{g/g}^a$	Ice cream		Cookies		Breakfast cereal		Milk chocolate	
	Rec., %	RSD _r , %	Rec., %	RSD _r , %	Rec., %	RSD _r , %	Rec., %	RSD _r , %
30	69	3.8	69	9.2	76	11.5	45	13.4
60	70	8.3	67	9.2	81	4.4	45	13.4
120	60	3.5	61	17.3	76	1.3	40	3.1
Mean	66		66		78		43	

^a $n = 6$.

Table 5. Recovery of peanut in ice cream and cookies (data of a workshop study)

ELISA kit	Commodity	Suspension spike level, $\mu\text{g/g}$	Mean recovery, % ^a	RSD _r recovery, % ^a
Neogen	Ice cream	12	99	6.3
		24	98	3.2
	Cookies	12	90	6.9
		24	90	3.5
Bio-Kit	Ice cream	12	99	6.2
		24	97	3.4
	Cookies	12	90	6.8
		24	89	3.5

^a $n = 10$.

10 $\mu\text{g/g}$ standard in an individual kit is equivalent to 10 $\mu\text{g/g}$ peanut material, but does not actually contain 10 $\mu\text{g/g}$ water-soluble peanut proteins. The actual amount of water-soluble peanut protein in these standards is not available from the manufacturer and cannot be determined, since the standards themselves may contain protein stabilizers. Each kit has its own extraction procedure and extraction buffer and, thus, the amount of extractable peanut proteins is highly dependent on the efficiency of the extraction method, which may vary from kit to kit. It is not possible to measure the amount of water-soluble peanut protein in this extract, because the extraction buffers themselves all contain some kind of protein additive. The standardization of different kit methods is necessary. In this study, the use of the NIST Peanut Butter to validate method performance of the different kits was a first effort toward this goal.

Performance of the spiking technique using peanut butter suspension was demonstrated in a 2002 U.S. Food and Drug Administration (FDA)/American Grocery Association Food Allergen Workshop, Jamaica, NY, September 4–6, 2002. Forty participants were divided into 2 groups, with 5 subgroups in each group. Four commercially available kits, Veratox for Peanut Allergen Test, Ridascreen Peanut Kit, Bio-Kit Peanut Protein Assay Kit, and ProLis Peanut Pak (Pro-Lab Diagnostics, Ontario, Canada) were used. Duplicate test samples of ice cream and cookies were spiked at 12 and 24 $\mu\text{g/g}$. Each test sample was analyzed in duplicate (a total of 10 analyses for each test sample). Results of this study are shown in Table 5. The Veratox for Peanut Allergen kit and the Bio-Kit Peanut Protein Assay Kit gave similar recoveries ranging from 89 to 99% at peanut spiked levels of 12 and 24 $\mu\text{g/g}$. Workshop participants were unsuccessful using the Ridascreen kit because of inexperience with the difficult-to-handle repetitive pipettors. The standards in ProLis Peanut Pak are calibrated to detect water-soluble peanut proteins and not peanut material. Subsequently, data obtained using this kit could not be used for calculation of recovery.

Performance of the kits used corresponded to the time of the study. The performance characteristics are likely to change

with time. Our approaches for method evaluation should still be applicable.

Conclusions

The SRM 2387 Peanut Butter obtained from NIST was quite satisfactory for preparing stable homogeneous suspension of peanut material suitable for use as standard in testing ELISA kits. This standard is stable at 22° and 4°C for 5 weeks and is stable >5 weeks at –20°C. It was used successfully for analysis of ice cream, breakfast cereal, cookies, and chocolate in recovery studies at levels of 6–120 μg peanut/g food.

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