

Determination of Protein Levels in Soy and Peanut Oils by Colorimetric Assay and ELISA

JOSEPH E. JABLONSKI, TONG-JEN FU, and LAUREN S. JACKSON

U.S. Food and Drug Administration, National Center for Food Safety and Technology, 6502 S. Archer Rd, Summit-Argo, IL 60501

STEVEN M. GENDEL

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

Analytical methods are needed for measuring the levels of protein from allergenic food transferred into cooking oil. A simple method for determination of total protein in cooking oils was developed. Oil was extracted with phosphate-buffered saline with 0.05% Tween (PBST) and the extracts were partitioned with hexane to remove residual oil. Total protein in the PBST extracts was assayed with bicinchoninic acid (BCA), micro-BCA, reducing-agent compatible BCA and CB-X™ kits. These methods were used to measure recovery of protein from peanut butter spikes of soy and peanut oil in the range of 50–1000 ppm. Recoveries were generally above 70%. However, the BCA and micro-BCA assays were subject to interference and enhanced color formation which were probably due to co-extracted antioxidants present in oil. The reducing agent-compatible BCA and CB-X protein assays reduced interference and gave lower protein values in crude, cold-pressed, and refined peanut oils. Heating oil to 180°C before extraction also reduced interference-induced color enhancement. A commercial ELISA test kit was also used to measure peanut protein in oil spiked with peanut butter. Recovery of peanut residues measured by ELISA was significantly decreased when the peanut butter-spiked oil was heated to 180°C compared to unheated oil. Recovery of spiked peanut butter protein measured by the buffer extraction-colorimetric method was not decreased in heated oil. The method developed here could be used to determine protein levels in crude and refined oil, and to assess the potential for allergen cross-contact from reused cooking oil.

allergenic foods (milk, egg, peanut, tree nuts, fish, shellfish, wheat, and soy) in the United States was reported in detail (2). The majority of food allergies are classified as IgE-mediated (3). Antigen binds to IgE on mast cells and basophils, causing the release of inflammatory mediators, resulting in allergic reaction and in some cases anaphylaxis (4). The rate and occurrence of peanut allergy is increasing according to a recent summary (5); the incidence of peanut allergy in schoolchildren of North America and the United Kingdom is now above 1% (6). Peanut allergy accounts for the majority of fatalities caused by anaphylactic reactions (7).

Allergic responses to peanut are often due to exposure to undeclared peanut allergens present in food. Per capita consumption of peanuts and processed peanut products in the United States was estimated at 6.6 lb by the U.S. Department of Agriculture (USDA) for 2005 (8). Consumption of peanuts in the United States is 51% as peanut butter, 21% snack peanuts, cleaned in-shell, and 19% in candy, according to data from USDA for 2006 (9). Peanut oil consumption was 136 000 metric tons in the United States for marketing year 2007/2008 (10). Some studies show that exposure to peanut oil, specifically crude oil, causes allergic reaction in sensitized subjects (11, 12). The British Dietetic Association has recommended that peanut-allergic individuals avoid consuming crude peanut oils (13). Studies have also shown that allergic and immunologic reaction does not occur with consumption of refined peanut oil (14, 15). Allergenicity of refined vegetable oils (peanut and soy) was reviewed by Crevel et al. (16).

Refined oils go through processing steps, such as alkali-refining, degumming, bleaching, and deodorizing, which greatly reduce the protein content of the oil (17). Reported levels of proteins in peanut oils have ranged from the hundreds of ppm for crude oils to ppb levels in refined oils (15, 16, 18). The variable levels of protein reported in crude and refined cooking oils, and the variability in human response to food allergens have made setting threshold limits for allergenic proteins in oils problematic (2, 19).

Potential for protein contamination of refined oils exists when the oil is reused for cooking purposes. Cooking oil may be reused multiple times in commercial and industrial settings.

Food allergy affects 1–3% of the world's population and 4–6% of children, according to published estimates (1). Incidence of food allergy to the eight most common

It is unclear if reuse presents a risk for allergic consumers. Proteins from allergenic foods may be extracted into the oil and may contaminate subsequent batches of nonallergenic food (5, 20). This type of cross-contamination may result in undeclared allergens appearing in a food product. The level of protein contamination in re-used oil remains to be determined. To evaluate the risk of reused cooking oils, analytical methods that can quickly and accurately detect proteins in oil are needed.

Some methods for measuring protein in cooking oils rely on buffer or water extraction, followed by colorimetric assays (15, 21, 22). One method used cold solvent precipitation and filtration followed by acid hydrolysis and amino acid analysis by HPLC-fluorescence detection (23). Radioimmunoassay inhibition has been used to quantify protein in phosphate-buffered saline (PBS) extracts of cooking oils (20). ELISA assays have been applied to measure peanut residues in peanut and soy oils (24, 25). There is evidence that heat processing of food changes the binding capacity of protein allergens to antibodies and modifies the results of ELISA assays (26). This paper presents a simple buffer extraction method to determine protein levels in soy and several varieties of peanut oils. It also compares results from four colorimetric assays used for the measurement of protein levels in buffer extracts of crude, cold-pressed, and refined cooking oils. Peanut butter was used as a spiking agent to compare recovery of peanut proteins from oil using the buffer extraction-colorimetric method and a commercially available ELISA kit. Spiking studies also simulated cross-contact of cooking oil that may occur when peanuts are roasted or fried in oil. The effect of heating the oil to typical frying temperature on peanut protein recovery was also investigated by both techniques. Colorimetric protein assays are susceptible to matrix interferences which could lead to over- or underestimation of the true value. Dye-binding assays are more susceptible to interference from detergents, whereas redox-complexation assays are more susceptible to interference from reducing agents and lipids (27). The results presented show that methods for measurement of protein in cooking oils must be used with care and consideration for matrix interference.

Experimental

Chemicals and Reagents

Phosphate-buffered saline with 0.05% Tween 20 (PBST) was prepared from packets, or tablets (Fluka Ultra Grade) from Sigma (St. Louis, MO). Deionized water was from an in-house system (Crossbow, Glenwood, IL). Hexane was HPLC grade from Fisher (Fairlawn, NJ). Bovine serum albumin (BSA) standard, 2 mg/mL in PBS, was from Pierce (Rockford, IL).

Samples

Smucker's Natural Creamy Peanut Butter, Wesson 100% Natural Vegetable (Soybean) Oil, Jewel 100% Pure Peanut Oil, and Hollywood Enriched Gold Peanut Oil were obtained

from local grocery stores. Crude Roasted Peanut Oil was kindly donated by Golden Peanut Co. (Alpharetta, GA).

Total Protein and ELISA Assay Kits

Bicinchoninic acid (BCA), Micro-BCA, and Reducing-Agent Compatible (RAC)-BCA total protein kits were obtained from Pierce. The CB-XTM total protein assay kit was from G-Biosciences (St. Louis, MO). The ELISA assay was the Veratox Peanut Kit from Neogen (Lansing, MI).

Equipment and Instrumentation

Model SWB25 shaking bath was from Thermo-Haake (Karlsruhe, Germany). Model 4430 Innova shaking incubator was from New Brunswick Scientific (Edison, NJ). ELx808 plate reader with KC4 software was from Biotek Instruments (Winooski, VT). Statfax plate reader was from Neogen. Amicon Molecular Weight Filters, 10 000 cutoff, were from Millipore (Cork, Ireland). Cool-Touch Deep Fryer was from DeLonghi (Saddlebrook, NJ). HH509R Meter and Temperature Probe was from Omega Engineering, Inc. (Stamford, CT). JAC Model 2010 Ultrasonic sonicating water bath was from Jeiotech (Seoul, Korea). Model 5810R Centrifuge and A-4-62 rotor were from Eppendorf (Hamburg, Germany).

Preparation of Oil Spikes

Oil was spiked with peanut butter by accurately weighing (± 0.01 mg) peanut butter into the cap of a glass bottle. Oil was weighed into the tared bottle to produce the desired protein content based on 25% protein content of the peanut butter from the BCA assay, which matched protein content on the label. The cap was placed on the bottle. The samples were shaken vigorously to homogenously suspend the peanut butter in the oil. Samples were then sonicated and intermittently shaken to produce a homogenous distribution of peanut butter in the oil. Typically, 200 mg peanut butter would be added to 250 g oil to produce a 200 ppm protein spike. Lower level spikes were prepared by diluting with oil to the desired protein level. Samples were stored in refrigerators (4–7°C) until used. Prior to weighing for analysis, oil samples were warmed to 35°C in a sonicating bath, and intermittently shaken until the peanut butter was uniformly suspended.

Preparation of Protein Reference Solutions

PBST was spiked with peanut butter by accurately weighing (± 0.01 mg) peanut butter into the cap of a glass bottle. PBST was added to the tared bottle to produce the desired protein content based on 25% protein assay of the peanut butter. The cap was placed on the bottle, and the samples were shaken vigorously to homogenously suspend the peanut butter in the buffer mixture. Samples were then sonicated and alternately shaken to produce a homogenous distribution of peanut butter in the buffer. Typically, 80 mg peanut butter would be added to 100 mL PBST to produce a 200 ppm protein spike.

Heat Processing of Oil Samples

Glass bottles containing unspiked and peanut-butter-spiked oil were placed in the Cool-Touch fryer with caps loose. Wesson soy oil was added to the fryer to a level just above the level of oil in the sample bottle. The fryer lid was closed, and a temperature probe was placed in the oil. The fryer temperature was set to maximum 370°F (188°C), and after 20 min, a maximum temperature in the range of 174–182°C was achieved. Samples were typically heated for 10 min after the maximum temperature was reached. Total heating time was approximately 30 min. Bottles were carefully removed and allowed to cool at room temperature. Samples were stored at 5–7°C until used.

Oil Extraction

Oil samples (25 g) were weighed into 50 mL polypropylene tubes on a top-loading balance and 6 mL PBST was added. Tubes were horizontally placed in the Innova 4430 shaker set at 180 rpm and 35°C, and extracted for 30 min. Samples were centrifuged at 3000 rpm for 30 min at 4°C, and then warmed at room temperature for at least 10 min. A 9.5 in. Pasteur pipet was used to withdraw the aqueous phase and any sedimented solids and to transfer them to a 15 mL polypropylene tube. The extraction with PBST was repeated to give a nominal total of 12 mL aqueous extract. The aqueous extract was partitioned with 1 mL hexane for a few minutes by shaking the tubes horizontally placed in the shaker at 150 rpm. Samples were centrifuged at 3000 rpm for 5 min at 25°C in a swinging bucket rotor. The volume of aqueous phase was measured from the graduations on the 15 mL centrifuge tube and recorded to the nearest 0.1 mL. A 9.5 in. Pasteur pipet was used to withdraw the aqueous phase and any sedimented solids and to transfer them to a clean 15 mL polypropylene tube. Dilutions were made with PBST as necessary.

Ultrafiltration

For some of the samples, the 12 mL PBST extracts were filtered and concentrated through 10 000 molecular weight cut-off (MWCO) filtration tubes to volumes of 0.2–0.5 mL. These extracts were rinsed three times with 6 mL aliquots of PBST in the filter tube. The rinsed and concentrated extract was removed from the filter with a Pasteur pipet and diluted to 1 mL with PBST.

Protein Assays

The standard BCA, RAC-BCA, and CB-X assays were used with BSA standards in the range of 50–1000 ppm prepared in PBST. The micro-BCA assay was used with BSA standards in the range of 2–40 ppm. A 0 ppm blank was run with each set of standards. The assays were run according to manufacturer instructions in the 96-well plate format. Duplicate readings were made for each standard and for each dilution of the oil extracts. KC4 software (Biotek Instruments) was used to convert absorbance measurements into ppm protein using the standard curve, and accounted for the measured volume of aqueous extract and any dilution factors.

The absorbance readings from the BSA standards were fit to a 2nd order calibration curve. Reagent blanks (PBST processed without oil) were run with each sample set to correct for background in oil samples.

ELISA Assay

The Veratox peanut ELISA kit was used as directed by the manufacturer. Oil (5 g) or PBST reference solution (5 mL) was used as the sample. The reference solution contained the same quantity of protein as the spiked oil sample. Peanut butter (80 mg) was suspended in 100 g oil or 100 g PBST to give a protein concentration of 200 ppm. The peanut standards provided in the kit were in the range of 2.5–25 ppm plus a blank. The wells were read at 650 nm with a Statfax (Neogen) plate reader.

Statistics

All statistical calculations including means, SDs, and *t*-tests were performed with Microsoft Excel™ software.

Results and Discussion

Total Protein in Peanut Butter

A standard BCA assay was used to determine the total protein content of peanut butter used for the spiking experiments. The mean protein value determined was 24.9% protein, which corresponded closely to the protein content (25%) as stated on the nutrient label of the jar of peanut butter. Therefore, the nominal value of 25% was used to calculate the protein level in spiked oil samples.

Recovery of Peanut Butter Protein from Oil: Colorimetric Assay

Table 1 shows recoveries of peanut protein spiked into Jewel 100% refined peanut oil measured by BCA, RAC-BCA, and CB-X colorimetric assays. The control sample protein values were corrected for reagent blank values, and the spiked sample protein values were net protein-corrected for protein in corresponding control samples. The highest recovery was obtained with the RAC-BCA method: 89.8 and 79.7% for the 50 and 200 ppm spikes, respectively. Use of the standard BCA assay gave lower recoveries, 71.5 and 73.9% for the 50 and 200 ppm spikes, respectively. The apparent protein level in the control (unspiked) refined peanut oil decreased from 17.24 to 7.41 ppm when the RAC-BCA method was used rather than the standard BCA assay. The CB-X kit gave the lowest apparent recoveries, 58.2 and 50.9% for the 50 and 200 ppm spikes, respectively. The CB-X assay also gave the lowest apparent protein value (1.27 ppm) in the control oil. The high standard deviation observed for the CB-X assay of control refined peanut oil was due to the large difference among the three measurements: 0.42, 3.38, and 0.00 ppm. These values are below the LOQ defined by the standard range for the CB-X assay (10–1000 ppm). Because CB-X assay uses a precipitation step prior to color complex formation, the low recovery may be due to incomplete precipitation of the peanut proteins used for spiking.

Table 1. Comparison of standard BCA, RAC-BCA, and CB-X protein assays on recovery of peanut butter protein spiked into Jewel 100% pure peanut oil

Sample	Protein mean, ppm, $\pm$ SD ($n = 3$)		
	Standard assay	RAC-BCA assay	CB-X assay
Control	17.24 $\pm$ 1.25	7.41 $\pm$ 1.06	1.27 $\pm$ 1.84
50 ppm spike	35.75 $\pm$ 3.89	44.9 $\pm$ 6.05	28.3 $\pm$ 4.94
Recovery, %	71.50	89.80	56.30
200 ppm spike	147.83 $\pm$ 18.38	159.37 $\pm$ 13.17	100.75 $\pm$ 8.98
Recovery, %	73.90	79.70	50.40

Table 2 compares results from the BCA and RAC-BCA colorimetric protein assays on PBST extracts of control soybean oils and 1000 ppm peanut protein spikes of soybean oil. The RAC-BCA assay, which is less prone to interference by reducing agents than the BCA assay, gave a protein value of 14.77 ppm in the control soybean oil, compared to 86.38 ppm as measured by the standard BCA protein assay. Recoveries of peanut protein from spiked oil were 85 and 67% measured by the RAC-BCA and standard BCA methods, respectively. The higher recovery from the RAC-BCA was significant at $P < 0.00013$ measured by t -test comparison of means (Excel Software).

Color development from the Cu^{2+} to Cu^+ redox reaction and complexation of bicinchoninic with protein requires incubation at 37°C for 30 min with the BCA assay, and 120 min with the micro-BCA assay (27). The BSA calibration standards formed the colored complex slowly over the required incubation period. Early in this study we observed that some oil extracts produced a strong color change immediately upon addition of the BCA or micro-BCA working reagent to extracts. Interference from co-extracted antioxidants may be the likely cause of the immediate color formation in some oils. The RAC-BCA kit, which is designed to minimize interference from antioxidants, did not produce immediate color formation and showed reduced background interference (Table 2). Residual lipid traces that escape the hexane extraction may also contribute to the interference (28).

Table 2. Comparison of standard BCA with reducing RAC-BCA protein assay for recovery of peanut butter spiked into soybean oil

Sample	Protein mean, ppm, $\pm$ RSD, %	
	Standard BCA assay	RAC-BCA assay
Control	86.38 $\pm$ 6.92, $n = 3$	14.77 $\pm$ 40.51, $n = 3$
1000 ppm spike	669.76 $\pm$ 2.28, $n = 4$	853.73 $\pm$ 5.33, $n = 4$
Recovery, %	67.0	85.4

Table 3. Effect of filtering PBST extracts through 10 000 MWCO prior to standard BCA assay on recovery of peanut butter protein spiked into Jewel 100% peanut oil

Sample	Protein recovered, ppm, $\pm$ SD	Recovery, %
Unfiltered control ($n = 3$)	17.24 $\pm$ 1.25	—
Unfiltered 50 ppm spike ($n = 3$)	35.57 $\pm$ 3.89	71.5
Unfiltered 200 ppm spike ($n = 3$)	147.83 $\pm$ 18.38	73.9
Filtered control ($n = 3$)	3.80 $\pm$ 0.59	—
Filtered 51.6 ppm spike ($n = 2$)	28.63 $\pm$ 2.92	55.5
Filtered 103 ppm spike ($n = 2$)	59.08 $\pm$ 8.41	57.3

Attempts were made to remove potential small molecule interferences co-extracted with protein by ultrafiltration. The PBST extracts were filtered, rinsed, and concentrated through 10 000 MWCO filtration tubes. Table 3 shows protein levels in Jewel 100% peanut oil with and without filtration/concentration, determined by the standard BCA method. The filtered control samples contained 3.80 ppm protein compared to 17.24 ppm for the unfiltered control extracts. Recoveries of peanut butter protein spiked at 51.6 and 103 ppm were 55.5 and 57.3%, respectively. These recoveries are lower ($P < 0.04$) than those obtained for unfiltered extracts spiked at 50 and 200 ppm (Table 3). The value of 3.80 ppm found in the filtered/concentrated control should be due only to protein. Antioxidants found naturally in vegetable oil or added as preservatives with molecular weight $< 10\,000$ Da should have been removed by the filtration treatment. Peptides less than the MWCO would also be removed. The RAC-BCA assay of oil sample gave 7.41 ppm (Table 1). Taken together, the values of 3.80 and 7.41 ppm from the assays modified to minimize interference from reducing compounds indicate that a small but measurable amount of protein is present in this refined peanut oil. Confirmatory techniques, such as gel electrophoresis, HPLC, or proteomics analysis, would help to verify the presence of protein in these samples.

Protein Quantitation in Commercial Peanut Oils

Table 4 shows apparent protein levels in three varieties of peanut oil: crude, cold-pressed, and refined, as measured by PBST extraction and four different colorimetric assays. As expected, the levels of apparent protein in the crude peanut oil were high; 415.98, 271.41, and 69.17 ppm as measured by the standard BCA, RAC-BCA, and CB-X assays, respectively.

The levels of apparent protein in cold-pressed peanut oil were 209.43, 122.07, and 4.12 ppm, as measured by the standard BCA, RAC-BCA, and CB-X assays, respectively. The low-protein value obtained from the CB-X assay may be a result of incomplete precipitation during the first step of the extraction procedure. The BCA assays do not use a protein precipitation step before color complex formation.

Table 4. Total protein analysis of three peanut oils (crude, cold-pressed, and refined) by different colorimetric assays after PBST extraction

Oil type	Brand	Protein mean, ppm, $\pm$ SD ($n = 3$)			
		Standard BCA assay	RAC-BCA assay	CB-X assay	Micro-BCA assay
Refined	Hollywood enriched	10.00 $\pm$ 1.67	6.78 $\pm$ 0.09	Not run	26.01 $\pm$ 0.85
Cold-pressed	Barr	209.43 $\pm$ 8.81	122.07 $\pm$ 6.50	4.12 $\pm$ 0.64	Not run
Crude	Golden peanut	415.98 $\pm$ 50.02	271.41 $\pm$ 44.66	69.17 $\pm$ 26.48	Not run

Apparent protein levels in the refined peanut oil were 10.00, 6.78, and 26.01 ppm, as measured with the standard BCA, RAC-BCA, and micro-BCA assays, respectively. The micro-BCA assay used a standard curve which bracketed a smaller concentration range (2–40 ppm) compared to the BCA and RAC-BCA assays (50–1000 ppm). The micro-BCA assay should therefore give more accurate values at low protein levels than the standard BCA or RAC-BCA assay. However, the more sensitive micro-BCA is also more likely to be affected by the interfering substances than are the standard BCA and RAC-BCA assays. This higher sensitivity to protein and interference could have produced the higher apparent protein value, 26.01 ppm, in the refined enriched peanut oil.

In the standard BCA and RAC-BCA assays, the refined enriched oil extracts gave A 562 nm readings that were between the absorbance of the blank and 50 ppm standard (lowest level calibration standard). Measurement of absorbance values near the blank absorbance value leads to questions regarding the method's LOQ. There are different methods for calculating and estimating method LOQ. For the purpose of this study, a simple approximation of the method LOQ is based on the lowest level of BSA standard included in the calibration curve. The low-level standard used for the micro-BCA assay was 2 ppm, whereas the other assays used 50 ppm for the low level calibration standard. Using these concentrations and the nominal extract volume of 12 mL and sample weight of 25 g, the method LOQ is approximately 1 ppm with the micro-BCA assay, and is 25 ppm for the other three colorimetric assays. In practice, the LOQ for the other three assays is lower than 25 ppm based on absorbance readings. For example, the mean of six 562 nm readings from blanks, 50, and 100 ppm standards in one BCA assay were 0.079, 0.166, and 0.236 absorbance units, respectively. The RSD values for the means were between 2.04 and 2.48%, indicating very high precision. Therefore, BCA absorbance readings below 0.166 (50 ppm standard) which are significantly different from the blank yield a quantifiable result.

AOAC defines LOQ as the mean value of the matrix blank reading plus 10 SDs of the mean, expressed in analyte concentration units (29). A true matrix blank (oil with zero level protein) was not available. In order to approximate this LOQ calculation, 10 aliquots from one bottle of Jewel 100% Pure refined peanut oil were analyzed. The mean protein level and SD were 15.07 $\pm$ 1.13 ppm from BCA analysis. With this matrix blank, the AOAC-defined LOQ value would be

26.34 ppm. In the case of a true matrix blank where there is zero level of protein in the oil, an estimate of LOQ would simply be 10 times the SD, which is 11.3 ppm. The practical LOQ for any protein assay will be determined by the accuracy and precision of the analyst in standard preparation and performance of the colorimetric assay. Protein values in samples that are near or below an established LOQ must be interpreted with caution.

As expected, the crude peanut oil contained the highest levels of protein. It is likely that the crude oil also contained the highest levels of interfering substances. Because the RAC-BCA assay is least susceptible to antioxidant interference, the 271 ppm total protein determined with the RAC-BCA assay for the crude oil is probably the most accurate measurement of true protein content for this oil. The CB-X assay also reduces background interference from reducing agents, but the precipitation step may be a source of protein loss. Crude peanut oil contained a considerable amount of solid-suspended particulate matter. Great care was taken to ensure that the particulate matter was homogeneously distributed before aliquots were weighed for analyses. Particulate matter present in the oil sample was considered to be part of the sample in all cases.

The Coomassie (Bradford) dye-binding protein assay is not subject to interference from reducing compounds (27). In our study, measurement of protein in peanut butter reference solutions with the Bradford assay gave much lower protein values than the BCA assay (data not shown). The CB-X assay is also a dye-binding assay and does not rely on a redox reaction to produce a color complex. The lower protein values and recoveries obtained with the CB-X assay may be a result of the same factors that caused low absorbance and underestimation of protein with the Coomassie assay, or from incomplete protein precipitation.

Two recent papers have published extensive data on protein content of cooking oils using an amino acid analyzer for quantitation. Ramazzotti et al. (30) analyzed refined peanut oil for protein by PBS extraction followed by dialysis. Quantitation of the extracts using colorimetric assays (such as BCA or Bradford) gave a value of 0.09 ppm protein with poor reliability, whereas the quantitative amino acid analysis gave a more consistent result with a value of 0.35 ppm protein. Both values were an order of magnitude lower than the value of 3.8 ppm found in the Jewel refined peanut oil after filtration through 10 000 MWCO and the BCA assay (Table 3). The

Table 5. Recovery of peanut butter protein spiked into soybean oil measured by PBST extraction and micro-BCA assay, with oil heated to 174–180°C after spiking

Sample	Protein mean, ppm, $\pm$ SD
Control oil	16.03 $\pm$ 3.75, $n = 7$
50 ppm spike	43.03 $\pm$ 7.53, $n = 4$
Recovery, %	86.1
200 ppm spike	191.05 $\pm$ 17.64, $n = 6$
Recovery, %	95.5

CB-X assay measured 1.27 ppm protein in refined peanut oil (Table 1). Martin-Hernandez et al. (31) analyzed cold-pressed unrefined peanut oil for protein by acetone/hexane precipitation, and centrifugation, quantitative amino acid analysis gave a value of 1.32 ppm protein. The CB-X assay measured 4.12 ppm protein in cold-pressed peanut oil used in this study (Table 4). Protein values obtained from these two published studies are lower than values obtained from the current study. However, the PBST extraction colorimetric assays used in this work gave protein values for refined and crude peanut oils in the range described by Crevel et al. (16). The different values obtained from various studies may be due to the differences in protein recovery using different extraction protocols or different matrix interferences. This highlights the need for additional comparative studies and further understanding of the matrix effects on the analytical results.

Recovery of Peanut Protein from Heated Oil: Colorimetric Assay

Frying oil may contain residual protein from fried foods. This protein has potential to transfer and contaminate other foods if the oil is reused. Therefore, it is useful to have a simple method for measurement of protein levels in oil used to cook allergenic foods. Table 5 shows recovery of peanut butter protein spiked into soybean oil and heated to 174–180°C in a deep-fryer before PBST extraction and micro-BCA colorimetric analysis. This study simulated cross-contact of roasting or cooking oil from peanuts. Recoveries were 86.1 and 95.5% for the 50 and 200 ppm spikes, respectively. The recoveries are net values, with the background in control and reagent blank samples subtracted. Apparent protein measured in the heated soybean oil control was 16.03 ppm compared with 86.38 ppm in unheated soybean oil measured by standard BCA (Table 2).

Table 6 shows protein levels in unheated and heated soybean oil as measured by PBST extraction and the standard BCA colorimetric assay. After heating at 174–180°C the measured protein level in the control oil decreased from 86.38 to 12.65 ppm. Recoveries of peanut protein spiked into oils at a level of 1000 ppm were 67 and 73% in the unheated and heated soybean oil, respectively. Heating did not significantly ($P < 0.156$) affect protein recovery from peanut butter-spiked

Table 6. Recovery of peanut butter protein spiked into soybean oil measured by PBST extraction and standard BCA assay, with unheated oil and oil heated to 174–180°C after spiking

Sample	Protein mean, ppm, $\pm$ SD	
	Heated	Unheated
Control	12.65 $\pm$ 0.48, $n = 4$	86.38 $\pm$ 5.97, $n = 3$
1000 ppm spike	729.62 $\pm$ 71.86, $n = 3$	669.76 $\pm$ 15.28, $n = 4$
Recovery, %	73.0	67.0

oil in this experiment. The recovery values are net values, with control and reagent blank values subtracted.

Vegetable oils contain a variety of naturally occurring and synthetic antioxidants, such as vitamin E (α -tocopherol) and TBHQ (tert-butylhydroquinone; 32–34). Allowable levels of synthetic antioxidants in U.S. foods are regulated by the U.S. Food and Drug Administration (35). However, it is beyond the scope of this paper to discuss in detail the antioxidant profile of vegetable oils and their effect on the BCA protein assay. Lipids are also known to interfere with BCA colorimetric protein assays (28). The heat-induced degradation of lipid and/or antioxidants through oxidation process could explain the lower level of apparent protein found in the heated oil samples (36). The data presented here clearly show that peanut and soybean oils contain nonprotein components that cause BCA assay color formation. This fact must be considered when a protein assay with a redox step, such as BCA, is used in the analysis of oil extracts.

The apparent protein levels found in heated soybean oil were 16.03 and 12.65 ppm by micro-BCA and standard BCA, respectively. These values are similar to the level measured by the RAC-BCA in unheated oil, again suggesting that the RAC-BCA assay was least affected by antioxidant interference. The RAC-BCA was probably the most accurate of the assays used in this work for determination of protein in oil. The European Food Safety Authority (EFSA) summarized data from clinical trials that measured allergic responses of subjects with soy allergy to soybean oil (37). The soy oil test substance was a highly refined blend containing a mean of 0.279 ppm protein found by microborate extraction and ATTO-TAGTM assay. The largest dose of oil contained approximately 12 μ g protein. The EFSA concluded that highly refined soybean oil is not very likely to trigger a severe allergic response in susceptible individuals under the conditions of production and use stated by the applicant. The protein levels found in our highly refined soy oil, as measured by PBST-colorimetric assay, were more than an order of magnitude higher than those found in the EFSA sample. This could be due to the difference in extraction buffer (PBST versus microborate), or to the fact that colorimetric assay overestimates protein extracted from oil even with precautions taken to eliminate interferences.

Table 7. Recovery of peanut butter protein spiked into soybean oil measured by ELISA; and effect of heating oil after spiking with 200 ppm protein from peanut butter

Sample matrix	Mean recovery, %, $\pm$ SD
Soybean oil, unheated	93.3 $\pm$ 2.4, $n = 3$
Soybean, heated	15.0 $\pm$ 6.1, $n = 3$
PBST extract of soybean oil, heated	15.5 $\pm$ 4.6, $n = 4$

Recovery of Peanut Butter Protein from Oil: ELISA Assay

The Veratox ELISA kit determines total peanut material. The ppm values determined from the ELISA plate reader were multiplied by 0.25 to give protein ppm based on 25% protein content of peanut butter. Recovery was calculated based on the 200 ppm peanut butter protein spiked to the oil. A control (unspiked) oil sample gave a reading of 0.1 ppm, which is below the ELISA method LOQ. The Veratox ELISA kit for peanut has a method LOQ of 2.5 ppm.

Table 7 shows a summary of average recoveries measured by ELISA from peanut protein-spiked soybean oil which had been heated to 174–180°C and the spiked unheated soybean oil samples. The recovery of peanut protein from the heated soybean oil was much lower (15.0%) than the recovery from unheated oil (93.3%). Table 7 also shows ELISA analysis of the PBST extracts of heated, spiked soybean oil used for the micro-BCA assay in Table 5. The 200 ppm spiked heated oil extracts had mean recovery of 15.5% as measured by ELISA compared with a recovery of 95.5% as measured by the micro-BCA assay (Table 5). The low-protein recovery measured by ELISA in the heated oil is probably due to a change in the immunoreactivity of the peanut antigens since the total protein recovery measured by colorimetric assay was 95.5%. Thermal denaturation of protein has been shown to affect ELISA results for egg proteins as reported by Faeste et al. (38). Therefore, residual peanut protein contained in used frying oil may show decreased detectability by ELISA measurements. The results presented here clearly show that peanut protein heated in oil is less detectable by the Veratox ELISA kit than is unheated peanut protein.

Limitations of using buffer extraction and colorimetric measurement of protein from oil include protein solubility in the buffer and presence of co-extracted compounds that interfere with the color formation reaction. Despite these limitations, colorimetric methods can be a quick and useful technique to determine protein in cooking oil.

Conclusions

Protein levels in cooking oils may be measured with a simple procedure described here involving PBST extraction and colorimetric detection/quantitation. However, interpretation of the data from colorimetric procedures must

be made with care. The BCA assay and the more sensitive micro-BCA protein assay are subject to interference from antioxidants or lipids, which cause anomalously high protein measurements. Interferences can be reduced through the use of other colorimetric assays such as RAC-BCA and CB-X. Based on recovery data and analysis of heated and unheated oils, of the four colorimetric assays evaluated, the RAC-BCA assay gives the most accurate results for protein measurement in cooking oils. Small-molecule interferences can be removed from buffer extracts of vegetable oils through filtration. However, filtration may also cause loss of protein. The LOQ from these simple colorimetric assays ranged from 1 to 25 ppm, depending on the calibration standard range, colorimetric assay, and background interference. Oils used to fry peanut-containing foods are a risk for cross-contact if they are reused. However, the ELISA kits can underestimate the amount of protein allergen in oil after frying. This study demonstrated that the Veratox kit gave a much lower recovery of peanut protein for heat-treated oil samples than that measured with the extraction/colorimetric assay. The colorimetric protein method described here could be used to assess the level of protein in refined oils used for cooking and in crude vegetable oils. In addition, the method can be applied to the determination of potential for allergen cross-contact from reused cooking oil.

Acknowledgments

We are grateful to the Golden Peanut Co., Alpharetta, GA, for kindly providing the sample of crude peanut oil.

References

- (1) World Health Organization (2006) *Infosan Information Note No. 3/2006 – Food Allergies*, Geneva, Switzerland
- (2) Buchanan, R., Dennis, S., Acheson, D., Assimon, S.A., Beru, N., Bolger, P., Carlson, D., Carvajal, R., Copp, C., Falci, K., Garber, E., Harden, E., Kane, R., Kvenberg, J., Luccioli, S., Park, D., Raybourne, R., Troxell, T., & Vierk, K. (2008) *J. Food Prot.* **71**, 1043–1088
- (3) Chelhide, M. (2007) *Curr. Opin. Allergy Clin. Immunol.* **3**, 264–268
- (4) Keet, C.A., & Wood, R.A. (2007) *Immunol. Allergy Clin. North America* **27**, 193–212
- (5) Taylor, S.L., & Hefle, S.L. (2005) *Food Technol.* **59**, 40–43, 79
- (6) Sicherer, S.H., & Sampson, H. (2007) *J. Allergy Clin. Immunol.* **120**, 491–503
- (7) Bock, S.A., Munoz-Furlong, A., & Sampson, H.A. (2001) *J. Allergy Clin. Immunol.* **107**, 191–193
- (8) USDA Economic Research Service (2005) *Food Availability Spreadsheets*, <http://www.ers.usda.gov/data/foodconsumption/foodavailspreadsheets.htm>, accessed January 2008
- (9) USDA Economic Research Service (2005) *Food Availability Spreadsheets*, <http://www.ers.usda.gov/data/foodconsumption/foodavailspreadsheets/htm>, accessed January 2008

- (10) USDA Economic Research Service (2007/2008) *Production, Supply and Distribution Online*, <http://www.fas.usda.gov/psonline>, accessed January 2008
- (11) Hourihane, J.O., Bedwani, S.J., Dean, T.P., & Warner, J.O. (1997) *Br. Med. J.* **314**, 1084–1088
- (12) Olszewski, A., Pons, L., Moutete, F., Aimone-Gastin, I., Kanny, G., Moneret-Vautrin, D.A., & Gueant, J.L. (1998) *Clin. Exp. Allergy* **28**, 850–859
- (13) McAlister, L., Pollard, G., Carter, C., & Bussel, G. (1995, Updated 1999) *The British Dietetic Society: Peanut Allergy: Information for Dietitians*, <http://www.bda.uk.com/Downloads/peanutallergy.pdf>, accessed March 2008
- (14) Peeters, K., Knulst, A.C., Rynja, F.J., Bruijnzeel-Koomen, C., & Koppelman, S.J. (2004) *J. Allergy Clin. Immunol.* **113**, 1000–1001
- (15) Teuber, S.S., Brown, R.L., & Haapenen, L.A. (1997) *J. Allergy Clin. Immunol.* **99**, 502–507
- (16) Crevel, R.W.R., Kerkhoff, M.A.T., & Konnig, M.M.G (2000) *Food. Chem. Toxicol.* **38**, 385–393
- (17) Norris, F.A. (1982) in *Bailey's Industrial Oil and Fat Products*, Vol. 2 (4th Ed.), D. Swern (Ed.), John Wiley & Sons, New York, NY, pp 253–314
- (18) Hoffman, D.R., & Collin-Williams, C. (1994) *J. Allergy Clin. Immunol.* **4**, 801–802
- (19) Taylor, S.L., Hefle, S.L., Bindslev-Jensen, C., Bock, S.A., Burks, A.W., Christie, L., Hill, D.J., Host, A., Hourihane, J.O'B., Lack, G., Metcalfe, D.D., Moneret-Vautrin, D.A., Vadas, P.A., Rance, F., Skrypec, D.J., Trautman, T.A., Yman, I.M., & Zeiger, R.S. (2002) *J. Allergy Clin. Immunol.* **109**, 24–30
- (20) Keating, M.U., Jones, R.T., Worley, N.J., Shively, C.A., & Yunginger, J.W. (1990) *J. Allergy Clin. Immunol.* **86**, 41–44
- (21) Klurfeld, D.M., & Kritchevsky, D. (1987) *Lipids* **22**, 667–668
- (22) Zitouni, N., Errhali, Y., Metche, M., Kanny, G., Moneret-Vautrin, D.A., Nicholas, J.P., & Fremont, S. (2000) *J. Allergy Clin. Immunol.* **106**, 962–967
- (23) Hidalgo, F.J., Alaiz, M., & Zamera, R. (2001) *Anal. Chem.* **73**, 698–702
- (24) Yeung, J.M., & Collins, P.G. (1996) *J. AOAC Int.* **79**, 1411–1416
- (25) Porras, O., Carlsson, B., Fallstrom, S.P., & Hanson, L.A. (1985) *Int. Arch. Allergy Appl. Immunol.* **78**, 30–32
- (26) Poms, R.E., & Anklam, E. (2004) *J. AOAC Int.* **87**, 1466–1474
- (27) Krohn, R.I. (2004) in *Handbook of Food Analytical Chemistry*, Vol. 1 (1st Ed.), R.E. Wrolsted, E.A. Decker, S.J. Schwartz, & P. Sporna (Eds), John Wiley & Sons, Hoboken, NJ, pp 77–113
- (28) Kessler, R.J., & Fanestil, D.D. (1986) *Anal. Biochem.* **159**, 138–142
- (29) *AOAC Peer-Verified Methods Program Manual on Policies and Procedures* (1998) <http://www.aoac.org/vmeth/PVM.pdf>, accessed March 2008
- (30) Ramazzotti, M., Mullinacci, N., Pazzagli, L., Moriondo, M., Manao, G., Vincieri, F.F., & Degl'Innocenti, D. (2008) *Food Chem. Toxicol.* **46**, 3383–3388
- (31) Martin-Hernandez, C., Benet, S., & Ladislav, O. (2008) *J. Agric. Food Chem.* **56**, 4348–4351
- (32) Reische, D.W., Lillard, D.A., & Eitenmiller, R.R. (2002) in *Food Lipids* (2nd Ed.), C.C. Akoh & D.B. Min (Eds), Marcel Dekker, Inc., New York, NY, pp 517–542
- (33) Krishna, A.G.G., & Prabhakar, J.V. (1994) *J. Am. Oil Chem. Soc.* **71**, 1245–1249
- (34) Chu, Y.H., & Hsu, H.F. (1999) *Food Chem.* **66**, 29–34
- (35) *Food Additives Permitted for Direct Addition to Food for Human Consumption in Code of Federal Regulations Title 21, Part 172, Subpart B, Food Preservatives*, revised as of April 1, 2008
- (36) Ceppanen, C.M., & Csallny, A.S. (2006) *J. Am. Oil Chem. Soc.* **83**, 121–127
- (37) Scientific Opinion of the Panel on Dietetic Products, Nutrition and Allergies (2007) *EFSA J.* **570**, 1–9
- (38) Faeste, C.K., Løvberg, K.E., Lindvik, H., & Egaas, E. (2007) *J. AOAC Int.* **90**, 427–436