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Effect of Processing on Recovery and Variability Associated with Immunochemical Analytical Methods for Multiple Allergens in a Single Matrix: Sugar Cookies

Sefat Khuda,[†] Andrew Slate,[§] Marion Pereira,[†] Fadwa Al-Taher,[#] Lauren Jackson,[⊗] Carmen Diaz-Amigo, [⊥] Elmer C. Bigley, III,[†] Thomas Whitaker,[§] and Kristina M. Williams*[,†]

[†]U.S. Food and Drug Administration, Laurel, Maryland 20708, United States

[§]North Carolina State University, Raleigh, North Carolina 27695, United States

[#]Illinois Institute of Technology, Bedford Park, Illinois 60501, United States

[⊗]U.S. Food and Drug Administration, Bedford Park, Illinois 60501, United States

[⊥]Eurofins CTC, Hamburg, Germany

Supporting Information

ABSTRACT: Among the major food allergies, peanut, egg, and milk are the most common. The immunochemical detection of food allergens depends on various factors, such as the food matrix and processing method, which can affect allergen conformation and extractability. This study aimed to (1) develop matrix-specific incurred reference materials for allergen testing, (2) determine whether multiple allergens in the same model food can be simultaneously detected, and (3) establish the effect of processing on reference material stability and allergen detection. Defatted peanut flour, whole egg powder, and spray-dried milk were added to cookie dough at seven incurred levels before baking. Allergens were measured using five commercial enzyme-linked immunosorbent assay (ELISA) kits. All kits showed decreased recovery of all allergens after baking. Analytical coefficients of variation for most kits increased with baking time, but decreased with incurred allergen level. Thus, food processing negatively affects the recovery and variability of peanut, egg, and milk detection in a sugar cookie matrix when using immunochemical methods.

KEYWORDS: *allergen detection, ELISA methods, food allergen, incurred reference material, thermal processing*

INTRODUCTION

Among allergenic foods, peanut, egg, and milk elicit >80% of the food hypersensitivity responses affecting both children and adults.[1] The number of affected individuals is increasing, possibly due to changes in consumption habits, the environment, or greater consumer awareness of food-related allergic reactions.[2−4] Strict avoidance of foods suspected of containing allergens decreases the potential risk of oral exposure, and therefore allergic individuals must rely on the accuracy of ingredient labels. Unintended exposure, however, can still occur by consumption of food products containing undeclared allergens from mislabeled raw ingredients, from allergens that are inadvertently introduced during the manufacturing process, or from ambiguous or "may contain" labeling practices. Most adverse events occur after the consumption of processed food or a meal containing undeclared allergenic ingredients rather than by the consumption of a single allergenic food product.[5−9] Therefore, there is a critical demand for commercial kits capable of detecting allergens in processed foods or allergenic ingredients in a complex matrix.

Enzyme-linked immunosorbent assays (ELISAs) are the most commonly used analytical immunoassays. Various factors may negatively affect immunologic detection or quantitation of allergens in processed foods. Allergenic proteins may lose their solubility due to heat, chemical modification, enzymatic hydrolysis, or changes in pH. Covalent chemical modifications of the allergen may occur, as with protein−sugar reactions (e.g., the Maillard reaction),[10−13] or allergenic proteins may self-aggregate or aggregate with other components of the food matrix, also reducing solubility. Certain components of the food matrix may block epitopes present on the allergenic protein, thus reducing the binding of assay antibodies used for allergen capture and/or detection. Changes in the structure of the allergenic protein could also decrease antigenicity, or interaction with components of the matrix could create neoepitopes.[14−16] Cross-reacting epitopes may be present in the matrix components themselves, causing false-positive immunoassay results.[9,12] The physical form of the food matrix also affects sample homogeneity, which ultimately affects allergen extraction efficiency and quantitation.[13,17,18]

Most previous studies of the effects of processing on allergen detection focused on single allergenic foods, such as dry-roasted peanuts; peanut oil; autoclaved peanuts; heated, boiled, and autoclaved egg powder; heated milk; and pure milk allergenic proteins.[19−26] Some studies, however, have used processed foods containing allergenic ingredients in a complex matrix.[27−34] Allergenic proteins with processing-related phys-

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icochemical and structural alterations may still evoke a response in sensitive individuals,[21,35−37] despite a loss in immunoreactivity. Validated analytical methods can protect sensitive individuals from contaminated foods or accidental consumption by providing sufficient data to accurately label foods and ensure proper cleaning of shared production lines and allow for retrospective evaluation of complaint samples by regulatory agencies.

Some earlier studies used spiked samples to evaluate commercially available ELISA kits for various food matrices.[17,38−40] The results of these studies varied by test kit, protein concentration, food matrix, and type of allergenic protein. Spiked samples are useful for determining some matrix effects, but are not appropriate for studying the effects of processing on method performance. These and other studies[17,27,33,34,41,42] demonstrated the necessity for using matrix-specific incurred standards in the evaluation of the performance of ELISA detection methods. Accuracy and variability are critical measures in the evaluation of the performance of any analytical method. The performance of ELISA kits may differ due to differences in antibody specificity, calibration methods, extraction buffer composition, extraction procedures, and differences between reporting units for expression of the results.

Due to the impracticality of using standards containing individual allergens for all possible food matrices, detection of multiple allergens in a single processed matrix was evaluated. Some studies using incurred samples for multiple allergen testing have been performed,[30,43] most recently for evaluating liquid chromatography−tandem mass spectroscopy methods.[44,45] In the present study, we selected sugar cookies as a matrix to determine whether peanut, milk, and egg allergens could be detected simultaneously in one model food. This matrix was selected on the basis of the simplicity of the recipe, the absence of ingredients that could cross-react with kit antibodies, and the ability to introduce multiple allergenic foods into the cookie without affecting the texture or consistency of the final product. The study aimed to evaluate the accuracy and precision of commercial ELISA kits for evaluating incurred sugar cookies for detection of these allergens and to determine the effect of processing on detection. This work will provide important insight to address some of the critical issues associated with quantitative analysis of allergens in processed foods using immunoassays.

MATERIALS AND METHODS

Food Samples. Sugar cookie dough and cookies baked at 190 °C for 25 and 30 min were studied. All control cookie dough (no peanut, egg, or milk) and incurred cookies were produced at the U.S. Food and Drug Administration, Institute for Food Safety and Health (Bedford Park, IL, USA).

Equipment for Preparing Food Samples. The equipment used for cookie preparation included as Hobert Legacy Mixer, model 120, 12-quart mixer with B flat beater attachment (Troy, OH, USA); a KitchenAid Mixer; model K5SS, heavy duty; 5-quart capacity (KitchenAid, Shelton, CT, USA); a Delonghi convection oven, model AR1070; and food processors with separate bowls and blades for grinding samples.

Cookie Ingredients. The following ingredients were used for cookie preparation: H&R all purpose flour (ConAgra Mills, Omaha, NE, USA), double-acting baking powder (Clabber Girl Corp., Terre Haute, IN, USA), Arm & Hammer pure baking soda (Church & Dwight Co, Princeton, NJ, USA), Kirkland pure vanilla extract (Costco Wholesale, Seattle, WA, USA), Carlini all-vegetable shortening (Aldi, Inc., Batavia, IL, distributor), and Domino premium pure cane sugar (local supermarket).

Reference Materials. Reference allergens used to make incurred samples were nonfat dry milk, NIST SRM 1549 (National Institute of Standards and Technology, Gaithersburg, MD, USA); spray-dried whole egg powder, NIST RM 8445 (National Institute of Standards and Technology); and light-roasted peanut flour, 12% fat light roast, product 521271, lot 109FA (Golden Peanut Co., Alpharetta, GA, USA).

Cookie Dough Recipe. The cookie dough was formulated to contain 1375 g of flour, 908 g of shortening, 1200 g of sugar, 23.1 g of baking soda, 9.1 g of baking powder, 20 g of vanilla extract, and 50 g of water.

Preparation of Incurred Samples. Control cookie dough was prepared by mixing the dry ingredients at low speed in a Hobart mixer for 2 h. Liquid ingredients were added, and the dough was mixed for an additional 20 min. For the incurred samples, a flour premix consisting of 5000 ppm (1 ppm = 1 μg/g) of nonfat dry milk, egg powder, and peanut flour was prepared and added at the appropriate level to the remaining dry ingredients, followed by mixing at slow speed for 2 h. Liquid ingredients were then added, and the incurred dough was mixed for an additional 20 min at low speed. The dough was formed into balls, placed on aluminum foil-covered baking sheets, and baked for 25 or 30 min at 190 °C. Cookies were weighed before and after baking. Water loss from the cookies baked for 25 or 30 min was minimal (<2%) and was therefore not considered in our statistical evaluation. Cookies were ground in a food processor prior to sampling. A dedicated "allergen-free" food processor was used for the 0 ppm cookies.

Test Kits. The commercial test kits used in this study were RIDASCREEN FAST peanut, egg, casein, and RIDASCREEN β-lactoglobulin (BLG) kits, R-Biopharm (RB) (Washington, MO, USA); Veratox peanut, egg, and total milk allergen quantitative test kits, Neogen (NE) Corp. (Lansing, MI, USA); Morinaga (MO) peanut, egg, and milk (casein and BLG) protein ELISA kits, Crystal Chem (Downers Grove, IL, USA); Tepnel (TE) BioKits, peanut, egg, casein, and BLG assay kits (Neogen Corp.); and ELISA Systems (ES) peanut, egg, casein, and BLG residue kits from BioMerieux (Durham, NC, USA).

Analytical Methods. The characteristics of all commercial kits used in this study are listed in Supplemental Table 1 of the Supporting Information. Allergen extraction and ELISA procedures were performed by following the manufacturers' instructions provided. ELISA results were determined spectrophotometrically using a SpectraMax M5 plate reader, and data were analyzed using Softmax Pro 5.3 software (Molecular Devices Corp., Sunnyvale, CA, USA). For quantitation, standard curves were created using the manufacturer's recommended curve fit or by using a four-parameter logistic calibration curve fit if none was recommended.

Experimental Design. The experimental design of this study was similar to that described previously for the detection of peanut proteins in foods.[17] A balanced nested design was used to measure peanut, egg, and milk (casein and BLG) proteins using ELISA test kits from five manufacturers. Four samples of cookie dough and baked cookies at each incurred level were extracted according to the kit instructions. For each sample, four aliquots were used to quantitate the allergen concentration (4 samples × 4 aliquots per sample = 16 total aliquots per incurred level). A total of 336 analyses were performed for each kit for each allergen using each of the three cookie preparations (dough, baked for 25 min, baked for 30 min; 4 samples × 4 aliquots × 7 incurred concentrations × 3 cookie preparations). A total of 5040 analyses were performed for peanut, egg, and casein (336 analyses × 5 commercial kits × 3 allergens) and 1344 analyses for BLG (336 analyses × 4 commercial kits).

Statistical Analysis. Each allergen-specific kit was evaluated for accuracy and precision. Accuracy was defined as the closeness of the mean measured allergen value to the true (incurred) value and is expressed as percent recovery (mean measured value/incurred value × 100). The relationship between measured and incurred values was determined by regression analysis and expressed graphically as

measured (M) versus incurred protein values (P). Measured values refer to allergen levels quantitated following the instructions of each test kit, and incurred protein values were calculated on the basis of the protein content of each of the allergen reference materials used for cookie preparation. Variance (standard deviation squared, SD^2), a measure of precision, was determined using the Proc Nested procedure in SAS.[46] The total variance was partitioned into sampling variance, which refers to the concentration differences among the four samples, and analytical variance, which refers to the concentration differences among the four aliquots of each sample. The sampling and analytical coefficients of variation [CV% = 100(sample or analytical SD of measured value/mean measured value)] were also calculated for each test kit and allergen as an additional measure of variability.

RESULTS AND DISCUSSION

Analysis of Cookie Dough for Peanut, Egg, and Milk Allergens. A major purpose of this study was to develop incurred standards for multiple food allergens in a single matrix. The protein level contributed by each allergen in the incurred cookies was calculated as described in Table 1.[47,48] Results from

Table 1. Calculated Peanut, Egg, and Milk Protein Content at Indicated Incurred Levels

	content (ppm) at incurred level of peanut flour[a], spray-dried whole egg,[b] and nonfat dry milk[c]						
	0 ppm	2.5 ppm	5 ppm	10 ppm	25 ppm	100 ppm	500 ppm
peanut	0	1.26	2.52	5.04	12.6	50.4	252
egg	0	1.2	2.4	4.8	12	48	240
casein	0	0.72	1.44	2.88	7.2	29	144
BLG	0	0.09	0.18	0.36	0.9	4	18

[a]Partially defatted light roast peanut flour. Protein: N × 5.46 = 50.39% (N = nitrogen). [b]NIST 8445. Protein: N × 6.25 = 48%. [c]NIST 1549. Protein: 36%.[47] Estimated protein content assuming casein is 80% and BLG is 10% of total milk protein.[48]

each kit were compared to the protein content of each allergen at the specified incurred level. Mean measured concentration and percent recovery (accuracy) for each test kit, and at each known allergen concentration, are shown in Supplemental Table 2 of the Supporting Information and Figure 1 for peanut, egg, casein, and BLG.

Peanut. The measured concentration of peanut protein in incurred cookie dough varied among the different test kits at all incurred levels. The ES, MO, and TE kits underestimated the incurred peanut protein content at all incurred levels. When averaged across all incurred levels, the recoveries of these three kits for cookie dough were 11.0% (ES), 19.6% (MO), and 12.1% (TE) (Figure 1A). The levels measured using the NE kit were slightly lower than each of the incurred levels, with a recovery of 81.2% when averaged across all incurred levels. For the RB kit, the measured values were slightly higher than the incurred level, with minimal background at the zero-incurred level. Recovery averaged across all incurred levels for the RB kit was 101.8%. On the basis of recoveries at the lowest incurred levels (1.26 and 2.52 ppm peanut protein), it is unlikely that the MO, ES, and TE kits would be able to detect peanut protein in the cookie dough matrix at the limits of quantitation (LOQs) claimed by the manufacturers. This raises the potential for false-negative results at lower incurred peanut protein levels for the MO, ES, and TE kits.

Egg. The MO, NE, and RB kits overestimated the incurred level of egg protein in cookie dough, with recoveries of 296.3% (MO), 355.9% (NE), and 299% (RB), when averaged across all incurred levels (Figure 1B). The measured levels of egg allergen using the ES kit were similar to the incurred levels with a mean recovery of 102.2% across all incurred levels. The performances of the TE and ES kits were comparable at lower incurred levels, but the TE kit overestimated the egg protein content at higher incurred levels (48 and 240 ppm egg protein), with a mean recovery of 114.7% across all incurred levels. The TE, RB, ES, and NE kits are based on antibodies specific for one or more egg white proteins, whereas the MO kit detects whole egg protein. Although the mean of most measured levels of egg protein for all kits was higher than the incurred levels, all kits would be able to detect egg at the claimed LOQs in the cookie dough matrix.

Milk Casein. Using the TE kit, the detected levels of casein in cookie dough were comparable to the calculated casein content of the incurred samples, although this kit had a higher background at the 0 ppm level than the other kits. The ES and NE kits overestimated the level of casein at all incurred levels with mean recoveries of 219.9% (ES) and 272.3% (NE) (Figure 1C). Although the mean recovery for the RB kit across all incurred levels was acceptable at 117.7%, the RB kit underestimated the casein content at the three lowest fortified levels (1.44 ppm, 74.2%; 2.88 ppm, 62.7%; and 7.20 ppm, 48.3% recovery) while overestimating casein at the higher fortified levels. The measured concentration of casein in cookie dough by the MO kit was low compared to the other kits; mean recovery at all incurred levels was 70.8%. It is questionable whether the MO and RB kits would be able to detect casein at the LOQs claimed by these manufacturers in the cookie dough matrix, because the reported LOQs for casein are 0.3 ppm for the MO kit and 0.5 ppm for the RB kit. The lowest incurred level for casein in this study was 0.72 ppm; thus, incurred levels at or below the reported LOQs of these kits are required before this conclusion can be drawn.

Milk BLG. Mean BLG values measured using the ES kit were close to the actual levels, particularly at the high and low incurred levels. The MO kit overestimated BLG protein at all incurred levels, whereas the RB and TE kits detected BLG only at the highest level, and even then recovery was poor [18.7% (RB) and 1.6% (TE)]. The mean recoveries of the ES, MO, RB, and TE kits at all incurred levels were 79.9% (ES), 308% (MO), 18.7% (RB), and 1.6% (TE) (Figure 1D). On the basis of these results, the RB and TE kits were not able to detect BLG in the cookie dough matrix at the LOQs indicated by the kit manufacturers (0.2 ppm for the RB kit and 2.5 ppm for the TE kit).

These results indicate that an incurred cookie dough matrix containing peanut, egg, casein, and BLG could be used to evaluate the ability of ELISA kits to detect these allergens. The results also indicate that the kits differed in their performance, which is expected on the basis of differences in (1) kit reagents, (2) statistical representation of the standard curve, (3) expression of results (i.e., casein vs nonfat dry milk; peanut protein vs total peanut), and (4) assay formats (capture vs competitive).

The functional relationship between measured and incurred levels in cookie dough was determined for each kit and each allergen by regression analysis (Supporting Information, Supplemental Figure 1). A linear relationship exists between measured (M) and incurred protein levels (P) such that the accuracy of the kit can be estimated by examining the slope of each plot. The closer the slope is to 1.0, the more accurate the kit. A slope equal to 1.0 represents the most accurate (i.e.,

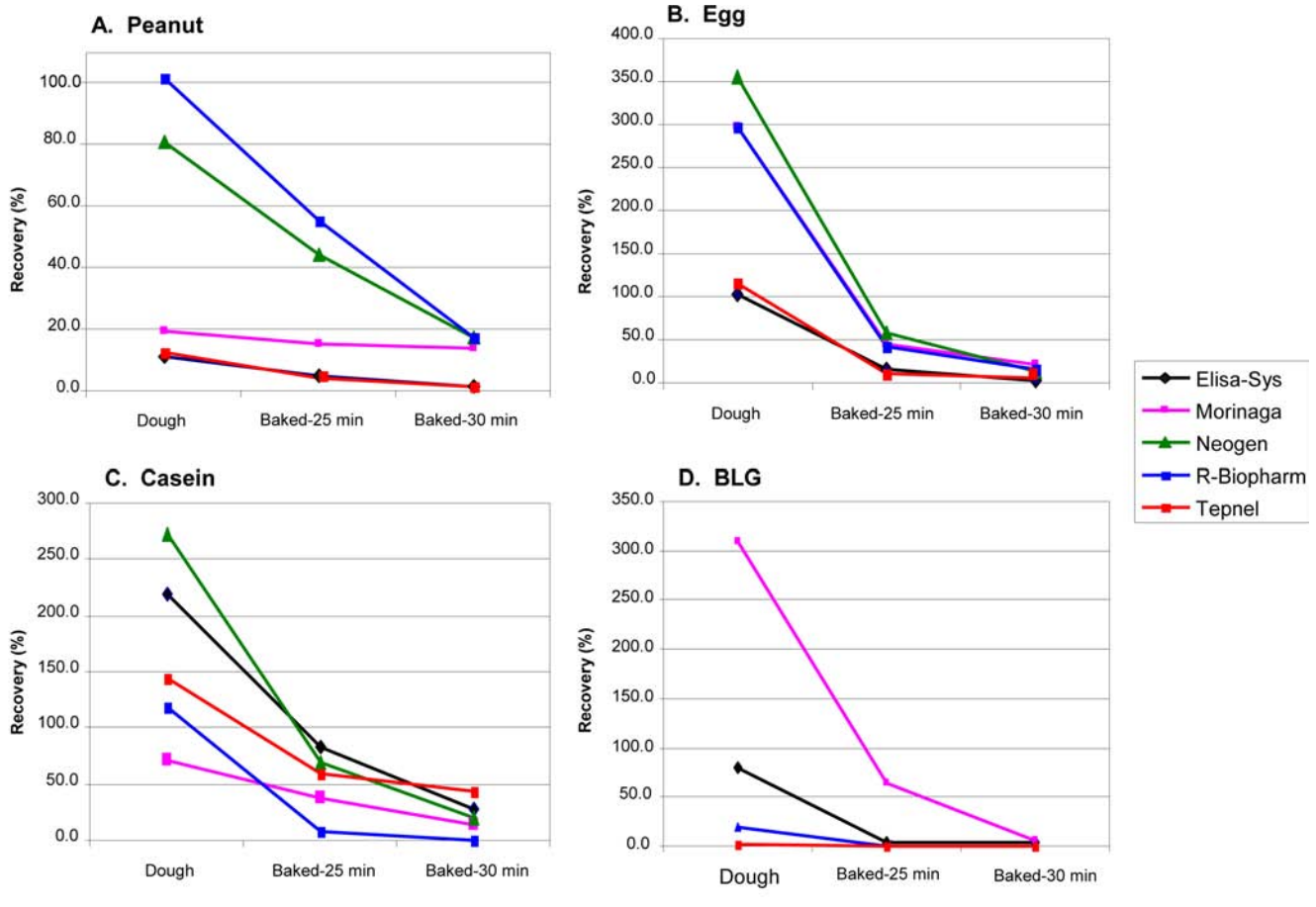

Figure 1. Mean percent recovery of all incurred levels in cookie dough and processed (baked for 25 or 30 min) cookies for (A) peanut, (B) egg, (C), casein, and (D) BLG (incurred level used to calculate recovery although effect on true concentration due to baking is unknown).

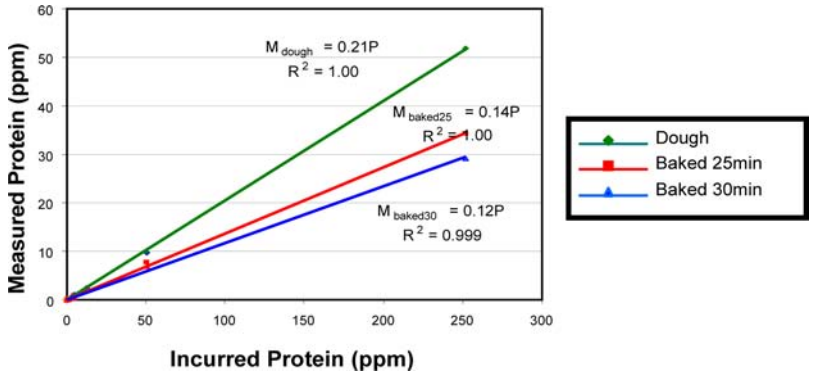

Figure 2. Correlation between measured protein (M) and incurred protein concentration (P) as determined by linear regression analysis for peanut allergen in unprocessed (dough) and processed (baked for 25 or 30 min) cookies using the Morinaga (MO) peanut protein kit.

recovery = 100%). For example, in Figure 2 the slope of the regression analysis of the MO kit reveals that this kit underestimates the incurred peanut protein level of the cookie dough (slope = 0.21, or 21% of the incurred peanut protein level). On the basis of the slopes of the regression analyses, the accuracy of test kits for allergen detection in a sugar cookie dough matrix (with the corresponding percent recoveries) was determined (Table 2). Caution is required, however, when the accuracies of different kits are compared. In cases in which a kit underestimates the protein level, the accuracy based on the regression analysis might appear better than the accuracy of another kit that overestimates the protein level. For example, a kit producing a slope from the linear regression analysis of 0.25 (25% recovery) underestimates the actual allergen content, but is more accurate than a kit with a slope >1.75 (>175% recovery) because its value is closer to 1. Underestimation of protein level could result in a false-negative determination at lower incurred levels. In contrast, kits that overestimate the allergen content may be less accurate, but would still result in detection of the allergenic protein.

Effect of Processing on Allergen Detection in the Incurred Sugar Cookie Matrix. Cookie dough was baked at 190 °C for 25 or 30 min. These two baking times were chosen because cookies baked for <25 min were undercooked and

Table 2. Accuracy of Each Test Kit (and Corresponding Percent Recovery) Based on the Slope of the Linear Regression Analysis Relating Measured Protein Level (M) to Incurred Protein Level (P) for Each Test Kit and Each Allergen in Cookie Dough and Cookies Baked for 25 and 30 Min (All Intercepts Assumed To Be Zero)[a]

kit manufacturer[a]	cookie dough	cookies baked for 25 min	cookies baked for 30 min
		Peanut	
R-Biopharm	1.00 (100%)	0.60 (60%)	0.11 (11%)
Neogen	0.86 (86%)	0.38 (38%)	0.15 (15%)
Morinaga	0.21 (21%)	0.14 (14%)	0.12 (12%)
ELISA Systems	0.17 (17%)	0.08 (8%)	0.02 (2%)
Tepnel	0.18 (18%)	0.07 (7%)	0.00 (0%)
		Egg	
R-Biopharm	2.65 (265%)	0.46 (46%)	0.10 (10%)
Neogen	4.06 (406%)	0.57 (57%)	0.09 (9%)
Morinaga	2.98 (298%)	0.40 (40%)	0.15 (15%)
ELISA Systems	1.19 (119%)	0.14 (14%)	0.03 (3%)
Tepnel	1.74 (174%	0.19 (19%)	0.04 (4%)
		Milk (Casein)	
R-Biopharm	1.47 (147%)	0.02 (2%)	0.00 (0%)
Neogen	2.34 (234%)	0.58 (58%)	0.07 (7%)
Morinaga	0.93 (93%)	0.68 (68%)	0.04 (4%)
ELISA Systems	2.56 (256%)	0.23 (23%)	0.06 (6%)
Tepnel	1.01 (101%)	0.11 (11%)	0.03 (3%)
		Milk (BLG)	
R-Biopharm	0.15 (15%)	0.00 (0%)	0.00 (0%)
Morinaga	4.37 (437%)	0.49 (49%)	0.12 (12%)
ELISA Systems	1.12 (112%)	0.02 (2%)	0.01 (1%)
Tepnel	0.09 (9%)	0.00 (0%)	0.00 (0%)

[a]The closer that the slope is to 1.00 (100% recovery), the more accurate the test kit for a particular allergen. [a]Characteristics of each kit listed in Supplemental Table 1 of the Supporting Information.

dough-like, whereas cookies baked for >30 min were overbaked (beginning to burn). The mean measured values and percent recovery of the allergenic proteins for all test kits at all incurred levels are shown in Supplemental Table 2 of the Supporting Information. The recovery of allergens from cookie dough and baked cookies averaged across all levels is shown in Figure 1.

Peanut. Levels of detected proteins and percent recoveries of the proteins in the cookies decreased as a function of baking time. The negative impact of the length of baking time on peanut recovery using ELISA and polymerase chain reaction methods was also observed by Scaravelli et al.[32] using a peanut incurred cookie matrix. For all peanut ELISA kits, percent recovery of peanut proteins after 30 min was <18% when averaged across all incurred levels. The decrease in recovery after baking was not as dramatic for the MO kit compared with the other kits (13.7% after 30 min), but the peanut protein recovery from the cookie dough for this kit was initially low (19.6%). This finding suggests that the baking process had less of an effect on the recovery of peanut protein with the MO kit, possibly because the sample extraction buffer used in this kit contains additives (sodium dodecyl sulfate and 2-mercaptoethanol) that improve the extraction of denatured proteins. The ELISA kits with the highest mean peanut protein recoveries from cookies baked for 30 min were NE (17.4%) and RB (17.1%). Compared with the recoveries of peanut protein from the cookie dough [81.2% (NE) and 101.8% (RB)], the baking process had a more negative effect on recovery using these kits. These results indicate that none of the kits were able to detect peanut protein at the kit LOQs in the baked cookie matrix, raising the potential for false-negative results.

Egg. No test kit adequately quantitated egg protein in baked cookies in terms of the mean measured concentrations and percent recovery at all incurred levels. As with the detection of peanut protein, and as reported previously using a baked peanut butter cookie matrix,[33] detection of egg protein in incurred samples was dependent on the baking time. The detected levels of egg protein were drastically reduced following 30 min of baking time, with kit recoveries ranging from 3.5 to 20.5% when averaged across all incurred levels. All kits were able to detect egg at the three highest incurred levels (12, 48, and 240 ppm egg protein) after baking, but at measured levels that were considerably lower than the calculated incurred levels. Egg protein recoveries in cookies baked for 30 min were greater using the MO kit than the other kits, which was probably due to improved solubility of the target proteins provided by the reducing−denaturing components of the MO extraction buffer.[28,33] The detection of egg protein was severely reduced after baking using the MO and all other kits, however, as previously observed.[33,43] On the basis of the results of the present study, none of the kits were able to detect egg protein at the kit LOQs in cookies baked for 30 min, increasing the potential for false-negative results in the baked cookie matrix.

Milk Casein and BLG. The detection of casein and BLG levels was also reduced in the cookie dough after baking. The recoveries for casein ranged from 7.5 to 83.2% after 25 min of baking and from 0.6 to 42.6% after 30 min of baking, when averaged across all incurred levels for all kits. The results indicate that baking did not affect casein detection by the ELISA kits as dramatically as peanut and egg protein detection. The TE kit had the best recoveries at the lowest incurred levels. The TE results were suspect, however, because this kit had a background reading at the zero incurred level that was not significantly different ($p > 0.05$) from that of the lowest incurred level. The decrease in casein detection after thermal processing was reported previously using incurred cookies[34,43] and pastry dough.[49] These previous studies reported that the negative impact on casein detection varied depending on the thermal processing method,[49] the processing time length,[34] and the ELISA kit.[43]

For BLG, mean recoveries for all kits at all incurred levels ranged from 0 to 64.8% in cookies baked for 25 min. In cookies baked for 30 min, BLG was close to undetectable with mean recoveries ranging from 0.0 to 6.2%. These results are not surprising considering the heat sensitivity of BLG.[22,23,37]

The functional relationship between the measured and incurred protein values for cookies baked for 25 or 30 min was determined for each kit and each allergen by linear regression analysis (Figure 2 and Supplemental Figure 1 of the Supporting Information). On the basis of this analysis, the accuracy of test kits for detecting allergens in the baked cookies was negatively affected by the baking process. For each kit, the accuracy determined from the slopes of the linear regression analyses for the cookie dough and baked cookies, respectively, is summarized in Table 2. The differences in the ability of the ELISA kits to accurately measure an individual allergen at or near the incurred level after processing could be due to differences in kit characteristics. Together, these data suggest that the length of the baking process negatively affects the accurate quantitation of peanut, egg, and milk proteins in the cookie dough matrix.

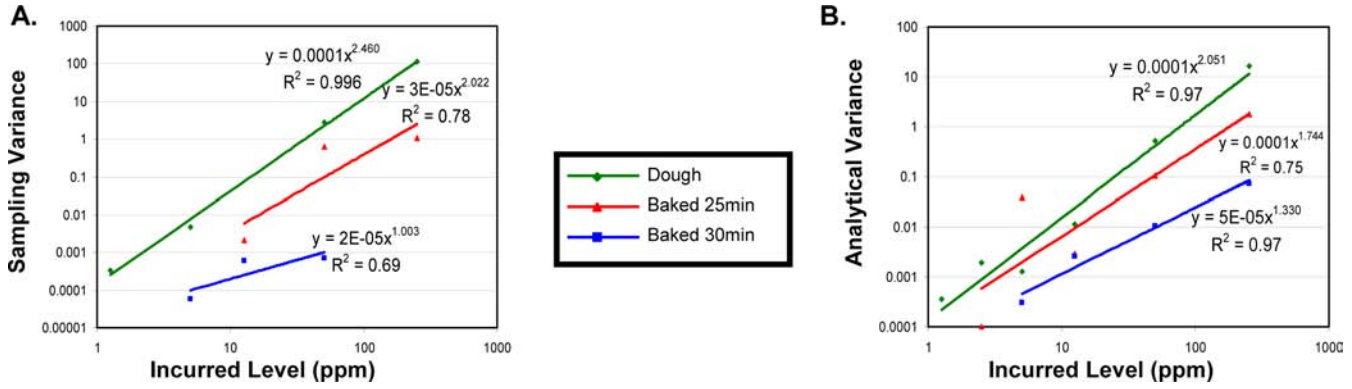

Figure 3. Regression analysis of (A) sampling and (B) analytical variance for the Neogen (NE) test kit for peanut protein in cookie dough and processed (baked for 25 or 30 min) cookies. Sampling and analytical variances increase with incurred allergen concentration.

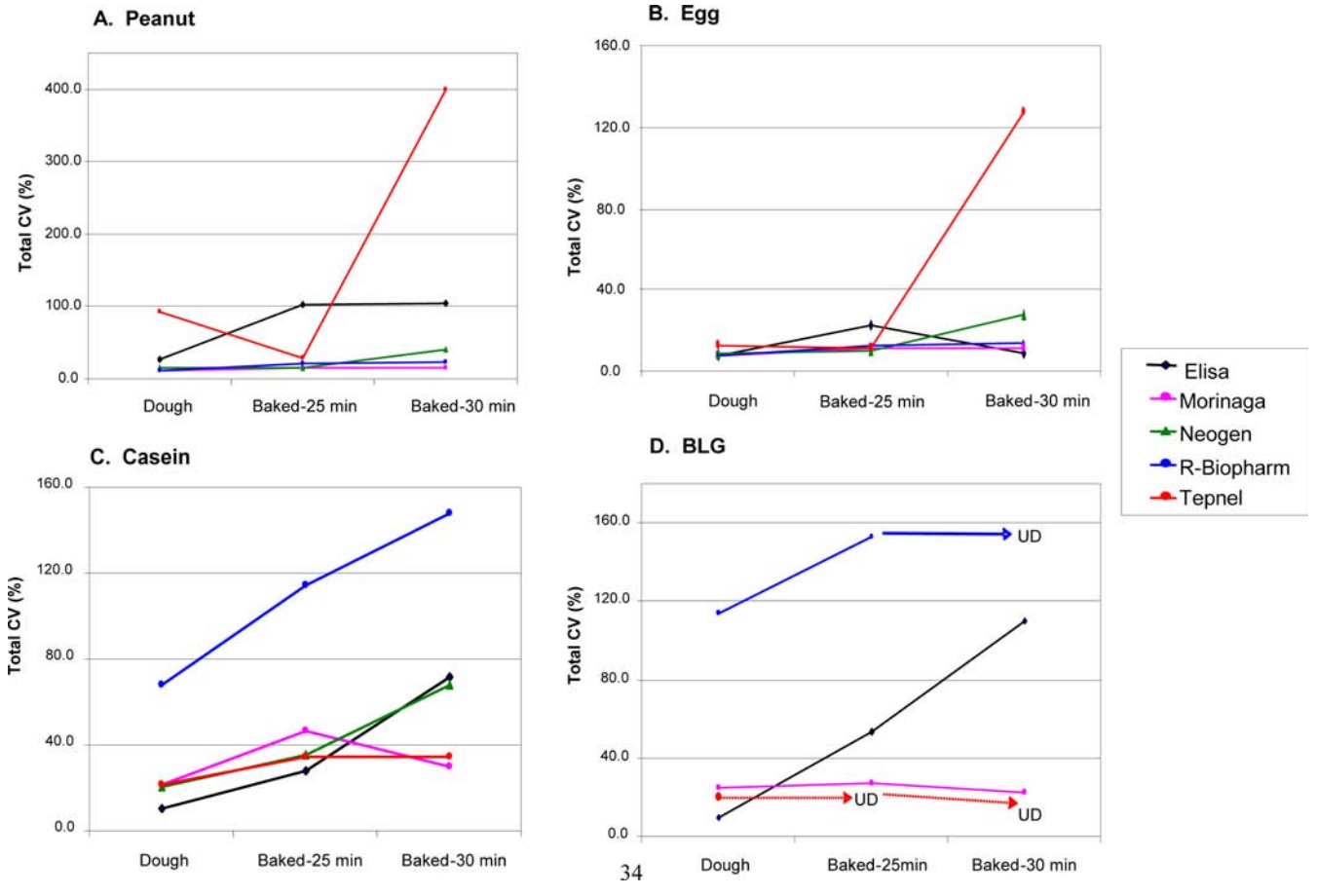

Figure 4. Total coefficient of variation (CV) averaged across all incurred levels for each kit and each allergen in cookie dough and processed (baked for 25 or 30 min) cookies (UD = undefined).

When the recovery of processed samples is evaluated, the results must be interpreted with caution. Technically, recovery can only be determined if the analytes (in this case, different allergenic proteins) are not destroyed by the processing conditions. Some allergenic proteins are altered by various processing conditions, including heat, rendering them non-allergenic.[23,36] Other proteins may be altered to the point of being undetectable by a specific antibody, but retain their allergenicity.[21,35,36] Thus, if the initial concentration of any allergenic protein is reduced by baking, then recovery should be based on the new level present after baking. Changes in the proteins during processing in a complex matrix are not completely understood, thus limiting the ability to measure a "true" percent recovery in processed samples. Additionally, samples were developed that were intended to have incurred levels close to the LOQs of most of the test kits. In a few cases, measured levels of allergens were below the LOQ indicated by the manufacturer, but these data were still included in the statistical calculations for comparative purposes.

Sampling and Analytical Variation Associated with Quantitation of Peanut, Egg, and Milk Allergens in Processed and Unprocessed Cookies. The sampling and analytical variances and sampling and analytical CVs from the nested design are taken as statistical measures of precision or

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variability associated with sampling and test kit performance, as described previously.[2] The calculated variance and CVs for peanut, egg, and milk allergens for the five test kits are shown in Supplemental Tables 3 and 4 of the Supporting Information, respectively. Sampling variability represents differences in the measurements of the allergens among the four samples at each incurred level. Analytical variability represents the difference in measurements of the allergens among the four aliquots taken from each of the four sample extracts at each incurred level.

Increased sampling and analytical variance of all test kits was observed as incurred allergen levels increased in cookie dough. To illustrate the increase in variance with incurred protein level, the sampling and analytical variances associated with using the NE test kit for peanut are shown in full-log plots in Figure 3 (Supporting Information, Supplemental Figure 2 for all kits). Because these plots are approximately linear, they can be represented by the regression equation $y = ax^b$, where y is the variance, x is the incurred allergen concentration, and a and b are constants determined from the regression analysis. The linear relationship between variance and incurred concentration is similar for processed samples (baked for 25 or 30 min) for most kits when plotted in a full-log scale, but for most kits, sampling and analytical variance were lower for baked cookies than for cookie dough. This is due to the fact that for many of the baked samples, especially at the lower incurred levels, the detectable levels of allergen are very low or zero.[50]

For the same reason, the CVs of most kits increased following baking, because the smaller the measured amount, the larger the CV. The calculated sampling, analytical, and total CVs are summarized in Supplemental Table 4 of the Supporting Information for all test allergens. The sampling CVs were mostly independent of incurred allergen level and processing conditions for all kits, with a few exceptions. Mean sampling CVs of all incurred levels (except 0 ppm) in processed and unprocessed cookies indicate good precision for most ELISA kits. The range in mean sampling CVs for all incurred levels and by all peanut kits for dough and baked cookies was from 5.8 to 29.9%; that for egg kits was from 4.6 to 27.5%; that for casein kits was from 8.2 to 40.5%; and that for BLG kits was from 6.5 to 58.8%. The range of mean sampling CVs of all incurred levels of all methods for all test allergens in incurred cookie dough was within an acceptable range,[46] between 7.5 and 19.3%. The lower variability in sampling CVs for cookie dough indicates that the samples were adequately homogenized before the ELISA analysis. Overall, the sampling CVs were best for all of the manufacturers' egg kits, ranging from 4.6 to 27.5% for cookie dough and baked cookies. Among all the test kits, the MO kit had the lowest total variation for peanut, casein, and BLG when averaged across all incurred levels in both unprocessed and processed cookies.

Analytical CVs were higher at the lower incurred levels for all test allergens and were also generally higher in processed cookies for all test kits, although there were some exceptions. This observation becomes more important when the sampling and analytical CVs were averaged across all incurred levels (except 0 ppm) for all of the test kits. The analytical CVs depended on the type of test kit used and the recovery associated with the kit. The MO kit had consistent mean analytical CVs for all test allergens in both processed and unprocessed cookies. The consistency of analytical CVs of the MO kit could be due to differences in the sample extraction procedure, in that the MO kit uses an extraction solution that contains denaturing and reducing agents. Total CV results had a pattern similar to analytical CVs (Figure 4 and Supplemental Table 4E of the Supporting Information). These results suggest that there is variation associated with both sampling and analytical procedures but that analytical variation contributes more to the total variation in this matrix.

Accuracy (recovery) and precision (variability) of commercial ELISA test kits for the quantitation of allergens in the incurred sugar cookies were determined. In some cases, the most accurate kit, based on regression analysis, for quantitation of a single allergen did not have the best precision. In this study, analytical variation contributed more than sampling variation to overall variability for most kits. This finding suggests that kit-specific standard calibrators, antibodies, extraction buffers and procedures, and data analysis may render one kit more precise for the detection of one allergen versus another in a single matrix. Because the results of some kits were expressed in different units from others (i.e., casein vs milk proteins), comparisons made using actual protein content of the incurred materials were more useful for determining accuracy and precision.

Results from baked cookies revealed that most kits were not accurate (very low recovery) for quantitating any of the tested allergens after baking; allergen recovery was drastically reduced for all allergens after 30 min of baking. This is a critical point, because some allergenic proteins that are undetectable by ELISA retain their allergenicity in sensitized patients.[10,11,49] For this reason, the choice of a kit with better accuracy is more prudent than that of a kit with better precision. The key issues associated with accuracy and precision can be addressed by using appropriate processed standards, determining appropriate extraction procedures to be used with processed foods, and developing antibodies capable of recognizing allergenic proteins having epitopes that may be altered through various processing conditions. The availability of defined reference materials for use in different matrices is critical for the harmonization of method validation protocols and food industry allergen control strategies.

Despite the fact that most consumed foods are processed, few studies have examined the impact of processing on individual allergenic proteins. An understanding of the impact of processing conditions on thresholds for eliciting responses in allergic individuals is crucial, both for mitigating risk to the allergic consumer and for management of the food production chain. Additionally, a better understanding of the effects of processing on allergen structure in a specific matrix, as it relates to immunogenicity and protein solubility, will result in improved assays and more consistent assay results. The use of analytical methods with very high specificity, such as mass spectrometry, can offer accurate quantitation and additional structural information on allergen protein-derived peptides in model-processed foods. The bioavailability of these proteins following consumption by an allergic consumer still needs to be determined. The limitations of various allergen assays should be considered before the selection of a method for a particular purpose. An understanding of these limitations will lead to improved detection methods that will enhance the confidence of allergic consumers when making food choices and the food industry when developing quality control procedures.

■ ASSOCIATED CONTENT

Supporting Information

Supplemental Tables 1−4 and Supplemental Figures 1 and 2. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: kristina.williams@fda.hhs.gov.

Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS USED

BLG, β-lactoglobulin; CV, coefficient of variation; ELISA, enzyme-linked immunosorbent assay; LOQ, limit of quantitation.

■ REFERENCES

(1) Sicherer, S. H.; Sampson, H. A. Food allergy. *J. Allergy Clin. Immunol.* **2010**, *125*, 116−125.

(2) Sicherer, S. H.; Muñoz-Furlong, A.; Sampson, H. A. Prevalence of peanut and tree nut allergy in the United States determined by means of a random digit dial telephone survey: a 5-year follow-up study. *J. Allergy Clin. Immunol.* **2003**, *112*, 1203−1207.

(3) Kagan, R. S.; Joseph, L.; Dufresne, C.; Gray-Donald, K.; Turnbull, E.; Pierre, Y. S.; Clarke, A. E. Prevalence of peanut allergy in primary-school children in Montreal, Canada. *J. Allergy Clin. Immunol.* **2003**, *112*, 1223−1228.

(4) Simonte, S. J.; Ma, S.; Mofidi, S.; Sicherer, S. H. Relevance of casual contact with peanut butter in children with peanut allergy. *J. Allergy Clin. Immunol.* **2003**, *112*, 180−182.

(5) Furlong, T. J.; DeSimone, J.; Sicherer, S. H. Peanut and tree nut allergic reactions in restaurants and other food establishments. *J. Allergy Clin. Immunol.* **2001**, *108*, 867−870.

(6) Bahna, S. L. Adverse food reactions by skin contact. *Allergy***2004**, *78* (Suppl. 59), 66−70.

(7) Ramirez, D. A., Jr.; Bahna, S. L. Food hypersensitivity by inhalation. *Clin. Mol. Allergy* **2009**, *7*, 4.

(8) Grimshaw, K. E.; King, R. M.; Nordlee, J. A.; Hefle, S. L.; Warner, J. O.; Hourihane, J. O. Presentation of allergen in different food preparations affects the nature of the allergic reaction − a case series. *Clin. Exp. Allergy* **2003**, *33*, 1581−1585.

(9) Pele, M.; Brohée, M.; Anklam, E.; Van Hengel, A. J. Peanut and hazelnut traces in cookies and chocolates: relationship between analytical results and declaration of food allergens on product labels. *Food Addit. Contam.* **2007**, *24*, 1334−1344.

(10) Maleki, S. J.; Chung, S. Y.; Champagne, E. T.; Raufman, J. P. The effects of roasting on the allergenic properties of peanut proteins. *J. Allergy Clin. Immunol.* **2000**, *106*, 763−768.

(11) Chung, S. Y.; Champagne, E. T. Association of end-product adducts with increased IgE binding of roasted peanuts. *J. Agric. Food Chem.* **2001**, *49*, 3911−3916.

(12) Van Hengel, A. J. Food allergen detection methods and the challenge to protect food-allergic consumers. *Anal. Bioanal. Chem.* **2007**, *389*, 111−118.

(13) Poms, R. E.; Anklam, E. Effects of chemical, physical, and technological processes on the nature of food allergens. *J. AOAC Int.* **2004**, *87*, 1466−1474.

(14) Harris, J. *Proceedings of the International Workshop on Food Allergy: Chemical and Technological Aspects*, Nov 6−8, 2001; EUR 20241 EN, European Commission, Joint Research Centre: Ispra, Italy, 2002; p 65.

(15) Sen, M.; Kopper, R.; Pons, L.; Abraham, E. C.; Burks, A. W.; Bannon, G. A. Protein structure plays a critical role in peanut allergen stability and may determine immunodominant IgE-binding epitopes. *J. Immunol.* **2002**, *169*, 882−7.

(16) Berrens, L. Neoallergens in heated pecan nut: products of Maillard-type degradation? *Allergy* **1996**, *51*, 277−278.

(17) Whitaker, T. B.; Williams, K. M.; Trucksess, M. W.; Slate, A. B. Immunochemical analytical methods for the determination of peanut proteins in foods. *J. AOAC Int.* **2005**, *88*, 161−174.

(18) Taylor, S. L.; Nordlee, J. A.; Niemann, L.M..; Lambrecht, D. M. Allergen immunoassays-considerations for use of naturally incurred standards. *Anal. Bioanal. Chem.* **2009**, *395*, 83−92.

(19) Beyer, K.; Morrow, E.; Li, X.-M.; Bardina, L.; Bannon, G. A.; Burks, A. W.; Sampson, H. A. Effects of cooking methods on peanut allergenicity. *J. Allergy Clin. Immunol.* **2001**, *107*, 1077−1081.

(20) Fu, T. J.; Maks, N.; Banaszewski, K. Effect of heat treatment on the quantitative detection of egg protein residues by commercial enzyme-linked immunosorbent assay test kits. *J. Agric. Food Chem.* **2010**, *58*, 4831−4838.

(21) Davis, P. J.; Smales, C. M.; James, D. C. How can thermal processing modify the antigenicity of proteins? *Allergy* **2001**, *56* (Suppl. 67), 56−60.

(22) Monaci, L.; Tregoat, V.; van Hengel, A. J.; Anklam, E. Milk allergens, their characteristics and their detection in food: a review. *Eur. Food Res. Technol.* **2006**, *223*, 149−179.

(23) Negroni, L.; Bernard, H.; Clement, G.; Chatel, J. M.; Brune, P.; Frobert, Y.; Wal, J. M.; Grassi, J. Two-site enzyme immunometric assays for determination of native and denatured β-lactoglobulin. *J. Immunol. Methods* **1998**, *220*, 25−37.

(24) Monaci, L.; van Hengel, A. J. Effect of heat treatment on the detection of intact bovine β-lactoglobulins by LC mass spectrometry. *J. Agric. Food Chem.* **2007**, *55*, 2985−2992.

(25) Fenaille, F.; Parisod, V.; Tabet, J. C.; Guy, P. A. Carbonylation of milk powder proteins as a consequence of processing conditions. *Proteomics* **2005**, *5*, 3097−3104.

(26) Zeleny, R.; Schimmel, H. Towards comparability of ELISA results for peanut proteins in food: a feasibility study. *Food Chem.* **2010**, *123*, 1343−1351.

(27) Poms, R. E.; Agazzi, M. E.; Bau, A.; Brohee, M.; Capelletti, C.; Nørgaard, J. V.; Anklam, E. Inter-laboratory validation study of five commercial ELISA test kits for the determination of peanut proteins in biscuits and dark chocolate. *J. Food Addit. Contam.* **2005**, *22*, 104−112.

(28) Faeste, C. K.; Løvberg, K. E.; Lindvik, H.; Egaas, E. Extractability, stability, and allergenicity of egg white proteins in differently heat-processed foods. *J. AOAC Int.* **2007**, *90*, 427−436.

(29) Tomková, K.; Cuhra, P.; Rysová, J.; Hanák, P.; Gabrovská, D. ELISA kit for determination of egg white proteins: interlaboratory study. *J. AOAC Int.* **2010**, *93*, 1923−1929.

(30) Matsuda, R.; Yoshioka, Y.; Akiyama, H.; Aburatani, K.; Watanabe, Y.; Matsumoto, T.; Morishita, N.; Sato, H.; Mishima, T.; Gamo, R.; Kihira, Y.; Maitani, T. Interlaboratory evaluation of two enzyme-linked immunosorbent assay kits for the detection of egg, milk, wheat, buckwheat, and peanut in foods. *J. AOAC Int.* **2006**, *89*, 1600−1608.

(31) Van Hengel, A. J.; Capelletti, C.; Brohee, M.; Anklam, E. Validation of two commercial lateral flow devices for the detection of peanut proteins in cookies: interlaboratory study. *J. AOAC Int.* **2006**, *89*, 462−468.

(32) Scaravelli, E.; Brohée, M.; Marchelli, R.; van Hengel, A. J. The effect of heat treatment on the detection of peanut allergens as determined by ELISA and real-time PCR. *Anal. Bioanal. Chem.* **2009**, *395*, 127−37.

(33) Diaz-Amigo, C. Towards a comprehensive validation of ELISA kits for food allergens: case 1—egg. *Food Anal. Methods* **2010**, *3*, 344−350.

(34) Diaz-Amigo, C. Towards a comprehensive validation of ELISA kits for food allergens. case 2—milk. *Food Anal. Methods* **2010**, *3*, 351−356.

(35) Mills, E. N.; Sancho, A. I.; Rigby, N. M.; Jenkins, J. A.; Mackie, A. R. Impact of food processing on the structural and allergenic properties of food allergens. *Mol. Nutr. Food Res.* **2009**, *53*, 963−969.

(36) Sathe, S. K.; Sharma, G. M. Effects of food processing on food allergens. *Mol. Nutr. Food Res.* **2009**, *53*, 970−978.

(37) Restani, P.; Ballabio, C.; Di Lorenzo, C.; Tripodi, S.; Fiocchi, A. Molecular aspects of milk allergens and their role in clinical events. *Anal. Bioanal. Chem.* **2009**, *395*, 47−56.
(38) Park, D. L.; Coates, S.; Brewer, V. A.; Garber, E. A.; Abouzied, M.; Johnson, K.; Ritter, B.; McKenzie, D. Performance tested method multiple laboratory validation study of ELISA-based assays for the detection of peanuts in food. *J. AOAC Int.* **2005**, *88*, 156−160.
(39) Williams, K. M.; Westphal, C. D.; Shriver-Lake, L. C. Determination of egg proteins in snack food and noodles. *J. AOAC Int.* **2004**, *87*, 1485−1491.
(40) Keating, M. U.; Jones, R. T.; Worley, N. J.; Shively, C. A.; Yunginger, J. W. Immunoassay of peanut allergens in food-processing materials and finished foods. *J. Allergy Clin. Immunol.* **1990**, *8*, 41−44.
(41) Lee, P. W.; Niemann, L. M.; Lambrecht, D. M.; Nordlee, J. A.; Taylor, S. L. Detection of mustard, egg, milk, and gluten in salad dressing using enzyme-linked immunosorbent assays (ELISAs). *J. Food Sci.* **2009**, *74*, 46−50.
(42) Whitaker, T. B.; Trucksess, M. W.; Giesbrecht, F. G.; Slate, A. B.; Thomas, F. S. Evaluation of sampling plans to detect Cry9C protein in corn flour and meal. *J. AOAC Int.* **2004**, *87*, 950−960.
(43) Dumont, V.; Kerbach, S.; Poms, R.; Johnson, P.; Mills, C.; Popping, B.; Tömösközi, S.; Delahaut, P. Development of milk and egg incurred reference materials for the validation of food allergen detection methods. *Qual. Assur. Saf. Crops Foods* **2010**, *2*, 208−215.
(44) Heick, J.; Fischer, M.; Kerbach, S.; Tamm, U.; Popping, B. Application of a liquid chromatography tandem mass spectrometry method for the simultaneous detection of seven allergenic foods in flour and bread and comparison of the method with commercially available ELISA test kits. *J. AOAC Int.* **2011**, *94*, 1060−1068.
(45) Heick, J.; Fischer, M.; Pöpping, B. First screening method for the simultaneous detection of seven allergens by liquid chromatography mass spectrometry. *J. Chromatogr., A* **2011**, *1218*, 938−943.
(46) *SAS/STAT Software: Changes and Enhancement through Release 6.12*; SAS Institute, Inc.: Cary, NC, 1997.
(47) Anderson, D. L. Neutron captures prompt gamma-ray activation analysis of meat homogenates. *J. Radioanal. Nucl. Chem.* **2000**, *244*, 225−229.
(48) Ansari, P.; Stoppacher, N.; Rudolf, J.; Schuhmacher, R.; Baumgartner, S. Selection of possible marker peptides for the detection of major ruminant milk proteins in food by liquid chromatography-tandem mass spectrometry. *J. Anal. Bioanal. Chem.* **2010**, *399*, 1105−1115.
(49) Downs, M. L.; Taylor, S. L. Effects of thermal processing on the enzyme-linked immunosorbent assay (ELISA) detection of milk residues in a model food matrix. *J. Agric. Food Chem.* **2010**, *58*, 10085−10091.
(50) Johansson, A. S.; Whitaker, T. B.; Hagler, W. M.; Giesbrecht, F. G.; Young, J. H.; Bowman, D. T. Testing shelled corn for aflatoxin, part I: estimation of variance components. *J. AOAC Int.* **2000**, *83*, 1264−1269.