



Enhanced quantitation of egg allergen in foods using incurred standards and antibodies against processed egg in a model ELISA

Ann V. Nguyen^a, Kristina M. Williams^a, Martine Ferguson^b, Daniel Lee^c,
Girdhari M. Sharma^a, Andrew B. Do^a, Sefat E Khuda^{a,*}

^a Center for Food Safety and Applied Nutrition, United States Food and Drug Administration, 8301 Muirkirk Road, Laurel, MD, 20708, United States

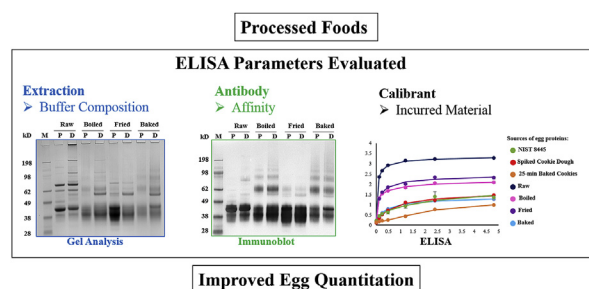
^b Center for Food Safety and Applied Nutrition, United States Food and Drug Administration, 5100 Paint Branch Parkway, College Park, MD, 20740, USA

^c Joint Institute for Food Safety and Applied Nutrition, Patapsco Building, Suite 2134, 5145 Paint Branch Parkway, College Park, MD, 20742, USA

HIGHLIGHTS

- Underestimation of egg from processed foods requires evaluation of critical Enzyme-Linked Immunosorbent Assay parameters.
- The advantage of incurred calibrators and anti-processed proteins antibodies for egg detection is demonstrated.
- These strategies will help to enhance labeling accuracy by increasing performance of egg detection methods.

GRAPHICAL ABSTRACT



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ABSTRACT

Underestimation of egg allergen from processed foods prompted the evaluation of critical Enzyme-Linked Immunosorbent Assay (ELISA) parameters: (1) extraction of egg proteins from a processed matrix; (2) use of anti-heat processed egg antibodies (Abs) on detectability of modified proteins, and (3) utilization of incurred material as standards. The relative affinity of two combinations of raw (R), boiled (B) and fried (F) Abs to unprocessed/processed egg proteins with or without matrix was determined from antibody (Ab) binding curves. In ELISAs using RBF-Abs and BF-Abs, denaturing buffer, and incurred standards, the Limit of Detection (LOD) and Limit of Quantitation (LOQ) were 0.47 and 0.25; and 1.58 and 0.85, respectively, and the linear range was 0–24 $\mu\text{g g}^{-1}$ egg protein. The recoveries of egg protein from cookies, cereal bar, and muffin (incurred levels 4.8–48 $\mu\text{g g}^{-1}$) with the developed ELISAs were in an acceptable range (50–130%). These ELISAs consistently detected more declared/undeclared egg proteins in market samples compared to assays using PBS for extraction. Overall, better assay performance was observed using BF-Abs. An ELISA combining anti-processed egg Abs, denaturing buffer, and incurred standards promises improved quantitation of egg proteins in processed foods.

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1. Introduction

The presence of trace amount of egg can trigger an allergic reaction in sensitive individuals [1]. Despite the enactment of the Food Allergen Labeling and Consumer Protection Act [2] and the

* Corresponding author. 8301 Muirkirk Road, Laurel, MD, 20708, United States.
E-mail address: sefat.khuda@fda.hhs.gov (S.E. Khuda).

Food Safety Modernization Act [3], the number of egg allergen-related recalls in the United States continues to rise [4]. Egg and egg-derived components are common ingredients in food products, increasing the potential for cross-contact due to inadequate cleaning of shared processing equipment between egg-containing and egg-free foods. Other common reasons for misbranding include mislabeling and mishandling of raw material, packaging error, etc. [5,6]. To comply with legislation and to protect allergic consumers, food industries rely on analytical methods to validate their quality control programs and to make critical labeling decisions.

Among the available methods for analysis of allergen residues in food, enzyme-linked immunosorbent assay (ELISA) continues to stand out as the method of choice for its sensitivity, specificity, ease of use and versatility. However, quantitation of egg from commercial products is complicated since undeclared egg is potentially present in numerous and varied matrices, such as bakery and snack foods, sauce, cheese, dairy, dressing, candy, etc. [4,7–11]. Previously, we surveyed a large number and wide variety of commercial bakery and snack food products and found discrepancies in the performance of commercial ELISA methods as well as a number of products that contained undeclared egg [12]. Low recoveries of egg proteins from various food matrices are common in the investigation of thermally processed foods [13,14].

The performance of quantitative ELISAs depends on the choice of extraction buffer, Ab, and the nature of the standards. Current commercially available ELISA kits for egg allergen come with unique combinations of the extractant, Ab, and calibrants, making it difficult to harmonize methods. In the evaluation of various extraction solutions and conditions, those with detergent and denaturant positively influenced the solubility of egg proteins in the presence or absence of food matrices but changing procedural conditions, such as time and temperature had negligible effect [15–19]. When examining the applicability of commercial ELISA kits for quantitation of egg in incurred model foods (cookies, cereal bar, muffin, chocolate), reduced recovery was obtained with all commercial test kits, including those employing extraction solutions containing additives such as surfactant and/or denaturant [20–22]. From these observations we hypothesized that the immunorecognition of the Abs toward processed proteins and the choice of appropriate calibrant for generation of a standard curve also positively impact the egg allergen quantitation in processed foods.

Current ELISAs are mostly based on polyclonal/monoclonal Abs specific for single or multiple egg white proteins [20,23,24]. However, some commercial foods are available that contain egg yolk. This poses a potential health risk, as some egg yolk proteins have been found to be allergenic and egg yolk accounts for almost 40% of the total protein in hen eggs [25]. False negatives may result from analysis of egg yolk-containing foods with assays targeting egg white components as protein markers for detection. Additionally, some egg allergenic proteins are more sensitive to heat processing effects than others, resulting in reduced antigenicity and increased potential for false negative results if those proteins were the target of the Ab [26]. Decisions to declare egg or not on food product labels are not dictated by the detection of specific allergens, be they major or minor, but rather by the presence of any egg protein in food. Since not all egg proteins are allergenic, gravitation towards detection of one or a few proteins constrains the assay to the physical and immunochemical characteristics of those proteins while overlooking the remaining egg proteins. Since the performance of an ELISA is dependent on Ab binding to its target in processed foods, thermal processing could have negative effects on egg protein quantitation. Higher affinities of Abs directed against processed or denatured egg proteins in processed food matrices has

been reported [15,19]. However, in recent studies, the recovery rates of egg proteins from baked foods were below 20% even with several commercial ELISAs that utilize Abs against thermally or chemically processed egg proteins [20,22]. Thus, the performance of egg ELISAs requires evaluation of additional assay components, including the use of naturally incurred calibration standards, for better representation the effects of processing in a food matrix on egg protein quantitation.

Critical to the performance of a quantitative ELISA assay is the nature of the calibrator used to generate a standard curve. The need for evaluation of incurred material for use as standards with ELISAs when quantitating allergenic proteins from processed foods has been previously reviewed [27,28]. Incurred standards account for the effects of processing and, potentially, food matrix effects whereas other standards, such as purified proteins, unprocessed reference materials or spiked samples fail to do so. AOAC Validation Procedures for Quantitative Food Allergen ELISA Methods: Community Guidance and Best Practices also emphasizes the use of incurred material as standard for best practice of validating developed analytical methods for food allergens [28].

This study was designed to evaluate the use of incurred model foods as standards and in-house established Abs generated against thermally processed egg (white and yolk) proteins in the performance of in-house developed sandwich ELISAs. The immunoreactivities of processed egg proteins with Abs against white and yolk processed under different conditions (raw, boiled, fried, or baked egg) were assessed. Two different Abs combinations directed against both egg white and yolk were used in the ELISAs for detection of proteins in whole egg: 1) raw, boiled, fried (RBF-Abs) and 2) boiled and fried (BF-Abs). We further show the advantage of using incurred foods as standards for quantitation, based on affinity and binding patterns of capture and detector Abs combinations with unprocessed/processed egg proteins in the presence and absence of matrix. The LOD, LOQ, detection range, cross reactivity and specificity of ELISAs were determined, as were the effects of extraction buffer composition on egg recovery from processed matrices. Recovery, precision, and repeatability were evaluated using several incurred model foods, including cookies, muffin, cereal bar and chocolate matrices. Applicability of the developed ELISAs for egg detection in common market foods containing declared egg (white or yolk alone) or no declared egg, with or without precautionary labeling, was also examined.

2. Materials and methods

2.1. Production of polyclonal Abs against raw and thermally processed egg

Previously established Abs against raw, boiled (100 °C, 10 min), and fried (200 °C, 30 s) egg white, boiled ovalbumin (OA) and ovomucoid (OM), and native OM [19] (Supplemental Information) were used. In addition, unprocessed/processed egg yolk antigens were prepared as described for egg white, but with an additional three rinses of hexane at 1:2 yolk/hexane ratio and air drying prior to extraction. Baked egg white and yolk antigens were prepared by heating at 177 °C for 25 min, in a convection oven (Thermo Scientific, Waltham, MA, USA). Polyclonal (rabbit) Abs against these egg antigens were produced in-house, following the previously described immunization protocol [19]. All animal protocols were approved by the Institutional Animal Care and Use Committee (IACUC) and rabbits were housed in an Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC)-accredited facility. Rabbit sera were stored at –20 °C until further use.

2.2. Samples for the study

2.2.1. Egg

Whole egg (white and yolk) or egg white were homogenized by blending (LB10, Waring Laboratory, Torrington, CT, USA), and thermally processed as above.

2.2.2. Model incurred foods

Cookies (baked at 190 °C for 25 or 30 min) and chocolate (tempered at 46 °C for 4 h) were incurred with spray-dried whole egg powder (NIST RM 8445: 48% protein; National Institute of Standards and Technology, Gaithersburg, MD, USA) [20,21]. Cereal bars (baked at 177 °C for 30 min) and muffins (baked at 177 °C for 48 min) were incurred with whole egg powder from Rembrandt Foods (50.67% protein; Rembrandt Foods, Rembrandt, IN, USA) ([22]). The muffin sample containing 100 $\mu\text{g g}^{-1}$ and cereal bar containing 1000 $\mu\text{g g}^{-1}$ whole egg powder, were finely ground, then mixed in blender for 5 min with allergen free samples to prepare lower incurred levels (Supplemental Information). Final incurred levels for all model foods were 0, 1.2, 2.4, 4.8, 12, and 48 $\mu\text{g g}^{-1}$ egg protein.

2.2.3. Commercial food samples

For the cross-reactivity study, 45 foods and food ingredients without egg were purchased from local grocery stores in Maryland. An additional 33 products were purchased to determine suitability of ELISAs for egg detection in market products.

2.3. Sample extraction

All of the above-mentioned samples were extracted at 1:10 ratio (1 g/10 mL for solid samples; 1 mL/9 mL for liquid samples) with phosphate-buffered saline (PBS) (Sigma-Aldrich, St. Louis, MO, USA) pH 7.4 for 1 h, or with denaturing buffer (20 mM $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, 1 M NaCl, 1% sodium dodecyl sulfate (SDS), and 0.1 M sulfite) overnight at room temperature (RT), then centrifuged at 3000 g for 20 min at 25 °C. Chocolate samples were melted in a water bath at 60 °C for 30 min prior to extraction. Extracts using denaturing buffer were used fresh for each experiment, and extracts in PBS were stored at –20 °C for use within a week.

2.4. Purification and conjugation of Abs

All Abs were purified from rabbit sera with a Protein G Agarose Kit (KPL, Gaithersburg, MD, USA). Detection Abs were conjugated with SureLINK Horseradish Peroxidase Conjugation kits (KPL, Gaithersburg, MD, USA) following quantitation by Pierce™ 660 nm (Thermo Scientific). Unlabeled and labeled Abs were used as capture and detection Abs, respectively, for ELISA assays.

2.5. Anion exchange chromatography

PBS or denaturing buffer extracts from raw, boiled, fried and baked egg white were buffer-exchanged with solvent A (20 mM Tris-HCl, pH 8.0) using PD-10 columns (GE Healthcare, Buckinghamshire, UK), then concentrated using 3 K centrifugal filters (Millipore, Cork, Ireland), and quantitated by Pierce™ BCA Protein Assay Kit (ThermoFisher Scientific, Waltham, MA, USA). A HiTrap Q HP (5 mL) column and Unicorn software (GE Healthcare, Little Chalfont, UK) was used with AKTA chromatography system (GE Healthcare). The column was equilibrated with 5 column volumes of solvent A, followed by injection of a 1 mL sample containing 12 mg of protein. After washing with 2 column volumes of solvent A, bound proteins were eluted in 20 column volumes with a linear gradient of 0–50% solvent B (20 mM Tris-HCl with 1 M NaCl, pH

8.0), and 5 column volumes of 100% solvent B. The flow rate was set at 4 mL min^{–1} and eluted proteins, collected in 4 mL fractions, were monitored by absorbance at 280 nm.

2.6. Sodium dodecyl Sulfate–Polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting

The protein fractions contained in the chromatography peaks, as determined by absorbance at 280 nm, were concentrated 10-fold and an equal volume of each sample was analyzed by SDS-PAGE using Precast NuPAGE® Bis-Tris 4–12% gels, (Life Technologies, Grand Island, NY, USA) under denaturing conditions. The gel was stained with SimplyBlue™ SafeStain (Life Technologies) for protein visualization. For Western blotting, unstained proteins were transferred onto nitrocellulose (Whatman GmbH, Dassel, Germany). Membranes were blocked for 1 h with 1x Detector Block Solution/0.1% Tween-20 (Seracare, Milford, MA USA), then incubated overnight with mixture of HRP-labeled Abs against native and boiled OA or native and boiled OM, diluted 1000-fold, or to 1 $\mu\text{g mL}^{-1}$, in blocking solution. In addition to in-house established Abs (Supplemental Information), anti-native OA Ab was commercially obtained (ThermoFisher Scientific). Proteins were visualized after 5 min development with CN/DAB Substrate (ThermoFisher Scientific) and images were captured by a G: Box Chemi XX6 and GeneSys software (Syngene, Frederick MD, USA).

2.7. Sandwich ELISA

The sandwich ELISA assay was optimized as follows: A Starwell Maxi Nunc Immuno Module (Thermo Scientific) was coated with 0.5 μg of each unlabeled Ab in 100 μL volume of 50 mM carbonate buffer pH 9.6 (Sigma-Aldrich) per well overnight at 4 °C. Subsequent incubations were carried out at RT. Plates were washed three times with PBS, then blocked with 300 μL per well of 1x Detector Block Solution/0.1% Tween-20 (Seracare) for 30 min. Plates were washed twice with washing buffer (PBS/0.1% Tween-20) and incubated with 100 μL of samples and standards for 1 h. When necessary, dilutions of samples were made in extraction buffer. Plates were then incubated for 30 min with 100 μL per well of HRP-labeled Abs diluted 1000-fold in blocking buffer (except anti-raw yolk and anti-raw white were diluted 2000-fold), then developed with 100 μL of SureBlue Reserve™ Microwell 3,3', 5, 5' – tetramethylbenzidine (TMB) Substrate (Seracare) for 20 min, with 4 washes with PBS-Tween 20 between each step. Finally, the reaction was stopped by the addition of 100 μL of 1 N HCl. The absorbance was measured at 450 nm using a SpectraMax M5 spectrophotometer, and data were analyzed using Softmax Pro 5.3 software (Molecular Devices Corp., Sunnyvale, CA, USA). Standard curves were generated using a 4-parameter logistic curve fit.

2.7.1. Immunodetection

Protein G purified Abs against white and yolk from each processing condition (raw, boiled, fried, or baked egg) were mixed in equal concentration and used as capture (unlabeled) or detection (labeled) Abs. Total protein content in all test whole egg extracts were determined by Pierce™ 660 nm protein assay with addition of ionic detergent compatibility reagent (IDCR) (Thermo Scientific) (Supplemental Table 1). Four sub-samples in six aliquots were analyzed from each protein source. All the samples were added at 1 μg per well to ELISA plates.

2.7.2. Ab binding curves

Two combinations of protein G-purified Abs against egg white and yolk were used: boiled, fried (BF), and raw, boiled, fried (RBF), with Abs in each mixture used in equal concentration. Three sub-

samples of unprocessed/processed whole eggs, 25-min baked incurred cookies, spiked cookie dough, and spray-dried whole egg powder NIST RM 8445 were extracted in PBS or denaturing buffer as described above. Extracts of spiked and incurred samples were added directly to ELISA plates. After extraction, incurred egg protein levels in cookies were 10-fold diluted (i.e. 0.12, 0.24, 0.48, 1.2, 2.4, 4.8 $\mu\text{g mL}^{-1}$). Whole egg and NIST 8445 extracts were diluted to comparable levels after determination of protein content by Pierce 660 nm with IDCR. Extracts from 48 $\mu\text{g g}^{-1}$ samples (1 g of 48 $\mu\text{g g}^{-1}$ sample extracted with 10 mL volume resulted in 4.8 $\mu\text{g mL}^{-1}$ egg protein) were diluted two-fold to obtain the 2.4 $\mu\text{g mL}^{-1}$ concentration. For incurred samples, 0 $\mu\text{g g}^{-1}$ egg protein was used as matrix blank, whereas for remaining sample sources, extraction buffer was used as blank. All samples were applied in triplicate to coated plates. A four-parameter logistic model was fit to the absorbance at 450 nm versus egg protein levels data for individual source and Abs-buffer combinations:

$$\text{Absorbance} = D + \frac{A - D}{1 + \left(\frac{x}{\text{EC}_{50}}\right)^b}$$

where A and D are minimum and maximum absorbance, respectively, b is the slope of the linear range in the sigmoidal curve, EC_{50} is the half maximal effective concentration ($\mu\text{g mL}^{-1}$), and x is the analyte concentration ($\mu\text{g mL}^{-1}$). The parameters were estimated using the analysis of Dose-Response Curves package in R [29].

2.7.3. LOD and LOQ determination for sandwich ELISAs using 25-min baked incurred cookies as standards

Seven sub-samples of cookies at incurred levels of 0, 1.2, 2.4 $\mu\text{g g}^{-1}$ were analyzed in triplicate. LODs and LOQs of ELISAs using different Abs-buffer combinations (BF and RBF-PBS; BF- and RBF-Denaturing) were calculated based on standard deviation of blank using basic formulas: $\text{LOD} = 3.3 \times s(0)$ (s(0) is the standard deviation of the response at 0 $\mu\text{g g}^{-1}$ level) and $\text{LOQ} = 10 \times s(0)$ [28].

2.7.4. Analysis of model processed foods and commercially available foods and food ingredients for egg content and cross-reactivity with developed ELISAs

For each food sample, five sub-samples in duplicate were analyzed with each Abs-buffer combination. Model foods contained incurred levels of 0, 1.2, 2.4, 4.8, 12, and 48 $\mu\text{g g}^{-1}$ egg proteins. The performance accuracy of the ELISAs for incurred model foods was evaluated via linear regression of measured protein ($\mu\text{g g}^{-1}$) on incurred values ($\mu\text{g g}^{-1}$). The performance precision was evaluated via a variance components analysis using PROC NESTED in SAS. The differences among ELISAs and food matrices in the mean percent recoveries were determined using Analysis of Variance (ANOVA) by PROC GLM in SAS. For the cross-reactivity experiment, undiluted samples with absorbance below that of 0 $\mu\text{g g}^{-1}$ cookie standard or LOD were considered negative.

3. Results and discussion

3.1. Solubility of processed major egg proteins in different extraction buffers

Because of the previously observed low yield of soluble egg proteins from processed foods [17,30], a fundamental objective for effective analysis of egg allergen is efficient extraction of egg proteins from complex processed food matrices. OA (43 kDa) and OM (28 kDa) represent about 60% of total egg white proteins, so the

examinations of heat processing effects were focused on these two proteins. OA is prone to aggregation during heat processing, which increases the difficulty of extraction, whereas OM is more thermostable [31]. PBS and denaturing buffer were selected to determine the effect of several heat processing procedures on the solubility of OA and OM as determined by elution profiles of extractants by anion exchange chromatography. These buffers were chosen based on previous studies comparing extraction buffers for egg proteins [15,17–19,32]. Proteins extracted in PBS or denaturing buffer were resolved by chromatography, resulting in profiles with two major peaks and several minor peaks (Fig. 1). Since the fractions were analyzed after one-step purification, it was expected that the fractions might contain some minor proteins in addition to the predominant ones. Chromatographic fractions of raw and thermally processed egg white extracts were resolved by SDS-PAGE and protein bands corresponding to the molecular weights of OA and OM were observed (Supplemental Figure 1A–1D). The baking process had the most negative impact on eluted proteins in chromatographic profiles. Identities of the fractions corresponding to OA and OM were further confirmed by Western blot analysis using anti-OA (raw + boiled) and anti-OM (raw + boiled) Abs (Fig. 2A–B for PBS buffer extracts; Fig. 2C–D for denaturing buffer extracts). The protein in each peak was assigned based on the stronger signal observed with specific Abs, overlooking weaker bands on the blots (possibly non-specific binding with other minor proteins). Using the PBS extract of raw egg, OA was identified as a large peak eluting at 60 mL. A smaller

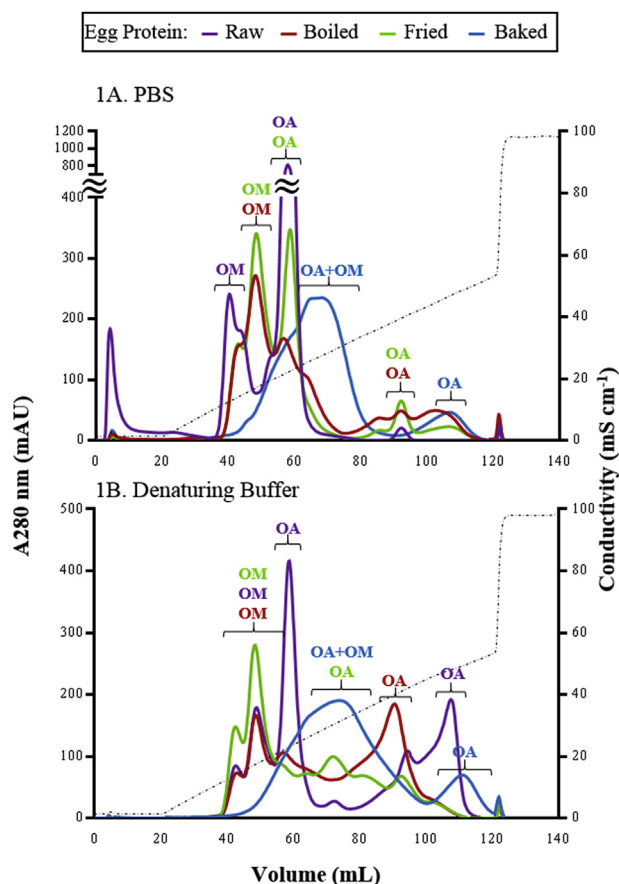
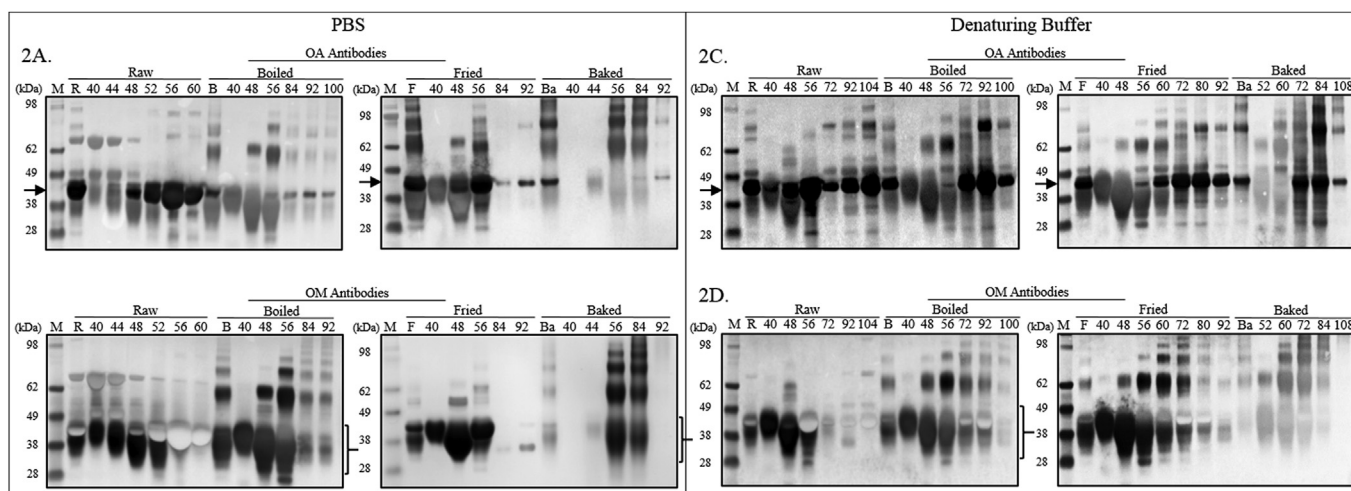


Fig. 1. Effect of processing and extraction buffers, 1A) PBS and 1B) denaturing buffer, on solubility of major egg allergens (OA and OM), by anion exchange chromatography. The text color of the identified proteins inside the graphs corresponds to the color of the respective profiles.



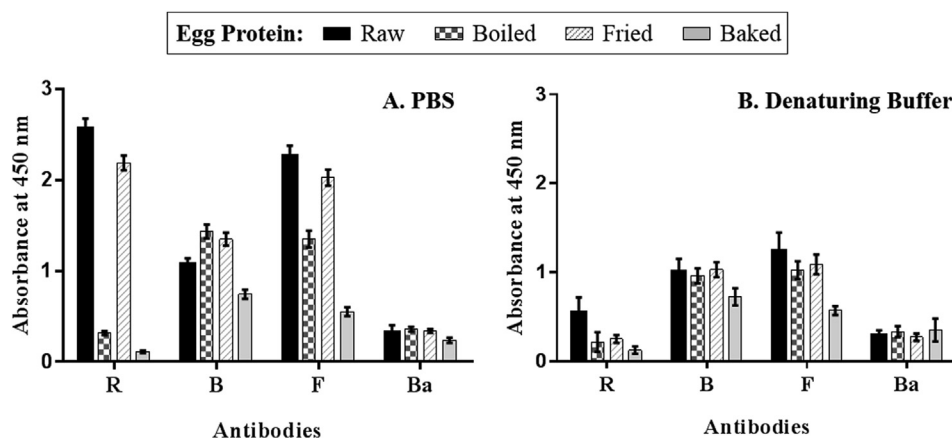


Fig. 3. Immunodetection of (A) PBS and (B) denaturing buffer-extracted egg proteins by sandwich ELISA using in-house established Abs against raw (R), boiled (B), fried (F) and baked (Ba) egg.

3.3. Selection of standard based on the Ab binding curves

The natures of the standard and food matrix have major influence on the results of ELISAs [27]. The use of unprocessed standards may lead to an underestimation of egg proteins in model processed foods analyzed by commercial ELISAs [20–22]. Commercial ELISAs often utilize native egg proteins without matrix to generate standard curves, whereas incurred material as a calibrant has the potential to be more representative of processed egg proteins in the milieu of a food matrix. We therefore evaluated the specificities of in-house established Abs with processed egg proteins in the presence or absence of food matrix. In this study we compared unprocessed/processed proteins from different egg sources (raw, boiled, fried, baked, NIST 8445, spiked cookie dough, and 25-min baked incurred cookies). Based on the results from the previous

immunodetection experiment (Fig. 3), mixtures of Abs against boiled and fried egg (BF) or raw, boiled and fried egg (RBF) were used. The response of the Abs-buffer combinations to increasing levels of proteins from different egg sources was illustrated in Fig. 4 (A–D). In a dose-response Ab binding curve, the estimated affinities, which correlate to sensitivity, can be described by the EC_{50} value – the concentration of antigen needed to reach half maximal response in the ELISA assay. Generally, the lower the EC_{50} value, the higher the estimated affinity of an Ab for a certain antigen. Differences in the Ab binding curves were observed based on the sources of egg proteins. In all four Abs-buffer combinations, the 25-min incurred cookies samples displayed a gradual increase in Ab binding response as protein concentration increased; this was further reinforced by having relatively high EC_{50} when compared to other egg sources. In contrast, proteins from other egg sources

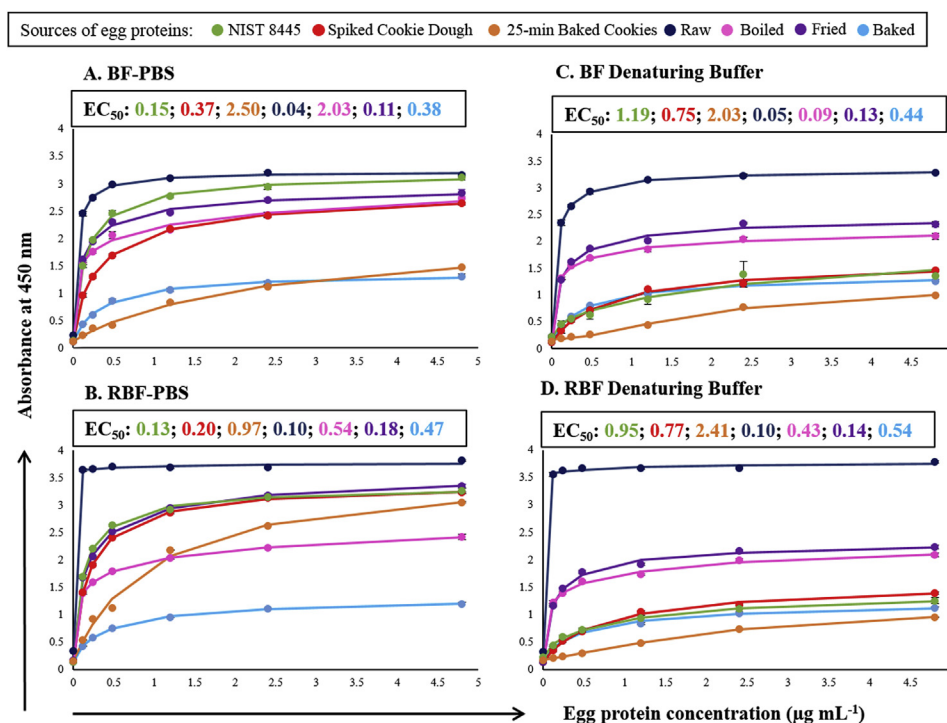


Fig. 4. Antibody binding curves and comparison of half maximal effective concentration (EC_{50}) showing response of unprocessed/processed egg proteins, with or without matrix, extracted in PBS or denaturing buffer using sandwich ELISAs with different Abs combinations (BF = boiled, fried; RBF = raw, boiled, fried).

manifested low EC_{50} s, with graphs showing steep increases in binding that reached saturation at very low antigen concentrations, indicating that these antigens had greater apparent affinity for the tested Abs-buffer combinations. The apparent affinity of an Ab-antigen reaction, as defined by EC_{50} , does not always correlate to appropriateness of such an antigen for use as a standard, because a standard with a much lower saturation point (low EC_{50}) than that of the analytical sample will lead to an artificially low protein estimation, and vice versa [27]. The dynamic range of the ELISA requires the ability to detect antigen at sufficient concentrations on either side of the 50% response for accurate estimates. Thus, the 25-min baked incurred cookies was concluded to be the most appropriate for use as standards for further ELISA development compared to the spiked cookie dough or other unprocessed/processed egg materials.

3.4. Performance of the developed ELISAs in detecting egg proteins using incurred model processed foods

3.4.1. Sensitivity

In the absence of a regulatory level for egg residue in foods, food manufacturers may take into consideration the ELISA assay limits to establish action levels as part of their quality control program [36], or to make critical labeling decisions that may consequently affect food choices and quality of life of allergic consumers [27]. In this study, four different ELISAs were developed utilizing two extraction buffers (PBS or denaturing buffer), two Abs mixtures (BF or RBF), and 25-min baked incurred cookies for generation of the standard curve. The LOD and LOQ for each ELISA was determined using the standard deviation of the blank (Supplemental Table 2) [28]. For ELISAs using Abs combinations BF, and RBF; in PBS buffer, the LODs were 0.06 and $0.12 \mu\text{g g}^{-1}$, and the LOQs were 0.21 and $0.41 \mu\text{g g}^{-1}$, whereas in denaturing buffer, the LODs were 0.25 and $0.47 \mu\text{g g}^{-1}$, and LOQs were 0.85 and $1.58 \mu\text{g g}^{-1}$, respectively. In the developed ELISAs of this study, the LODs and LOQs were within an acceptable range, as outlined in AOAC Standard Method Performance Requirements (SMPRs[®]) for Quantitation of Chicken Egg by ELISA-Based Methods [37]. The linear range of the incurred standards in each developed ELISAs was determined by liner regression of the incurred protein levels ($\mu\text{g g}^{-1}$) and absorbance at 450 nm (Fig. 5). Fig. 5A represents linear regression of standards ranging from 0 to $48 \mu\text{g g}^{-1}$ and Fig. 5B represents standards ranging from 0 to $24 \mu\text{g g}^{-1}$, and the linearity of the standard curves were assessed by goodness of fit value (R-squared). Regardless of Abs combinations, ELISAs using denaturing buffer had a linear range of 0– $24 \mu\text{g g}^{-1}$ ($R^2=0.99$)

compared to ELISAs using PBS which had lower range of linearity ($R^2 < 0.99$).

3.4.2. Recovery and accuracy

Thermal processing negatively impacts the recovery of egg proteins from foods using immunoassays [14,38]. The duration and degree of processing play a prominent role in the test outcome, as demonstrated by lower estimation of egg proteins in processed model foods analyzed with commercial ELISA methods [20–22]. Therefore, the performances of the developed ELISAs were evaluated using model processed foods (30-min baked cookies, cereal bar, muffin, and tempered chocolate) incurred with egg proteins ranging from 0 to $48 \mu\text{g g}^{-1}$. Regardless of the Abs combinations, the recoveries of egg proteins averaging across all incurred levels ranged from 60 to 210% in denaturing buffer and 11–271% in PBS in all test matrices (Supplemental Fig. 2). The assays utilizing extracts with PBS buffer showed remarkable variation in the recovery of egg proteins among test matrices, overestimating egg levels in tempered chocolate while underestimating egg levels in baked food matrices. Significant differences were observed between the mean percent recoveries utilizing denaturing and PBS buffers for all food matrices ($p < .0001$) except for the 30-min baked cookies matrix. The mean percent recoveries of individual tested incurred levels for all food matrices were analyzed (Table 1). In the analysis of PBS buffer extracts, the mean recoveries ranged from 8.8 to 60% for incurred levels 4.8 – $48 \mu\text{g g}^{-1}$ and similarly 5.4–68% for incurred levels 1.2 and $2.4 \mu\text{g g}^{-1}$ of cookies, cereal bar, and muffin with both assays. The mean recoveries were consistently high (168–350%) for tempered chocolate across all incurred levels. The overestimation of egg proteins at each incurred levels of tempered chocolate by PBS assays might be due to the lower temperature used during the tempering process compared to the temperature at which cookies were baked and/or non-specific oxidation of the TMB substrate by chocolate matrix components (polyphenols, tannins, etc.) [39]. In the analysis of denaturing buffer extracts using both Abs combinations, the mean recoveries ranged from 52 to 130% for incurred levels 4.8 – $48 \mu\text{g g}^{-1}$ of cookies, cereal bar, and muffin, with an overestimation at 1.2 and $2.4 \mu\text{g g}^{-1}$ incurred levels of cereal bar and muffin (140–411%), and underestimation at 1.2– $4.8 \mu\text{g g}^{-1}$ incurred levels of tempered chocolate (0–31%). Overestimations of 1.2 and 2.4 incurred levels in denaturing extracts of cereal bar and muffin indicated less accuracy of the assays, but the results were still positive at these levels. The denaturing buffer might reduce non-specific reactions in the analysis of chocolate by dissociating the protein-polyphenol complex [40]. Additional chemical such as polyvinylpyrrolidone inclusion in the

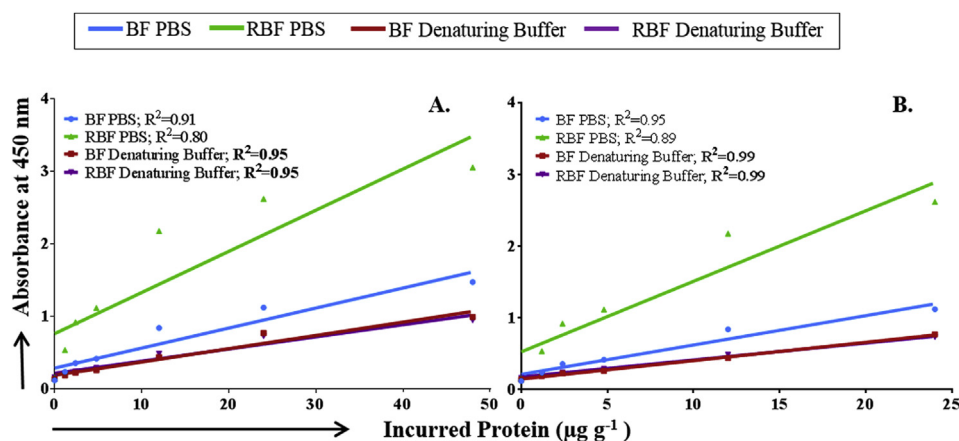


Fig. 5. Linear range of incurred cookies as standards in the developed ELISAs by regression analysis of incurred levels ranging from (A) 0–48 and (B) 0– $24 \mu\text{g g}^{-1}$ egg protein.

Table 1
Mean percent recovery for egg protein at indicated incurred levels using 30-min baked cookies, cereal bar, muffin, and tempered chocolate with developed ELISAs. Data represents mean recovery $\pm$ standard error (SE). BF = Abs against boiled, fried egg; RBF = Abs against raw, boiled, fried egg.

Developed ELISAs	Incurred Model Foods	Mean Recovery by incurred level (%)					
		0	1.2	2.4	4.8	12	48
BF PBS	30-min Baked Cookies	ND	44.0 $\pm$ 2.8	68.0 $\pm$ 6.0	46.2 $\pm$ 2.1	60.0 $\pm$ 4.7	43.6 $\pm$ 0.8
	Cereal bar	ND	59.5 $\pm$ 2.9	62.4 $\pm$ 4.1	50.0 $\pm$ 2.2	38.9 $\pm$ 2.3	29.4 $\pm$ 0.7
	Muffin	ND	9.3 $\pm$ 1.9	14.6 $\pm$ 2.8	8.8 $\pm$ 1.1	9.1 $\pm$ 0.9	13.2 $\pm$ 1.2
	Tempered chocolate	ND	235.7 $\pm$ 23.8	189.5 $\pm$ 12.6	176.3 $\pm$ 9.9	313.3 $\pm$ 15.8	272.3 $\pm$ 7.9
RBF PBS	30-min Baked Cookies	ND	40.2 $\pm$ 2.2	57.3 $\pm$ 1.8	38.0 $\pm$ 0.9	56.7 $\pm$ 2.7	45.2 $\pm$ 1.0
	Cereal bar	ND	5.4 $\pm$ 1.0	14.0 $\pm$ 0.7	10.4 $\pm$ 0.7	13.4 $\pm$ 0.3	16.0 $\pm$ 1.2
	Muffin	ND	17.8 $\pm$ 1.6	19.3 $\pm$ 0.9	17.1 $\pm$ 0.7	14.2 $\pm$ 0.3	11.2 $\pm$ 0.7
	Tempered chocolate	ND	301.3 $\pm$ 13.2	220.7 $\pm$ 9.8	168.6 $\pm$ 5.0	350.4 $\pm$ 17.3	314.6 $\pm$ 13.1
BF Denaturing Buffer	30-min Baked Cookies	ND	94.7 $\pm$ 20.6	69.8 $\pm$ 2.6	55.5 $\pm$ 3.9	61.9 $\pm$ 2.4	51.8 $\pm$ 1.9
	Cereal bar	ND	411.3 $\pm$ 13.4	223.1 $\pm$ 4.4	130.6 $\pm$ 13.5	101.3 $\pm$ 2.5	102.2 $\pm$ 2.0
	Muffin	ND	145.8 $\pm$ 5.7	140.9 $\pm$ 5.6	78.4 $\pm$ 2.2	81.3 $\pm$ 2.1	48.6 $\pm$ 1.4
	Tempered chocolate	ND	0.0 $\pm$ 0.0	17.1 $\pm$ 5.4	10.8 $\pm$ 3.1	63.1 $\pm$ 2.1	75.7 $\pm$ 1.5
RBF Denaturing Buffer	30-min Baked Cookies	ND	48.8 $\pm$ 18.2	70.6 $\pm$ 6.2	54.1 $\pm$ 4.8	56.1 $\pm$ 1.8	51.9 $\pm$ 1.7
	Cereal bar	ND	465.1 $\pm$ 13.8	243.5 $\pm$ 6.8	122.4 $\pm$ 1.0	113.3 $\pm$ 2.2	106.3 $\pm$ 1.7
	Muffin	ND	139.8 $\pm$ 13.8	200.5 $\pm$ 32.8	74.8 $\pm$ 2.9	72.6 $\pm$ 3.7	51.3 $\pm$ 3.2
	Tempered chocolate	ND	0.0 $\pm$ 0.0	31.2 $\pm$ 8.0	26.7 $\pm$ 4.8	72.4 $\pm$ 0.9	95.7 $\pm$ 3.1

extractant [41] and utilization of matrix specific standards could be considered to improve recovery of egg proteins from the chocolate matrix. For both extraction buffers, the RBF Abs combination, which includes Abs against raw egg proteins, improved recovery only slightly, which suggests that egg proteins in processed foods have undergone modification, and thus using native protein as an immunogen for Ab production is less than ideal.

The accuracy of the developed ELISAs was determined by linear regression of the mean measured value (M) on incurred levels (P) of egg protein (Fig. 6) [20,21]. Slope measures accuracy with a value of 1 indicating perfect accuracy (i.e. 100% recovery). ELISAs using denaturing buffer consistently had slopes closer to 1 compared to assays using PBS. In our previous studies examining the recovery of egg proteins from these model processed foods using commercial

ELISAs, the overall recoveries across all egg incurred levels of 30-min baked cookies, cereal bar, muffin, and tempered chocolate were <10%, 2%, <4%, and 60–300% with the test kits utilizing non-reducing extraction buffer, and 15%, 73%, 80%, and 76% with the test kit utilizing reducing extraction buffer, respectively [20–22]. The developed assays in this study utilizing extracts with denaturing buffer improved recovery of egg proteins for incurred levels 4.8–48 $\mu\text{g g}^{-1}$ from baked model foods and met the recovery performance requirement of the AOAC SMPRs[®] for Quantitation of Chicken Egg by ELISA-Based Method (50–150%) for most test matrices [37]. Thus, ELISAs using denaturing buffer and incurred standards can be effectively used to analyze similarly processed foods.

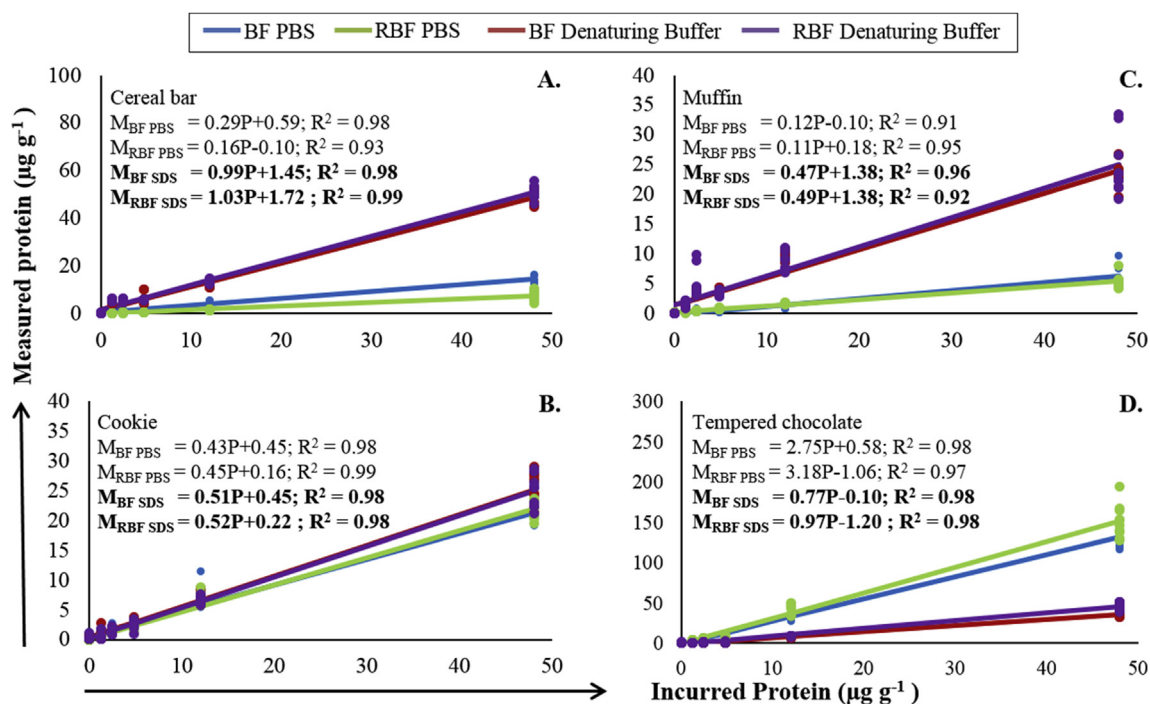


Fig. 6. Accuracy of ELISAs for detection of egg proteins in incurred model foods as measured by the slope of linear regression between measured (M) and incurred (P) protein values. A slope equaling 1 indicates 100% recovery. Slopes of linear regression are bolded for the more accurate ELISAs.

3.4.3. Precision

The precision of developed ELISAs was evaluated by partitioning the total variance into its variance components (sampling and analytical variance) for test food matrices. The sampling variance is represented by the concentration differences among the five subsamples and analytical variance (repeatability) is represented by the concentration differences between the two aliquots of each sample and their coefficients of variation (CVs) (Table 2). The variances were partitioned after adjusting for variability due to the incurred level, as an increase in variance could be the result of increased recovery amounts. The analytical and sampling CVs of the assays were within an acceptable range (i.e. < 20%) except for the PBS buffer assays using RBF and BF Abs combinations, which had poor precision for cereal bar (analytical CV = 29.6 and sampling CV = 40.3) and muffin (analytical CV = 51.3 and sampling CV = 27.6), respectively. These lower precision levels also correspond to the lowest mean percent recovery for the above food matrices. Examining the sampling and analytical variance by incurred levels, low precision was observed in all developed ELISAs utilizing both buffers at levels 0–2.4 $\mu\text{g g}^{-1}$, which improved at levels 4.8–48 $\mu\text{g g}^{-1}$ (Supplemental Table 3). When compared to ELISAs employing denaturing or PBS buffer, both current methods with denaturing buffer showed better performance for all the tested model processed foods, indicating the positive influence of naturally incurred material as standards and Abs against processed proteins.

3.5. Specificity and applicability of the developed ELISAs

The specificities of the developed sandwich ELISAs were investigated by analyzing undiluted extracts from 45 food ingredients that were selected based on AOAC Guidance [28]. Most of the tested samples did not show cross-reactivity (<LOD) in ELISAs with PBS buffer. When tested with ELISAs using denaturing buffer, about half of the tested samples did not cross-react (<LOD). Minor cross-reactivity (LOD-LOQ and 1–5 $\mu\text{g g}^{-1}$) was observed for most of the remaining samples, including peanut, as full-strength extracts, which became non-reactive after a 2-fold dilution. (Table 3). These differences in results might be resulted from the buffer composition effect on the epitopes of extracted proteins instead of specificity of the Abs. The observed low cross-reactivity examining raw single ingredients with denaturing buffer may not apply in the intended use of these ELISAs for analysis of complex processed foods. All four assays responded to a small group of ingredients (chicken, turkey, and duck) showing highest signal with chicken, because of the presence of proteins of similar amino acid sequences shared among avian species, such as α -livetn found in egg yolk, feather, and meat [25,42]. Since the Abs used in this study were generated against whole chicken egg, it was not unexpected that non-specific reactions would occur with these related proteins.

Table 3

Cross-reactivity of foods and food ingredients in the developed ELISAs. In PBS buffer, BF LOQ: 0.21 and RBF LOQ: 0.41; In Denaturing buffer, BF LOQ: 0.85 and RBF LOQ: 1.58; In PBS buffer, BF LOD: 0.06 and RBF LOD: 0.12; In Denaturing buffer, BF LOD: 0.25 and RBF LOD: 0.47. ND = Not Detected.

Food/Food ingredient	Measured Egg Protein in PBS buffer ($\mu\text{g g}^{-1}$)		Measured Egg Protein in Denaturing buffer ($\mu\text{g g}^{-1}$)	
	BF	RBF	BF	RBF
Pumpkin seed	ND	ND	LOD-LOQ	ND
Sunflower seed	ND	ND	2	2
Linseed	ND	ND	2	1
Sesame	ND	ND	2	4
Poppy Seed	ND	ND	2	3
Pine nut	ND	ND	1	3
Walnut	ND	ND	1	2
Cashew	ND	ND	1	LOD-LOQ
Pecan	ND	ND	1	ND
Almond	ND	ND	26	25
Peanut	ND	ND	5	4
Hazelnut	ND	ND	1	ND
Brazil nut	ND	ND	1	ND
Pistachio	ND	ND	ND	ND
Macadamia nut	ND	ND	ND	ND
Chestnut	ND	ND	ND	ND
Chick pea	ND	ND	1	LOD-LOQ
Kidney beans	ND	ND	1	ND
Lentils	ND	ND	ND	ND
Split peas	ND	ND	ND	ND
Peas	ND	ND	ND	ND
Lima Beans	ND	ND	2	4
Soy Bean	ND	ND	1	2
Adzuki beans	ND	ND	1	2
Brown rice	ND	ND	2	3
White rice	ND	ND	LOD-LOQ	LOD-LOQ
Buckwheat	ND	ND	ND	ND
Corn	ND	ND	ND	ND
Oats	ND	ND	1	2
Barley	ND	ND	1	2
Rye	ND	ND	LOD-LOQ	ND
Wheat	ND	ND	ND	ND
Pork	ND	ND	LOD-LOQ	ND
Beef	ND	ND	ND	ND
Turkey	3	3	2	7
Chicken	7	7	16	131
Octopus	ND	ND	ND	ND
Fish	ND	ND	ND	ND
Shrimp	ND	ND	LOD-LOQ	2
Duck	0.7	0.5	2	7
Kiwi	ND	ND	1	LOD-LOQ
Cocoa	ND	ND	ND	ND
Coconut	ND	ND	ND	ND
Milk	ND	ND	ND	ND
Bovine gelatin	ND	ND	ND	ND

Table 2

Precision of ELISAs to detect egg protein in indicated model foods. Precision measured by sampling and analytical variances and CVs aggregated across all incurred levels (1.2, 2.4, 4.8, 12, 48 $\mu\text{g g}^{-1}$ egg proteins).

Extraction Buffer	Incurred Model Foods	BF ELISAs		RBF ELISAs	
		Analytical CV (%)	Sampling CV (%)	Analytical CV (%)	Sampling CV (%)
PBS	30-min Baked Cookies	10.1	14.9	6.4	14.1
	Cereal bar	3.0	17.4	40.3	29.6
	Muffin	51.3	27.6	26.7	21.4
	Tempered chocolate	7.3	19.0	17.0	21.5
Denaturing Buffer	30-min Baked Cookies	16.8	14.7	7.2	20.2
	Cereal bar	2.7	13.3	10.9	7.5
	Muffin	4.9	15.2	0.0	34.8
	Tempered chocolate	4.0	14.9	3.9	22.9

Commercial Food Samples		Measured Egg Protein in PBS buffer ($\mu\text{g g}^{-1}$)		Measured Egg Protein in Denaturing buffer ($\mu\text{g g}^{-1}$)		
		BF	RBF	BF	RBF	
Declared Egg (component)	Bread Roll (yolk)	1 $\pm$ 0.1	1 $\pm$ 0.0	43 $\pm$ 1.5	56 $\pm$ 3.3	
	Cracker (yolk)	404 $\pm$ 65.2	247 $\pm$ 56.7	394 $\pm$ 18.5	601 $\pm$ 180.9	
	Wafer (yolk)	326 $\pm$ 51.9	180 $\pm$ 53.6	402 $\pm$ 19.0	323 $\pm$ 12.6	
	Macaroon (white)	7242 $\pm$ 280.1	6400 $\pm$ 638.0	4716 $\pm$ 55.6	5218 $\pm$ 59.8	
	Cake (white)	38508 $\pm$ 1493.1	29291 $\pm$ 763.5	14761 $\pm$ 2189.4	13036 $\pm$ 959.7	
	Bread (white)	561 $\pm$ 14.7	346 $\pm$ 42.4	2119 $\pm$ 88.3	1514 $\pm$ 80.7	
	Bread roll (white)	202 $\pm$ 15.1	118 $\pm$ 11.7	3614 $\pm$ 114.8	2418 $\pm$ 172.9	
	Biscotti (white)	440 $\pm$ 33.1	349 $\pm$ 66.4	1409 $\pm$ 29.5	1064 $\pm$ 45.2	
	Croissant (whole)	ND	ND	ND	ND	
	Bread roll (whole)	233 $\pm$ 20.8	232 $\pm$ 39.0	1581 $\pm$ 144.9	1171 $\pm$ 134.3	
	Cookie (whole)	2451 $\pm$ 216.2	2386 $\pm$ 259.3	1762 $\pm$ 77.4	1934 $\pm$ 105.8	
	Cookie (whole)	202 $\pm$ 32.5	169 $\pm$ 22.0	335 $\pm$ 27.3	242 $\pm$ 18.5	
	Bread (whole)	778 $\pm$ 67.7	485 $\pm$ 38.3	1299 $\pm$ 74.5	1102 $\pm$ 134.6	
	Undeclared Egg Without Precautionary Labeling	Bread	8 $\pm$ 0.4	9 $\pm$ 0.1	3502 $\pm$ 182.3	3424 $\pm$ 180.1
Tortillas		14 $\pm$ 0.8	12 $\pm$ 0.9	135 $\pm$ 41.3	123 $\pm$ 27.6	
Cookie		ND	ND	8 $\pm$ 0.7	8 $\pm$ 0.8	
Biscuits		848 $\pm$ 62.6	918 $\pm$ 66.7	2828 $\pm$ 226.4	6038 $\pm$ 1775.2	
Biscuits		5174 $\pm$ 122.6	5665 $\pm$ 275.3	10777 $\pm$ 580.2	18290 $\pm$ 1532.6	
Others		ND	ND	< LOQ	ND	
Pretzels		ND	ND	1 $\pm$ 0.3	< LOQ	
Bread		ND	ND	< LOQ	ND	
Cracker		ND	ND	ND	< LOQ	
Bread Roll		ND	ND	ND	ND	
Undeclared Egg With Precautionary Labeling		Bread	9 $\pm$ 0.5	7 $\pm$ 0.3	42 $\pm$ 4.2	30 $\pm$ 3.2
		Bread	2 $\pm$ 0.4	1 $\pm$ 0.1	1 $\pm$ 0.3	ND
		Cookie	14 $\pm$ 0.2	12 $\pm$ 0.3	3 $\pm$ 0.1	6 $\pm$ 0.5
		Muffin	276 $\pm$ 6.0	216 $\pm$ 6.3	9204 $\pm$ 589.9	7448 $\pm$ 815.0
	Bread	71 $\pm$ 10.8	39 $\pm$ 8.3	50 $\pm$ 21.8	40 $\pm$ 8.3	
	Biscuits	ND	ND	1 $\pm$ 0.4	ND	
	Bread	ND	1 $\pm$ 0.5	3 $\pm$ 0.3	ND	
	Cookie	1 $\pm$ 0.1	ND	ND	ND	
	Bread	ND	ND	ND	ND	
	Bread	ND	ND	ND	ND	

statistically consistent and improved quantitation of egg in processed foods can be obtained utilizing incurred standards, appropriate extractant, and Abs capable of recognizing processed egg proteins.

Disclaimer

The opinions here are those of the authors and do not necessarily reflect the view of the Food and Drug Administration or the Agency.

Declarations of interest

None.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.aca.2019.07.030>.

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1 Supplemental Information

2 *Preparations of raw and thermally processed egg antigens for production of polyclonal Abs*

3 Raw egg white: Grade A large eggs were purchased from local grocery store and kept at 4°C for
4 use within a week. The egg white was separated from the yolk by letting the egg white drip
5 through cheese cloth. The egg white was extracted in PBS at 1:2 (v/v) ratio in a shaking
6 incubator (4°C, 150 rpm) for 3 h. The supernatant was collected after centrifugation at 3000 g
7 (~2000 rpm) for 10 min at 4°C and dialyzed against PBS (800 ml for 24 h with first change
8 overnight) using 1 kDa Pre-wetted RC Tubing (Spectrum Laboratories, Inc., Rancho
9 Dominquez, CA, USA). Then, the supernatant was centrifuged at 10,000 g for 30 min and
10 filtered with 0.4 µm filter (GE Healthcare, Buckinghamshire, UK).

11 Raw egg yolk: The outside of the yolk sac was gently wiped with damped paper towel. Egg yolk
12 was defatted with hexane (1:2 v/v) by mixing for 5 min and centrifuged at 3000 g for 3 min. The
13 hexane was discarded, and the defatting procedure was repeated twice. The egg yolk was air-
14 dried then extracted in PBS at 1:2 (v/v) ratio in a shaking incubator (4°C, 150 rpm) overnight.
15 Afterward, the supernatant was dialyzed and filtered as described for raw egg white.

16 Boiled egg white: Eggs were boiled in water for 10 min, then egg white and yolk were separated.
17 When needed, the residual egg yolk was wiped from egg white. Egg white was homogenized
18 (LB10, Waring Laboratory, Torrington, CT, USA), then extracted with PBS (1:2 w/v) on a stir
19 plate with gentle stirring (4°C overnight). After centrifugation, the supernatant was dialyzed and
20 filtered as described above.

21 Boiled egg yolk: The boiled yolk was defatted as described for raw egg yolk, then extracted with
22 PBS (1:2 w/v) on a stir plate with gentle stirring (4°C overnight) followed by dialysis and
23 filtration.

24 Fried egg white: Raw egg white and yolk was separated as described above. Egg white was fried
25 in a frying pan, without oil, for 30 seconds on a hot plate (VWR, Radnor, PA, USA). The
26 temperature monitored by handheld infrared thermometer (Etekcity, Anaheim, CA, USA). Fried
27 egg white was homogenized using blender, then extracted with PBS (1:2 w/v) on a stir plate with
28 gentle stirring at 4°C overnight. After centrifugation, supernatant was dialyzed and filtered.

29 Fried egg yolk: Raw egg yolk was fried as described for fried egg white, then defatted, extracted
30 as described previously. Subsequently, the supernatant was dialyzed and filtered.

31 Baked egg white: Raw egg white and egg yolk were separated from fresh egg. To maintain the
32 consistency in all independent experiments, 40 mL egg white (approximately 1 egg white) was
33 baked in a 400 mL beaker at 177°C for 25 min in convection oven (Thermo Scientific Waltham,
34 MA, USA) then left to cool at room temperature. Baked egg white was homogenized using
35 blender and subsequently extracted with PBS (1:2 w/v) on a stir plate at 4°C overnight. The
36 supernatant was dialyzed and filtered.

37 Baked egg yolk: Raw egg yolk (40 mL $\approx$ 2 egg yolks) was baked as described for baked egg
38 white, then homogenized and extracted with PBS (1:2 w/v) on a stir plate at 4°C overnight. The
39 supernatant was dialyzed and filtered.

40 Unprocessed and processed OA and OM antigens: Purified OM (Type III-O trypsin inhibitor),
41 and OA proteins from chicken egg white were purchased from Sigma–Aldrich (St. Louis, MO,
42 USA) and further purified by high pressure liquid chromatography using a Waters Corporation

(Columbia, MD, USA) Protein Pak 125 size exclusion column. These proteins were dissolved in PBS (1 mg ml⁻¹) for use as native or boiled (10 min) antigen.

Model incurred foods

Cookies: The base ingredients were 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. For incurred samples, a flour premix consisting of 5000 µg g⁻¹ of egg powder (NIST RM 8445; National Institute of Standards and Technology, Gaithersburg, MD, USA; 48% protein) was prepared and added at the appropriate levels (corresponding to 1.2, 2.4, 4.8, 12, 48 µg g⁻¹ egg protein) to the remaining dry ingredients. The dry ingredients of control (egg-free) and incurred samples were mixed at slow speed in a Hobart mixer (Troy, OH, USA) for 2 h, then liquid ingredients were added, and mixed for an additional 20 min. The dough balls were baked for 25 or 30 min at 190°C on aluminum foil-covered baking sheets. Minimal (<2%) moisture loss from the cookies baked for 25 or 30 min was observed based on measured weights before and after baking and was therefore not included in statistical evaluation. Cookies were homogenized in a food processor prior to sampling. A dedicated “allergen-free” food processor was used for the control (0 µg g⁻¹) cookies [20].

Tempered chocolate: The base ingredient was Scharffen Berger semisweet 62% cacao chocolate, produced on a peanut-, egg-, and milk-free dedicated line (Scharffen Berger, Berkeley, CA, USA), and incurred with spray-dried whole egg powder-NIST RM 8445. The chopped pieces of dark chocolate in a bowl (Chocovision, Poughkeepsie, NY, USA) was melted for 30 min in a preheated (42°C for approximately 15 min) Revolution 2 tempering machine (Chocovision) with continuous mixing by a Teflon scraper. For incurred samples, a sample containing 500 µg g⁻¹

egg powder were made by mixing with the melted chocolate for 30 min in the tempering machine. Incurred samples corresponding to 1.2, 2.4, 4.8, 12, 48 $\mu\text{g g}^{-1}$ egg protein were made by mixing the 500 $\mu\text{g g}^{-1}$ sample with melted chocolate. Then, the control (egg-free) and incurred samples were tempered at 46°C for 4 h with continuous mixing to maintain homogeneity. Afterwards, the samples were cooled at room temperature for 2 h, then harden in the refrigerator for an additional 2 h before grinding in a food processor. Dedicated equipment was used to prevent cross-contact during sample production [21].

Cereal bar: The base ingredients were 12770 g of corn syrup, 5520 g of granulated sugar, 260 g of salt, 2860 g of canola oil, 8320 g of water, 49900 g of rolled oats, 9980 g of crisp rice, 1018 g of corn flour. The binder ingredients (corn syrup, sugar, canola oil, salt, water) were mixed in a steam jacketed kettle for 10 min at 52°C. For incurred 1000 $\mu\text{g g}^{-1}$ cereal bar, 100 g of whole egg powder (Rembrandt Foods, Rembrandt, IN, USA; 50.67% protein) was substituted for 100 g of corn flour used in the control, and combined in a Hobart mixer (Troy, OH, USA) for 10 min. Then, binder and all dry ingredients of control (egg-free) and incurred samples were homogenized using a spiral mixer (Peerless Food Equipment, Sidney, OH, USA). All cereal bar samples were baked in a Baxter OV300E (Orting, WA) rotating oven at 177°C for 30 min, then homogenized using Cuisinart DLC-X 20 cup food processor (Stamford, CT, USA) for 3 min [22]. Good Manufacturing Practices were followed to minimize cross-contact during sample production. The experimental incurred levels were prepared by homogenizing final 1000 $\mu\text{g g}^{-1}$ incurred with control sample (w/w) for 5 min in blender after baking, thus, moisture loss was not accounted in the statistical evaluation.

Muffin: The base ingredients for muffin were 9980 g of sugar, 5990 g of vegetable oil, 200 g of salt, 150 g of egg substitute powder, 9070 g of water, 200 g of vanilla extract, 15570 g of wheat flour, and 750 g of baking powder. For incurred $100 \mu\text{g g}^{-1}$ muffin, 10 g of whole egg powder (Rembrandt Foods) was substituted for 10 g of wheat flour used in the control. Sugar, salt, egg replacer powder, whole egg powder, vanilla extract, vegetable oil, and water were combined using a Single Sigma Arm Peerless SD mixer for 5 min, then the rest of the dry ingredients were added and mixed for an additional 5 min. Control (egg-free) and incurred samples (≈ 125 g batter per muffin) were baked at 177°C for 48 min in a four-shelf rotating oven (Reed, Kansas City, MO, USA), then homogenized using Cuisinart DLC-X 20 cup food processor (Stamford, CT, USA) for 3 min [22]. Good Manufacturing Practices were followed to minimize cross-contact during sample production. The experimental incurred levels were prepared by homogenizing final $100 \mu\text{g g}^{-1}$ incurred with control sample (w/w; dry) for 5 min in blender and moisture loss was not accounted in the statistical evaluation.

101 Supplemental Figure 1: SDS-PAGE analysis of anion exchange chromatography fractions for
102 PBS extracted (A) raw and boiled, (B) fried and baked egg white, and denaturing buffer
103 extracted (C) raw and boiled, (D) fried and baked egg white (R= Raw, B= Boiled, F= Fried, Ba=
104 Baked egg white extract; arrow indicates OA, bracket indicates OM).

105 Supplemental Figure 2: Comparison of the developed ELISAs utilizing incurred cookies (25-min
106 baked at 190°C) for calibration standards on the recovery of egg proteins from model food
107 matrices in PBS and denaturing buffer. Five independent samples were evaluated in duplicate.
108

109 Supplemental Table 1: Estimated protein content of native and processed egg proteins in HSB,
 110 Denaturing, Carbonate, Tris and PBS buffers by Pierce 660. Protein levels are expressed as
 111 mean $\pm$ standard deviation.

Whole Egg Sample	Carbonate, pH 9.5	HSB, pH 7.4	PBS, pH 7.4	Denaturing Buffer	Tris, pH 8
Raw	7384 $\pm$ 76	6627 $\pm$ 123	4524 $\pm$ 78	5914 $\pm$ 148	6331 $\pm$ 220
Boiled	591 $\pm$ 17	411 $\pm$ 5	653 $\pm$ 46	473 $\pm$ 34	540 $\pm$ 29
Fried	392 $\pm$ 9	343 $\pm$ 2	470 $\pm$ 51	684 $\pm$ 36	444 $\pm$ 57
Baked	556 $\pm$ 3	353 $\pm$ 22	474 $\pm$ 11	893 $\pm$ 12	447 $\pm$ 9

112
 113
 114 Supplemental Table 2: Observed values of Blank used for calculation of LODs and LOQs of the
 115 developed ELISAs.

Independent samples	PBS Buffer						Denaturing Buffer					
	BF			RBF			BF			RBF		
1	0.00	0.02	0.05	0.02	0.00	0.01	0.03	0.04	0.00	0.07	0.03	0.01
2	0.00	0.01	0.01	0.00	0.00	0.01	0.02	0.00	0.08	0.00	0.14	0.08
3	0.00	0.05	0.00	0.02	0.01	0.00	0.26	0.00	0.00	0.18	0.03	0.00
4	0.06	0.00	0.03	0.00	0.02	0.00	0.00	0.08	0.21	0.04	0.00	0.00
5	0.03	0.01	0.00	0.02	0.00	0.00	0.00	0.01	0.00	0.72	0.00	0.00
6	0.00	0.05	0.03	0.00	0.00	0.01	0.08	0.00	0.00	0.00	0.00	0.09
7	0.03	0.00	0.00	0.00	0.00	0.19	0.00	0.00	0.25	0.05	0.00	0.00

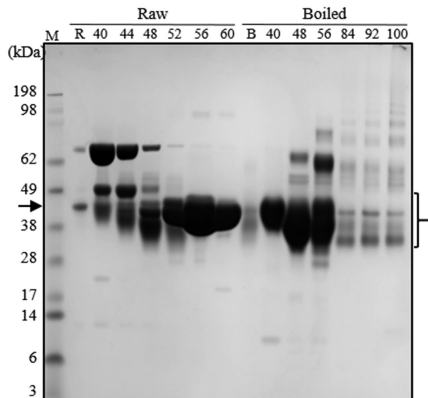
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Supplemental Table 3: Variability as measured by the sampling and analytical CVs associated with the developed ELISAs used to detect egg protein in 30-min baked cookies, cereal bar, muffin, and tempered chocolate at indicated incurred levels. CV% = 100

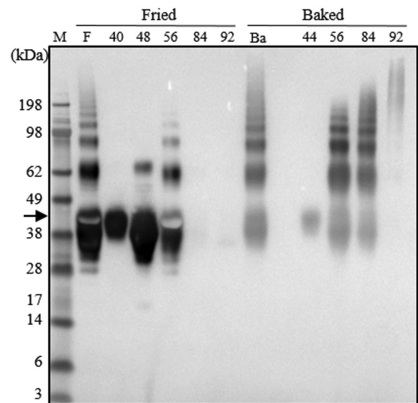
Developed ELISAs	Incurred Model Foods	0		1.2		2.4		4.8		12		48	
		Sampling CV (%)	Analytical CV (%)	Sampling CV (%)	Analytical CV (%)	Sampling CV (%)	Analytical CV (%)	Sampling CV (%)	Analytical CV (%)	Sampling CV (%)	Analytical CV (%)	Sampling CV (%)	Analytical CV (%)
BF PBS	30-min Baked Cookies	UD	UD	UD	25.07	UD	28.27	7.96	12.34	21.71	13.53	4.69	3.90
	Cereal bar	22.68	UD	15.11	6.33	UD	1.75	14.89	1.14	19.58	2.16	8.19	1.94
	Muffin	UD	UD	44.66	49.92	54.18	32.63	22.89	34.02	24.22	19.00	12.58	25.07
	Tempered chocolate	UD	UD	33.47	5.06	21.92	4.27	13.94	11.96	16.03	5.03	8.93	3.54
RBF PBS	30-min Baked Cookies	UD	UD	16.76	6.63	9.66	4.08	4.13	6.33	14.87	6.20	6.35	2.95
	Cereal bar	UD	0.00	33.00	46.92	11.37	11.98	17.58	11.76	6.33	5.49	14.83	20.32
	Muffin	43.31	UD	6.51	27.50	2.30	14.26	12.52	3.20	UD	7.40	13.01	16.31
	Tempered chocolate	72.10	UD	14.28	3.46	13.26	6.52	6.27	7.31	16.20	3.08	10.32	8.88
BF Denaturing Buffer	30-min Baked Cookies	UD	64.01	51.15	49.20	UD	15.29	16.30	15.70	11.14	6.39	7.32	9.05
	Cereal bar	UD	UD	10.86	1.18	6.45	1.52	34.71	1.20	7.68	2.85	6.22	1.45
	Muffin	UD	UD	11.37	6.11	13.27	1.90	9.22	2.16	8.42	2.58	9.38	3.06
	Tempered chocolate	UD	UD	UD	UD	67.61	76.14	93.52	21.70	9.99	4.33	6.59	1.47
RBF Denaturing Buffer	30-min Baked Cookies	44.90	UD	120.04	33.02	27.04	11.35	UD	32.26	10.37	2.88	10.53	2.73
	Cereal bar	86.68	UD	8.48	4.93	8.38	3.91	2.26	1.34	5.06	3.84	4.10	3.40
	Muffin	UD	UD	32.08	7.79	54.52	6.22	12.09	4.97	16.52	3.29	19.58	6.98
	Tempered chocolate	UD	0.00	UD	UD	51.96	64.59	8.27	55.89	3.76	1.28	10.87	1.22

(CV=sample or analytical standard deviation of measured value/mean measured value); UD = Undefined due to dividing by zero.

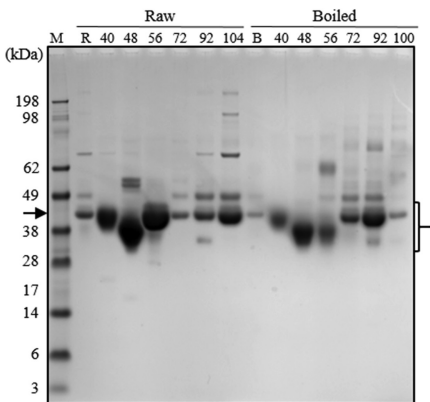
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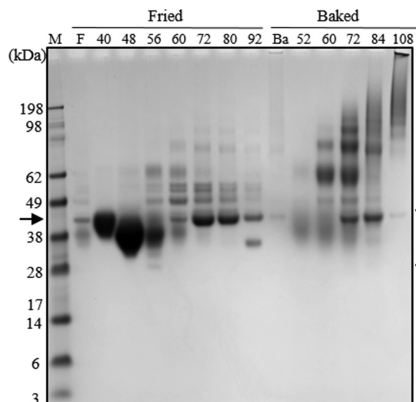
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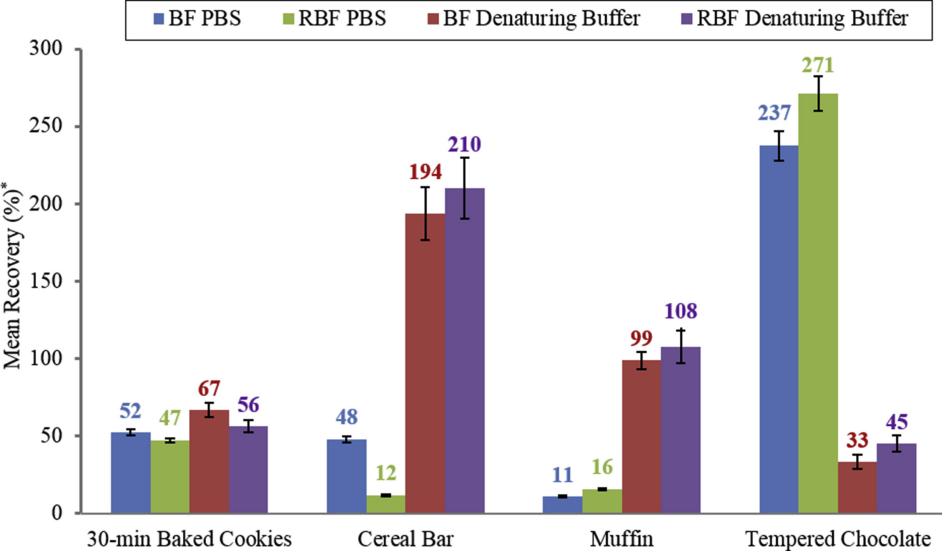


1C.



1D.





* Averaging across all incurred levels: 1.2, 2.4, 4.8, 12, 48 $\mu\text{g g}^{-1}$ egg proteins in all model foods