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Effect of Heat Treatment on the Quantitative Detection of Egg Protein Residues by Commercial Enzyme-Linked Immunosorbent Assay Test Kits

TONG-JEN FU,*,† NICOLE MAKS,‡ AND KATIE BANASZEWSKI‡

†U.S. Food and Drug Administration and ‡Illinois Institute of Technology, National Center for Food Safety and Technology, 6502 South Archer Road, Summit-Argo, Illinois 60501

This study examined the changes in the solubility of egg proteins as affected by different heat treatments and compared the performances of three commercial test kits for the quantitation of protein residues in heat-treated samples. National Institute of Standards and Technology (NIST) whole egg standard reference material #8415 and Henningsen spray-dried whole egg powder were subjected to heating in the presence of water at 60 and 100 °C, autoclaving for 5 or 10 min, or dry heating at 60−400 °C for 10 min. The amount of protein in the heated samples was assayed using the bicinchoninic acid total protein assay as well as egg-specific commercial enzyme-linked immunosorbent assay (ELISA) kits. Elevated heat resulted in a lower level of proteins extracted. Neogen's Veratox kit, which is reactive to multiple proteins in egg, greatly underestimated the amount of residual proteins in the boiled or autoclaved samples. Tepnel BioSystems' Biokits assay, which employs antibodies specific to a heat-stable marker protein (ovomucoid), registered a higher level of protein in these samples. Both test kits substantially underestimated the amount of residual proteins in samples dry-heated at temperatures >176 °C. The Morinaga test, using an improved extraction buffer, registered the highest level of protein in the heat-treated NIST samples but not the Henningsen samples. The underestimation by the commercial test kits was attributed to changes in the immunoreactivity of residual proteins after heat treatments and not the differences in the amount of protein extracted. These results suggest that thermal processing may affect the quantitative analysis of allergens and needs to be taken into account in the validation of commercial ELISA test kits.

KEYWORDS: Food allergen; egg; ELISA test kits; thermal treatment

INTRODUCTION

Hen's eggs are a major source of high-quality proteins and essential nutrients. Egg components provide certain desirable functional attributes, such as foaming, emulsification, coagulation, adhesion, and binding and have been widely used as ingredients in many food applications (*1*). Eggs are also one of the most common causes of food hypersensitivity in young children (*2*). It has been reported that two-thirds of children diagnosed with food allergies are reactive to eggs (*3*). Adverse reactions to eggs include atopic dermatitis, rhinitis, urticaria, and asthma (*4*). In some cases, life threatening or fatal anaphylactic reactions could occur (*5*). Strict avoidance of the offending food remains the only effective means to prevent the occurrence of allergic reactions (*4*, *6*).

Consumers rely on food labels to disclose the presence of allergenic ingredients. The enactment of the Food Allergen Labeling and Consumer Protection Act (FALCPA) requires food manufacturers to clearly disclose ingredients derived from eight major allergenic food groups, namely, peanut, milk, egg, wheat, soy, tree nuts, fish, and crustacean shellfish (*7*). However, because of the wide use of egg ingredients, unintended exposure to egg allergens in food could occur as a result of incomplete labeling, processing errors, or cross-contact contamination of shared processing equipment. According to the U.S. Food and Drug Administration, undeclared egg allergens accounted for the greatest number of recall actions between fiscal years 1999 and 2004 (*8*). Vierk et al. (*9*) reported that the major causes of product recalls are ingredient statement omissions/errors and manufacturing equipment cross-contact, which accounted for 51 and 40%, respectively, of the recalled products.

Food manufacturers have been increasingly vigilant in controlling allergen cross-contact (*10*, *11*). The use of appropriately sensitive detection methods helps to validate/verify allergen control measures. Sensitive detection methods are also needed by regulatory agencies to ensure compliance with food-labeling regulations (*12*). Enzyme-linked immunosorbent assays (ELISA) remain the most frequently used method for allergen detection. An increasing number of ELISA test kits have become commercially available (*13*−*15*). Many of these tests are designed to allow quantitative determination of protein residues in allergenic food where the presence of target proteins is detected by a colorimetric reaction following binding with a specific enzyme-labeled antibody. The concentration of the antigen is then interpolated from a

*To whom correspondence should be addressed. Tel: 708-728-4149. Fax: 708-728-4177. E-mail: tongjen.fu@fda.hhs.gov.

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standard curve generated with reference protein/food standards (*13*). An internal conversion factor is used by some test kits to convert the measured protein concentration to a more commonly used unit [e.g., ppm egg or egg white protein (EWP)].

Many commercial kits employ antibodies that are raised against protein extracts of whole foods and are thus reactive to multiple proteins. For example, the Neogen Veratox Quantitative Egg Allergen Test and the TECRA Egg Visual Immunoassay are reactive to total egg proteins. Other kits use antibodies that are raised against individual allergens and are therefore only reactive toward specific proteins in food. For example, the Tepnel BioSystems' Biokits Egg Assay employs antibodies specific to a marker protein, ovomucoid. The ELISA Systems' Egg Residue Microwell ELISA is specific to both ovomucoid and ovalbumin (*14*). Ovomucoid and ovalbumin are two of the most allergenic proteins in hen's egg (*16*). Ovalbumin, which is a glycoprotein with a molecular weight (MW) of 43 kDa and the predominant protein in egg white, accounts for 54% of the protein content. Ovomucoid is a highly glycosylated protein with a MW of 28 kDa and accounts for 11% of the protein content in egg white (*16*).

Allergen detection by ELISA depends greatly on whether the target proteins can be effectively recognized by antibodies used in the assay. Because quantitation is achieved via measurements of protein antigenicity, any changes in the antigenic property of the target proteins may influence assay results. Most foods are cooked or processed to a certain degree prior to consumption. Thermal treatments often lead to changes in the structures of proteins, which may then affect the recognition or binding of these proteins by antibodies used in the assay. How thermal processing may affect the quantitative analysis of allergens by commercial ELISA kits remains to be determined.

The extent of thermally induced changes may differ among different proteins. For example, while ovalbumin undergoes denaturation and aggregation during heating (*17*−*19*), ovomucoid is noncoagulable and retains part of its antigenicity and allergenicity after heating (*16*,*20*,*21*). It remains to be determined whether differences in the susceptibility of proteins to thermal denaturation can lead to variations in allergen quantitation among ELISA test kits that are reactive to different proteins.

A number of published studies have examined the performance of commercial ELISA kits for the detection of egg proteins in processed foods (*22*−*24*). It was observed that some test kits failed to detect egg proteins in thermally processed foods, while others exhibited reduced detection depending on the thermal processing conditions used (*22*, *23*). Whether the decrease in detectability was due to the inability to extract egg proteins from thermally treated foods or due to the changes in the immunoreactivity of the residual proteins after heat treatments has not been determined (*23*).

Recently, a new test kit (Egg Protein ELISA Kit, manufactured by Morinaga Institute of Biological Science, Yokohama, Japan) that uses an extraction buffer containing a surfactant and a reducing agent has been shown to exhibit a higher recovery of proteins from food that have been subjected to various manufacturing processes (*24*, *25*). How this test kit compares with others for quantitation of egg proteins in thermally processed food remains to be determined.

The goals of the current study were to determine the effects of different thermal treatments (both moist and dry heat) on the solubility of egg proteins and to compare the performance of three commercial test kits, Neogen's Veratox Egg Allergen Test, Tepnel Biosystems' Biokits Egg Assay, and Morinaga's Egg Protein ELISA Kit, for quantitation of protein residues in heat-treated egg samples. Whether there are differences in quantitation between ELISA kits that are reactive to total protein and those that are reactive to specific marker proteins (e.g., ovomucoid) was examined. The performance of the Morinaga test in comparison with the other tests for quantitation of heat-treated egg proteins was also evaluated. A NIST (National Institute of Standards and Technology) whole egg powder standard reference material (SRM #8415) and Henningsen Type W-1 spray-dried whole egg powder were used as the model food systems for this study.

MATERIALS AND METHODS

Materials. Whole egg powder standard reference material, SRM #8415, was purchased from the NIST (Washington, DC). Henningsen Type W-1 spray-dried whole egg powder was kindly provided by Dr. Steve Taylor (Food Allergy Research and Resource Program, University of Nebraska, Lincoln, NE). Phosphate-buffered saline (PBS, pH 7.4) and 2X Laemmli buffer were from Sigma-Aldrich Chemical Co. (St. Louis, MO). Coomassie Brilliant Blue R 250 was from Fisher Scientific Inc. (Pittsburgh, PA). Novex 10−20% Tricine Pre-Cast Mini gels (1.0 mm × 15 well) and other electrophoresis reagents were from Invitrogen Corp. (Carlsbad, CA). Mark 12 molecular weight marker was obtained from Invitrogen. Reagents used for sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE) analysis were obtained from Bio-Rad (Richmond, CA) and Invitrogen.

Moist Heat Treatment. Four milliliters of deionized (DI) water was added to each of the polyethylene tubes containing 1 g of NIST SRM #8415 or the Henningsen egg powder. The egg−water mixtures were heated in a water bath at 60 °C for 5 or 10 min or at 100 °C for 10 min or autoclaved at 121 °C for 10 min. Each of the unheated and heat-treated samples was divided into 1 mL portions for further extraction and analysis by the bicinchoninic acid (BCA) total protein assay (Pierce, Rockford, IL) or the three commercial test kits.

Dry Heat Treatment. Glass vials containing 1 g of NIST SRM #8415 or the Henningsen egg powder were heated in a muffle furnace (Barnstead International, Dubuque, IA) at 60, 100, 120, 176, 204, 232, or 400 °C for 10 min. After they were heated, each of the unheated and heat-treated samples was mixed with 4 mL of DI water before they were divided into 1 mL aliquots for further extraction and analysis by the BCA assay or the three commercial test kits.

Protein Quantification by BCA. The 1 mL egg−water aliquots of the unheated and heat-treated samples were further extracted in 9 mL of PBS overnight at 4 °C in a shaking incubator at 250 rpm. The amounts of extractable proteins were assayed by BCA per the manufacturer's instructions. Bovine serum albumin was used as the protein standard. For samples heated at 232 and 400 °C, prior to the BCA analysis, the PBS extracts were passed through protein desalting columns (Pierce) to remove the interfering brown pigments formed during heat treatments.

Protein Quantification by the Veratox Test. The Veratox Quantitative Egg Allergen Test manufactured by Neogen Corp. (Lansing, MI) uses a sandwich format and employs antibodies specific for unprocessed and heat-processed egg proteins. An unspecified egg protein preparation was used to formulate the kit standards for which the concentrations had been calibrated so that the assay result was reported as ppm whole dry egg with a quantitation range of 2.5−25 ppm. To determine the amount of egg in the moist- and dry-heated samples, the protocol recommended by the kit manufacturer was followed except that the amount of extraction buffer used was adjusted to accommodate the 1 g sample size. Briefly, the 1 mL egg−water mixtures were each extracted with 25 mL of prewarmed (60 °C) extraction buffer containing PBS plus 1/5 of a scoop of extraction additive (skim milk) in a shaking water bath at 60 °C for 15 min. The samples were centrifuged at 14000 rpm using an Eppendorf model 5810 R Centrifuge (Hamburg, Germany) for 5 min at room temperature. The supernatant was serially diluted to appropriate concentrations before it was subjected to ELISA analysis per the kit instructions. The egg concentrations in the samples were interpolated from the standard curve constructed with kit standards using Neogen's log/logit software.

Protein Quantification by the Biokits Test. The Biokits Egg Assay kit manufactured by Tepnel BioSystems, Ltd. (Deeside, United Kingdom) is a sandwich type enzyme immunoassay utilizing biotin−avidin enhancement. The assay used polycolonal antibodies specific for the major egg allergen ovomucoid. Kit standards contained 0.5−10 ng/mL ovomucoid,

Table 1. Normalized Concentrations of Egg or Egg Proteins in the Thermally Treated NIST Egg Powder (SRM #8415) as Determined by BCA as Well as the Three Commercial Test Kits[a]

		normalized concentrations in samples (%)[b,c]			
	heat treatment	BCA	Veratox	Biokits	Morinaga kit
moist heat	unheated	100.0 ± 1.5 A, 1	100.0 ± 14.4 A, 1	100.0 ± 5.0 A, 1	100.0 ± 6.4 A, 1
	60 °C, 5 min	93.0 ± 4.9 B, 1	87.3 ± 8.2 B, 1	96.7 ± 12.3 A, 1	98.0 ± 15.8 A, 1
	60 °C, 10 min	82.6 ± 10.8 C, 1	81.0 ± 14.3 B, 1	83.9 ± 8.6 B, 1	90.9 ± 19.8 A, 1
	100 °C,10 min	23.5 ± 2.6 D, 1	3.2 ± 1.2 C, 2	20.8 ± 17.4 C, 1	62.6 ± 20.8 B, 3
	autoclaved, 10 min	30.1 ± 3.6 E, 1	1.2 ± 0.5 C, 2	6.4 ± 3.5 D, 3	18.1 ± 8.5 C, 4
dry heat	unheated	100.0 ± 6.5 A, 1	100.0 ± 5.3 A, 1	100.0 ± 5.3 A, 1	100.0 ± 5.5 A, 1
	60 °C, 10 min	96.5 ± 5.3 A, 1	94.8 ± 12.7 A, 1	96.5 ± 9.7 A, 1	102.5 ± 17.6 A, 1
	100 °C, 10 min	95.1 ± 8.4 A, 1	100.2 ± 23.8 A, 1	92.8 ± 7.5 A, 1	94.7 ± 14.1 A, 1
	120 °C, 10 min	90.5 ± 8.8 A, 1	84.7 ± 16.6 A, 1	85.5 ± 10.2 B, 1	83.22 ± 27.7 B, 1
	176 °C, 10 min	51.7 ± 23.2 B, 1	19.4 ± 20.3 B, 2	23.7 ± 14.4 C, 2	62.9 ± 21.1C, 1
	204 °C, 10 min	25.1 ± 9.7 C, 1	11.0 ± 11.6 B, 2	14.8 ± 6.5 D, 2	34.5 ± 13.8 D, 1
	232 °C, 10 min	9.5 ± 2.7 D, 1	0.5 ± 0.3 B, 2	0.9 ± 0.6 E, 2	1.0 ± 0.4 E, 2
	400 °C, 10 min	5.8 ± 3.7 D, 1	0.002 ± 0.001 B, 2	0.005 ± 0.003 E, 2	0.004 ± 0.002 E, 2

[a] Each value represents the average ± standard deviation of the results obtained from five separate heat treatment trials. [b] Values in the same column that are followed by the same letter are not significantly different. [c] Values in the same row that are followed by the same number are not significantly different.

which was equivalent to 0.5−10 ppm EWP powder. The assay result was reported as ppm EWP with a detection range of 0.5−10 ppm. For our analysis, the 1 mL egg−water mixtures were extracted with 10 mL of kit extraction buffer (containing Tris buffer, 0.3 M NaCl, and fish gelatin, adjusted to pH 8.2) preheated to 60 °C. The samples were mixed in a shaker for 15 min at room temperature. One milliliter of the sample extract was removed and centrifuged at 10000*g* for 10 min at room temperature. The supernatant was collected and diluted 10-fold in the working diluent solution. The resulting extracts were further diluted in the working diluent to appropriate concentrations before they were subjected to ELISA analysis per the kit instructions. The egg protein concentrations in the samples were interpolated from the standard curve with a sigmoidal curve fit.

Protein Quantification by the Morinaga Test. The Egg Protein ELISA Kit manufactured by Morinaga Institute of Biological Science, Inc. (Yokohama, Japan) is a sandwich enzyme immunoassay targeting ovalbumin as a marker for detection of egg in processed or unprocessed food. The kit standards were prepared from an unspecified egg protein solution to attain a concentration range of 0.78−50 ng/mL, and the assay result was reported as mg/g or ppm egg protein content. For our analysis, the 1 mL egg−water mixtures were extracted with 19 mL of sample extraction buffer containing SDS and 2-mercaptoethanol. The mixture was mixed overnight in a shaking incubator (100 rpm) at room temperature. After centrifugation at 3000*g* for 20 min, the supernatant was diluted 20-fold with diluent I. This extract was then serially diluted in diluent II to the appropriate concentration for ELISA analysis. The egg protein concentrations in the samples were then determined from the standard curve using a sigmoidal curve fit.

SDS-PAGE Analysis. The protein profiles of the unheated and heat-treated samples were determined by SDS-PAGE. The PBS extracts of all samples without or with a 10-fold dilution (for the Henningsen samples) were mixed with equal volumes of 2X Laemmli buffer and boiled for 10 min. Fifteen microliters of the samples was loaded in a 10−20% tris-tricine mini-gel. The gel was run in an XCell SureLock Mini-Cell (Invitrogen) at a constant voltage (125 V) according to the manufacturer's instructions. Proteins were visualized by Coomassie Brilliant Blue staining.

Comparison of the Efficacy of Extraction Procedures. One gram of NIST SRM #8415 egg powder was mixed with 4 mL of DI water. The sample was either not heated, boiled, or autoclaved for 10 min before it was divided into 1 mL portions for further extraction. Additionally, 1 g of NIST egg powder was subjected to dry heating at 100, 176, and 232 °C before it was mixed with 4 mL of DI water and then divided into 1 mL portions for further extraction. The 1 mL unheated or heat-treated egg−water mixtures were subjected to one of the following extraction protocols: (1) extracted with 9 mL of PBS overnight at 4 °C in a shaking incubator at 250 rpm, (2) extracted with 9 mL of PBS (i.e., Veratox extraction buffer without the skim milk additive) for 15 min in a shaking water bath set at 60 °C and 150 rpm, and (3) extracted with 9 mL of Tris buffer with 0.3 M NaCl (i.e., Biokits extraction buffer without fish gelatin) preheated to 60 °C and shaken for 15 min at room temperature. The protein concentration in each of the extracts was determined by BCA.

Statistical Analysis and Experimental Design. The heating experiments were repeated three or five times for the Henningsen or the NIST egg powder samples, respectively. For each trial, duplicate egg powder samples were treated at each temperature. For all of the BCA and ELISA assays, at least duplicate wells were run for each sample. Differences in the normalized concentrations between samples treated at different temperatures were analyzed by analysis of variance using the OriginPro software (OriginLab, Northampton, MA). Fisher's LSD test was used for comparison of the means. In all cases, the level of significance was set at $P < 0.05$.

RESULTS

NIST Egg Powder. The effects of both moist heat (i.e., heating in the presence of water) and dry heat treatments on the quantitative detection of proteins in the NIST SRM #8415 egg powder by BCA and three commercial test kits were examined. It was observed that each of the tests gave different readings even for the unheated samples aliquoted from the same egg−water solutions (data not shown). To facilitate the comparison and the determination of the impact of heat treatments, for each test, the concentrations of the heat-treated samples were normalized against those of the unheated samples. **Table 1** lists the average values of the normalized concentrations based on results from five separate heat treatment trials.

Protein Quantitation by BCA. For the moist-heated samples, elevated temperatures resulted in a lower level of proteins extracted by PBS. While a greater than 80% yield in extractable proteins was observed after the samples were heated at the egg pasteurization temperature of 60 °C for 10 min, boiling for 10 min resulted in a more than 75% decrease in the amount of protein extracted (**Table 1**). The higher temperature and pressure associated with autoclaving did not result in an additional protein loss. SDS-PAGE analysis of the PBS extracts (**Figure 1**) revealed that the intensity of all protein bands in the boiled and autoclaved samples was greatly reduced. Ovomucoid was one of the proteins that remained visible in these samples. The banding pattern of the autoclaved samples was similar to that of the boiled samples, but the bands appeared more diffused, and the ovomucoid band was not as distinct.

For the dry-heated samples, the level of protein extracted in PBS also decreased with increasing temperatures, but the decrease occurred at a higher temperature (**Table 1**). Unlike boiling or autoclaving, dry heating at 100 or 120 °C did not significantly

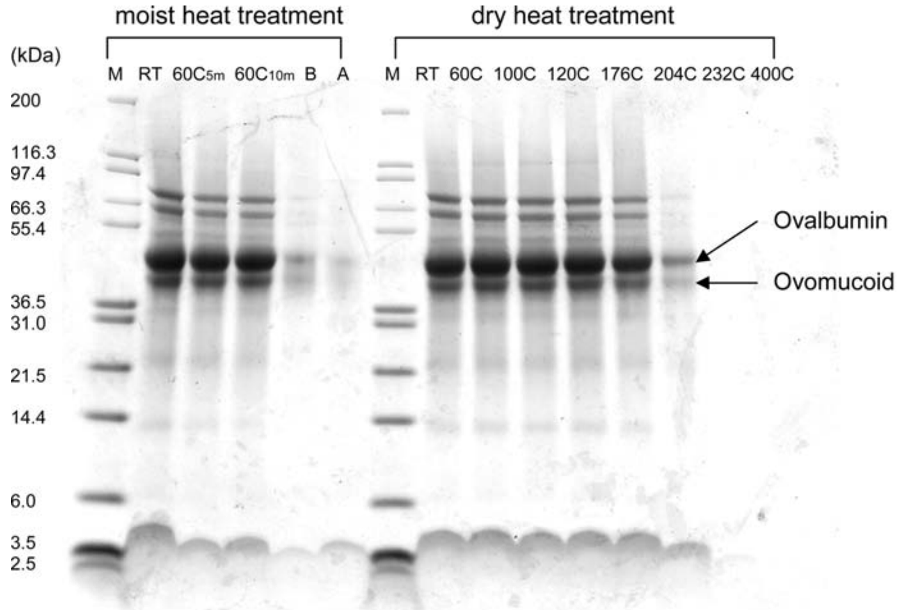

Figure 1. SDS-PAGE analysis of the proteins extracted in PBS from the NIST SRM #8415 egg samples that have been moist and dry heated at different temperatures. The ovalbumin and ovomucoid bands are indicated by the arrows (M, marker; RT, room temperature; B, boiled; and A, autoclaved).

Table 2. Comparison of the Efficiency of the Extraction Protocols Used by the BCA Assay (PBS/4 °C), Veratox Kit (PBS/60 °C without Skim Milk), and the Biokits Assay (Tris/0.3 M NaCl without Fish Gelatin)[a]

	actual or normalized protein concentrations in samples[b,c]					
	PBS/4 °C		PBS/60 °C		Tris/0.3 M NaCl	
	mg/mL	%	mg/mL	%	mg/mL	%
unheated	1.53 ± 0.08	100.0 ± 5.3 A, 1	1.72 ± 0.08	100.0 ± 4.8 A, 1	1.61 ± 0.02	100.0 ± 1.0 A, 1
boiled	0.37 ± 0.02	24.4 ± 1.2 B, 1	0.50 ± 0.02	28.8 ± 1.1 B, 1	0.43 ± 0.04	26.8 ± 2.7 B, 1
autoclaved	0.48 ± 0.02	31.3 ± 1.0 B, 1	0.57 ± 0.10	32.9 ± 6.1 B, 1	0.47 ± 0.00	28.8 ± 1.9 B, 1
dry heat, 100 °C	1.39 ± 0.17	90.6 ± 11.2 A, 1	1.65 ± 0.11	96.0 ± 6.1 A, 1	1.53 ± 0.08	95.0 ± 5.1 A, 1
dry heat, 176 °C	0.89 ± 0.17	58.1 ± 11.1 C, 1	1.06 ± 0.25	61.6 ± 14.7 C, 1	1.11 ± 0.15	69.0 ± 9.3 C, 1
dry heat, 232 °C	0.22 ± 0.01	14.3 ± 0.4 D, 1	0.27 ± 0.02	15.8 ± 1.0 D, 1	0.33 ± 0.02	20.3 ± 1.2 D, 2

[a] Protein levels in extracts were determined with the BCA assay. Each value represents the average ± standard deviation of results obtained from three replicate samples. [b] Values in the same column that are followed by the same letter are not significantly different. [c] Values in the same row that are followed by the same number are not significantly different.

affect the solubility of egg proteins. A significant protein loss was observed when samples were heated at 176 °C for 10 min. At 204 °C, the amount of protein detected was only ~25% of the amount found in the unheated samples. At 400 °C, the samples appeared burnt, and very little protein was detected. SDS-PAGE analysis revealed that while the protein profile in samples dry-heated at temperatures up to 120 °C remained largely unchanged, at 176 and 204 °C, a decrease in the intensity of all major protein bands was apparent (**Figure 1**). Ovalbumin and ovomucoid were among the most prominent proteins in the gel. At 232 or 400 °C, no distinct protein bands could be observed in the gel.

Protein Quantitation by Commercial Test Kits. For the NIST egg samples that were heated at 60 °C in water, all three test kits gave similar normalized concentrations as those determined by BCA (**Table 1**). However, for the boiled or autoclaved samples, the relative amounts of protein determined by the three test kits did not always reflect those determined by BCA. For the boiled samples, the Veratox test registered a reading that was 3.2% of the unheated samples. This was significantly lower than the amount of residual proteins determined by BCA (23.5%). The readings obtained by the Biokits assay were higher in some trials but lower in others, resulting in a large standard deviation (**Table 1**). The average value (20.8%) was similar to that determined by BCA but was much higher than that indicated by the Veratox test. The Morinaga kit registered a significantly higher level of proteins in the boiled samples than those measured by BCA or the other two tests. For the autoclaved samples, all three test kits underestimated the relative amount of proteins that could be detected by BCA, with the Veratox test showing a greater degree of underestimation. The Morinaga test registered the highest protein level among the three test kits, although the reading was still lower than that detected by BCA.

For samples dry heated at or below 100 °C, the normalized concentrations as determined by the three test kits generally reflected those measured by BCA (**Table 1**). However, for samples dry-heated at 176 or 204 °C, both the Veratox and the Biokits tests registered lower levels of protein than those determined by BCA. The Morinaga test registered the highest level of protein among all three test kits evaluated. At 232 °C or higher, all three kits greatly underestimated the level of protein present.

Comparison of the Efficacy of Extraction Procedures. To determine whether the deviation in protein quantitation between different methods was due to the differences in the amount of protein extracted, we compared the efficiency of the extraction protocols used by BCA (PBS/4 °C), the Veratox kit (PBS/60 °C without skim milk), and the Biokits assay (Tris/0.3 M NaCl without fish gelatin). Morinaga's extraction protocol was not included in the comparison because of the presence of interfering

Table 3. Normalized Concentrations of Egg or Egg Proteins in the Thermally Treated Henningsen Egg Powder as Determined by BCA as Well as the Three Commercial Test Kits[a]

	heat treatment	normalized concentrations in samples (%)[b,c] BCA	Veratox	Biokits	Morinaga kit
moist heat	unheated	100.0 ± 1.8 A, 1	100.0 ± 1.5 A, 1	100.0 ± 1.0 A, 1	100.0 ± 2.1 A, 1
	60 °C, 5 min	96.4 ± 3.6 A, 1	93.4 ± 10.2 A, 1	96.3 ± 2.4 B, 1	95.2 ± 3.2 B, 1
	60 °C, 10 min	92.7 ± 3.5 B, 1	88.2 ± 8.2 B, 1	86.9 ± 5.9 C, 1	93.2 ± 4.4 B, 1
	100 °C, 10 min	10.8 ± 1.9 C, 1	7.5 ± 1.8 C, 2	10.0 ± 0.6 D, 1	4.4 ± 0.8 C, 3
	autoclaved, 10 min	12.7 ± 4.2 C, 1	7.0 ± 2.2 C, 2	10.4 ± 0.4 D, 1	5.2 ± 0.4 C, 2
dry heat	unheated	100.0 ± 1.3 A, 1	100.0 ± 1.2 A, 1	100.0 ± 2.0 A, 1	100.0 ± 0.8 A, 1
	60 °C, 10 min	97.5 ± 1.6 A, 1	91.9 ± 6.9 A, 1	97.5 ± 1.9 A, 1	94.9 ± 4.6 A, 1
	100 °C, 10 min	95.6 ± 1.4 A, 1	86.2 ± 10.8 B, 1	92.3 ± 5.6 B, 1	87.4 ± 9.3 B, 1
	120 °C, 10 min	93.5 ± 3.5 A, 1	81.6 ± 14.4 B, 1	85.7 ± 5.8 C, 1	80.0 ± 10.8 C, 1
	176 °C, 10 min	53.4 ± 22.9 B, 1	12.4 ± 5.5 C, 2	10.9 ± 0.3 D, 2	11.0 ± 1.2 D, 2
	204 °C, 10 min	17.5 ± 6.8 C, 1	8.1 ± 2.7 C, 2	4.2 ± 4.9 E, 2	7.7 ± 0.2 D, 2
	232 °C, 10 min	4.4 ± 0.8 D, 1	0.9 ± 0.1 D, 2	0.4 ± 0.5 E, 2	1.0 ± 0.1 E, 2
	400 °C, 10 min	1.5 ± 0.5 D, 1	<0.001, D, 2	<0.001 E, 2	<0.001 E, 2

[a] Each value represents the average ± standard deviation of the results obtained from three separate heat treatment trials. [b] Values in the same column that are followed by the same letter are not significantly different. [c] Values in the same row that are followed by the same number are not significantly different.

proteins in its extraction buffer as indicated by SDS-PAGE analysis (data not shown).

Table 2 summarizes the actual and normalized concentrations of protein recovered from both the moist- and the dry-heated NIST egg samples using the three different extraction protocols. For each of the unheated or heat-treated samples, the actual amount of protein extracted in PBS after an overnight incubation at 4 °C was similar to those recovered using the other two protocols. All three extraction protocols also showed a similar % recovery for the heat-treated samples. For example, a recovery of 27−33% was observed for the boiled and autoclaved samples using the Veratox and Biokits extraction protocols. This value was not significantly different from that recovered using the BCA protocol (24−31%). For samples dry-heated at 176 °C, more than 60% of protein relative to the unheated samples could be recovered using the extraction protocols of both Veratox and Biokits assays as compared with the 58% recovery using the BCA protocol. At 232 °C, the Veratox and Biokits extraction buffers were able to recover approximately 16−20% of protein relative to the unheated samples, which was similar to the 14% level recovered using the BCA protocol.

Henningsen Egg Powder. The effect of heat treatment on the quantitative detection of egg proteins was also studied using the Henningsen spray-dried egg powder as the model food system. As was observed in the NIST samples, each of the tests gave different readings for the same unheated samples. The concentrations of the heat-treated samples were therefore normalized. **Table 3** lists the average values of the normalized concentrations of egg protein or egg in the heat-treated samples as determined by BCA and the three commercial ELISA test kits.

Protein Quantitation by BCA. The amount of protein in the unheated Henningsen egg powder/water mixture was determined to be approximately 66.5 ± 6.9 mg/mL, which was more than three times that determined for the unheated NIST samples (18.6 ± 2.9 mg/mL). Boiling of the Henningsen egg samples for 10 min caused a ~90% decrease in the amount of protein extracted (**Table 3**). Similar to the results of the NIST samples, autoclaving did not cause additional protein losses. For dry-heated samples, a significant protein loss occurred at 176 °C, and the level of extractable proteins decreased with increasing temperatures following a similar trend to that observed in the NIST samples. There were a greater number of protein bands visible in the SDS-PAGE gel of the Henningsen samples (**Figure 2**). The relative intensity of ovalbumin was much greater in the Henningsen samples than in the NIST samples. Other bands such as the one with MW between 66.3 and 97.4 kDa and the one with MW ~116.3 kDa were also much darker than the corresponding bands observed in the NIST samples. Boiling and autoclaving greatly reduced the amount and number of proteins visible in the gel. For the dry-heated samples, a significant decrease in the intensity of all protein bands occurred at 176 °C. At 232 °C or higher, very little protein was observed in the gel.

Protein Quantitation by Commercial Test Kits. For samples heated at 60 °C in water, all three commercial test kits gave similar normalized concentrations to those obtained by BCA (**Table 3**). For the boiled and autoclaved samples, while the Biokits registered similar values to those determined by BCA, both the Veratox and the Morinaga tests underestimated the amount of protein present in these samples. The Morinaga test gave the lowest recovery among the three test kits for the boiled samples. While correctly indicating the relative amount of proteins in samples dry-heated at temperatures up to 120 °C, all three test kits underestimated the amount of residual proteins in samples dry-heated at 176 °C or higher. The readings registered by the Morinaga test in these dry-heated samples were not different from those obtained by the other two test kits.

DISCUSSION

In the current study, we examined how heat treatments affected the solubility of egg proteins and studied factors that affect ELISA detection of protein residues in egg samples, including different heat treatments (moist and dry heat), antibody preparations (as presented in different commercial kits), and susceptibility of target proteins to thermal denaturation (ovomucoid vs total protein). The NIST SRM #8415 whole egg powder was chosen as the first model food system. Whole egg itself contains most of the components found in the typical human diet including proteins, carbohydrates, lipids, minerals, and other nutrients and thus serves as a good model system for the study of the effect of thermal processing on protein analysis in food. The NIST SRM #8415 has been used as a reference material for the development of a number of commercial test kits including the Biokits and the Morinaga tests (*26*, *27*) and has been used in a number of test kit validation studies (*23*, *28*). It contains 37.8% of protein, but Williams et al. (*23*) reported that only 24.8% of the total protein content was extractable in PBS. The low recovery was attributed to the cobalt-60 γ-radiation treatment applied during preparation

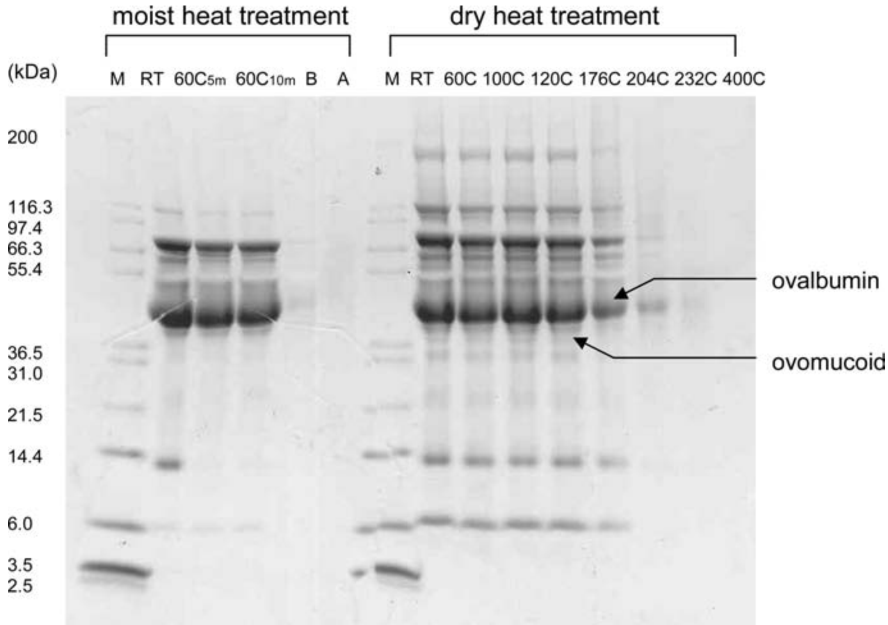

Figure 2. SDS-PAGE analysis of the proteins extracted in PBS from the Henningsen egg samples that have been moist and dry heated at different temperatures. Samples were diluted 1:10 in PBS prior to SDS-PAGE analysis (M, marker; RT, room temperature; B, boiled; and A, autoclaved).

of this material (*23*, *29*). The heat that was applied during drying of the egg preparation may also contribute to the denaturation of proteins. We have observed that the amount of extractable protein in NIST SRM #8415 was only about 28% of that found in the Henningsen egg powder. This result is consistent with that reported by Faeste et al. (*22*), in which the NIST preparation has only ~30% activity as compared with other commercially available whole egg powders.

As some of the proteins in NIST SRM #8415 may have been denatured, the suitability of this material for ELISA validation studies has been questioned (*23*). The Henningsen Type W-1 spray-dried whole egg powder has recently been adopted by NIST as a new egg standard (SRM #8445) intended for use in evaluating allergen test kits (*30*). We therefore also included the Henningsen egg powder in our evaluation.

This study showed that elevated heat resulted in a lower yield of extractable proteins. Boiling or autoclaving caused an approximately 70 or 90% decrease in the amount of protein extracted from the NIST or the Henningsen samples, respectively. SDS-PAGE analysis showed that ovomucoid was one of the proteins that remained in the extract of the boiled samples. It has been reported that ovomucoid remains soluble after boiling shell egg for 1 h (*31*), but other egg proteins including ovalbumin are coagulable and difficult to extract after heating (*22*, *32*, *33*).

Unlike boiling or autoclaving, dry heating at 100 or 120 °C did not significantly affect the solubility of egg proteins, indicating that the presence of water plays an important role in the aggregation of egg proteins. At higher temperatures (e.g., 204 °C), very few protein bands could be seen in the SDS-PAGE gels, but the ovomucoid band remained visible, again demonstrating the relatively high thermal stability of this protein.

In general, the changes in protein solubility seen in the Henningsen egg samples after different heat treatments followed a similar trend to that observed in the NIST egg powder, although the Henningsen samples showed a greater degree of protein loss at corresponding temperatures. This could be due to the presence of a greater percentage of heat-labile proteins in the Henningsen samples.

It was observed that different methods registered different readings even for samples aliquoted from the same preparation. Variations in protein quantitation among different commercial ELISA test kits have been reported in the literature (*22*, *34*, *35*). Normalization of the concentrations was done in this study so that the impact of heat treatments on the quantitative analysis of egg proteins by these methods could be compared. A similar approach was also used by Faeste et al. (*22*) in their evaluation of different commercial test kits.

The relative amounts of proteins in samples heated under mild conditions as determined by BCA were correctly indicated by all three ELISA test kits. However, in samples subjected to elevated heat (i.e., boiling, autoclaving, or dry heating at 176 °C or higher), the levels of residual proteins were not always indicated correctly by the Veratox or the Biokits tests. To explain this discrepancy, it helps to recognize that ELISA analysis of proteins consists of two steps: the extraction of proteins from the food matrix, followed by the quantitation of extracted proteins via an antigen−antibody reaction in comparison with the reaction of protein standards.

The differences in protein quantitation between the BCA and the commercial ELISA kits could be due to the differences in the amount of protein extracted or it could be due to the heat-induced changes in the immunogenicity of extracted proteins, thus affecting the proper recognition by the antibodies used in the test kits. We have shown that the extraction protocols used in BCA, Veratox, and Biokits assays recovered similar amounts of proteins from heat-treated NIST egg samples (**Table 2**). The skim milk and fish gelatin, which were included in the kit extraction buffers to prevent masking of target proteins by certain polyphenolic compounds in food (*36*), were omitted in this comparison to avoid interference of these proteins in the analysis. It has been reported that the addition of these additives may improve extraction efficacy (*36*), suggesting that the actual amount of proteins extracted by the Veratox or Biokits tests could be greater than the values indicated in **Table 2**. However, this possibility still would not explain the underestimation of residual proteins by these two test kits.

Therefore, the difference in protein quantitation between BCA and the Veratox or the Biokits tests is likely due to the changes in the immunogenicity of extracted proteins as a result of different heat treatments. The Veratox assay is reactive to multiple egg proteins. The immunoreactivity of many egg proteins, including ovalbumin, can be reduced by thermal treatments (*16*, *21*, *33*, *37*), and consequently, a weaker binding between the heat-treated proteins and the kit antibodies can occur. When interpolating from a standard curve that is typically generated from native proteins, this could lead to a lower level of quantitation.

The Biokits test, which employs antibodies specific to ovomucoid, registered a higher level of residual protein in the boiled or autoclaved samples than that observed in the Veratox kit (**Tables 1** and **3**). This could be explained by the fact that ovomucoid is relatively more resistant to thermal denaturation. Protein quantitation based on the interpolation of a standard curve constructed using ovomucoid would therefore be less affected by thermal treatment. It has been suggested that ovomucoid is a good marker for the detection of egg allergens in processed foods due to its high thermal stability (*21*). The use of a single protein that is immunochemically stable throughout food manufacturing (e.g., Ara h 2) as a marker for the detection of peanut traces in food has also been suggested by Westphal et al. (*35*).

It was observed that the amount of ovomucoid present in the boiled NIST egg samples varied between different trials. In some trials, the ovomucoid band in the SDS-PAGE gel was clearly visible, but in other trials, the ovomucoid band was not as distinct. This difference may have contributed to the large variation seen in the Biokits results for the boiled samples (**Table 1**).

Both the Veratox and the Biokits assays substantially underestimated the amount of residual proteins in NIST or Henningsen samples that were dry heated at temperatures > 176 °C (**Tables 1** and **3**). It could be that elevated heat affected the immunoreactivity of egg proteins so that they would not bind the kit antibodies properly and thus resulted in lower readings. Contrary to the results observed for the boiled and autoclaved samples, the readings obtained by the Biokits assay for these dry-heated samples were not higher than those obtained by the Veratox test. It could be that the dry heat affected the immunoreactivity of ovomucoid in the same way as it affected the proteins targeted by the Veratox test, but additional studies are needed to confirm this possibility.

The Morinaga test was developed to improve the detection of egg proteins in thermally processed foods. The kit employs SDS and 2-mercapethanol in the extraction buffer, which help to redissolve aggregated proteins. To allow proper recognition, the kit also employs antibodies that were specifically raised to recognize denatured proteins (*25*). Our study showed that for NIST samples that were boiled or dry-heated at 176 and 204 °C, this test kit generally registered higher levels of proteins than those determined by BCA or the other two test kits. The higher recovery is likely due to the enhanced extraction efficacy, although we were not able to experimentally confirm this because of the presence of proteins in the extraction buffer, which interfered with the ability to measure the extracted egg proteins (*22*). For samples that were autoclaved or dry-heated at 232 °C or higher, the Morinaga kit registered a lower level of residual proteins than that determined by BCA suggesting that, under these conditions, the enhancement in extraction efficacy was not able to compensate for the decrease in the immunoreactivity of extracted proteins and thus resulted in the underestimation of protein concentrations.

Contrary to the results seen in the NIST samples, the Morinaga test did not show improvement in the detection of protein residues in thermally treated Henningsen samples. It is not clear what contributed to this rather unexpected result. The Morinaga kits have been validated and calibrated using the NIST SRM #8415. As the amount and number of extractable proteins in the NIST preparation were much less than those found in the Henningsen preparation, it is not clear whether this difference could lead to the different results seen in this study. Additional work is needed to determine the actual cause of this discrepancy.

Commercial ELISA tests are used by food manufacturers for the detection of allergens in foods that are frequently subjected to varying degrees of thermal processing. There have been interests in the industry and the regulatory agencies to validate commercial test kits (*38*−*40*). Very often, the validation studies focus only on how well the test kits recover allergens that are spiked into food matrices without subjecting the food to actual processing conditions (*38*, *39*). Such an approach does not take into account the influence that processing conditions may have on ELISA quantitation of proteins. It is only until recently that food preparation conditions were considered as part of method validations (*40*, *41*). The current study identified the factors that may affect the quantitative analysis of egg proteins in thermally processed foods by ELISA test kits and provided insight into how heat-induced changes in the solubility and immunoreactivity of proteins may affect assay results. The information presented will help the design of future test kit validation studies and facilitate the development of improved ELISA methods. Because egg or egg components are used in many different food formulations, additional studies are needed to determine whether similar results will be obtained in other food matrices spiked with egg materials.

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