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Total Protein Determination Using Micro Plate Assay

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Total Protein Determination Using Micro Plate Assay

A Project

Submitted

To

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By

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Of

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DEDICATION

To my mother

Sharon A. Ruffatti

And to the memory of my father

Robert P. Ruffatti

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I owe my deepest gratitude to my parents Robert and Sharon Ruffatti for their love, support, and instilling the value of education and continual self-development.

The journey has been long with many obstacles along the way. I would like to my friends and family for the motivation to pull through and complete the masters program, especially my brother Ryan.

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Abstract

One of the largest challenges that food manufactures are facing today is the management of food allergens. Allergenic protein in trace amounts, part per million concentrations, will trigger a reaction is some individuals. Food manufacturers need to prevent allergen cross-contamination by performing adequate sanitation after production of an allergenic containing food. Allergen detection kits are used to determine if sufficient protein was removed from the equipment surfaces during sanitation. One kit on the market is the 3M™ Clean-Trace™ Surface Protein (Allergen) swab tests. The test qualitatively detects the presence of protein based on the biuret reaction and will yield a purple color if protein is present. The disadvantages of the swab test are the determination of the color is based subjective visual inspection and the quantity of protein present is unknown. In this project a quantitative method for reading 3M™ Clean-Trace™ Surface Protein (Allergen) swab tests kits was developed using a micro plate assay. Bovine serum albumin (BSA), egg whites, non-fat dry milk (NFDM), and soy isolate were applied to the swab test at various quantities and the absorbance of the test kit solution was measured at 560nm. With this quantitative approach it was possible to detect 2µg of BSA, 5µg of egg whites, NFDM, and soy isolate. The method provided increased sensitivity from the traditional visual color determination which detected 6ug BSA, 10ug of egg whites, 15ug of soy isolate and inconclusive results for the 15µg of NFDM. The use of this approach

will reduce excess cleaning of equipment surfaces for food manufacturers by providing a quantitative result for low quantities of protein, where the visual color determination is subjective or ambiguous.

Introduction

Food Allergens

One of the largest challenges that food manufactures are facing with today is the management of food allergens. Today at least 10-12 million Americans are affected by food allergies. Research estimates that exposure to food allergens cause 50,000 emergency room visits and 150-200 deaths due to anaphylaxis shock.[3] Studies have shown clear indication that the prevalence of food allergy has risen in recent years. Food allergens are caused by an Ig-E (antibody) mediated response. Protein from the offending food is seen as a foreign body by the immune system called an antigen. The antigen stimulates production of antibodies against the offending food. The antibodies will join with a mast basophil cell and create a sensitized cell. The next time the individual eats the offending food the sensitized cell recognizes the antigen (target protein) and the cell releases histamine, which triggers the allergic symptoms.[1] The IgE in a sensitized individual will bind to epitopes on the allergenic protein, which can be a linear stretch of amino acids or consist of amino acids that are not sequential, but within close proximity through the protein's three-dimensional structure.[11]

The most common foods that cause allergic reactions are peanut, tree nuts, milk, egg, soy, fish, shellfish, and wheat. These eight foods make up 90% of food allergen in the United States and are called the "Big Eight". Allergenic foods many contain several proteins with allergenic potential.[11] For example, peanuts have eight proteins with allergenic potential and in milk all proteins can be allergenic.[11] Currently studies are occurring around the world to discover and document proteins of allergenic importance. Currently, there is no cure for a food allergy and the only treatment is to follow a diet that avoids the offending food. Today it is unknown how much of a protein will cause a reaction in an individual because the sensitivity is different for every person. The presence of trace amount in part per million concentrations will trigger a reaction is some individuals.[1]

Effect of Food Allergens on Food Manufacturing

The U.S. government regulates the presence of the “big eight” allergens in manufactured food. The company could have liability if the food contains allergenic proteins that are not listed on the label.[1] Today the presence of unlabeled allergen is one of the main reason for food recalls. One way the risk of allergen contamination can be reduced is by the implementation of an adequate allergen sanitation program.[6] A company will often have specific sanitation procedures for clean equipment after producing an allergen containing product. Then the company will verify that the sanitation procedure is adequate in removing the presence of allergens. The first step in the sanitation verification process to ensure the equipment meets the standard of “visually clean” after

sanitation. The second step is the use of an allergen test kit for the presence allergenic protein. Protein can still be present on the equipment surface even if the equipment met the standard of “visually clean”. Today many companies collect samples from the equipment surfaces using sterile swabs and test for the presence of the target allergen. One question that is often asked by manufactures today is whether the standard of “visually clean” is enough to prevent cross contamination of allergenic proteins. Another major question is “How clean is clean enough?". According to food allergen experts the acceptable limits of cross-over food allergens should account for "the amount of the offending food that elicits mild objective symptoms in the most sensitive individual".[1] The problem is at this point in time the definitive threshold levels not yet been defined due to the considerable inter- and intra-individual variability in sensitivities between individuals.[1] Today many companies and organization are using a standard of five part per million for the limit of a target allergenic protein present in a food product. That number is only considered the limit because that is the detection limit of the most popular testing method for a food product, ELISA.

Allergen Test Kits

The second challenge comes into play when it is time to test the collected samples from the equipment surface. Key issues of allergen testing are cost, ease of use, availability of reagents human serum/animal serum, matrix effects: processing solubility, extractability interference, cross-reactivity, and comparative stability.[5] There are currently the several methods available for detecting the

presence of allergens: ELISA, general protein tests, ATP/bioluminescence tests, polymerase chain reaction (PCR) and MS methods are currently being developed.[5] The multitude of methods available for the detection of food allergens, many of which are commercially available, raises questions about their robustness and comparability.[11] It is recommended by FARRP (Food Allergy Research and Resource Program) to use a test kit that detects protein, since the food allergy is triggered by a protein in the food.[1] The most common method of testing is the use immunoassays kits, like ELISA (enzyme linked immunosorbant assay) and lateral flow strip tests. Some companies perform the testing in-house and other companies use contract labs. Immunoassays kits test specifically for an allergenic food like egg, milk, or peanut.[5] Test kits contain an antibody that is selective to a specific protein in the target food. The antibody in the kit and target fit together like a lock and key based on a combination of amino acid sequence and 3-D structure of the protein target.[6] The protein target does not need to possess allergenic potential to be used as the detection marker for allergenic ingredients in food products as long as the selected marker protein is highly abundant in the allergenic commodity.[11] On the other hand, false positive results can occur when the antibody in the kit responds to proteins present in other foods.[11] Therefore target selection in immunoassay test kits requires extensive research using protein databases and practical experiments designed to detect possible cross-reactivity.[11] An immunoassay test kit is not appropriate for all food products. The immunoassay aqueous system does not work well with oils. Retorted products may contain proteins that are insoluble because the high

pressure and high heat applied during processing causes structural changes to the proteins. Salad dressings may precipitate the target protein due to the low ph.[5] The properties of a sample need to be evaluated before performing an immunoassay test because adulteration of the protein's 3-D structure can yield a false negative result because the antibody will not detect the epitope on the target protein.[11] Processes that can affect the structure of a protein are heating, high pressure treatment, chilling, ultra-filtration, irradiation, hydrolysis and fermentation.[11] One example is the "roasting of peanut decreases the efficiency of protein extraction and thereby negatively impacts detection.[11]

Another test kit on the market place is general protein tests 3M™ Clean-Trace™ Surface Protein (Allergen) swab tests. The test is a swab method for detection of protein based on biuret reaction on equipment surfaces. The advantages are the swabs are easy to use, results are ready in 15 minutes, and it detects the presence of protein even if the 3-D structure has been altered during processing or sanitation. If protein is not present then the target allergen is not present because food allergens are proteins. The disadvantage of general protein detection is the protein source is unknown if a positive result is obtained. The use of general protein swabs is in debate because some experts believe the detection limit is not low enough and the detection limit is 20ppm.[5] The manufacturer of the swabs states the detection limit to be 3ug for Bovine Serum Albumin (BSA), but the limit will vary depending on the protein of interest.[14] One study compared general protein swabs (3M™ Clean-Trace™ Surface Protein (Allergen) swab tests) and ELISA kits for the testing of equipments surfaces. The study found the

sensitivity of the general protein swab test was greater than the ELISA kit for some allergenic foods.[12] Another disadvantage of the swab test is the amount of protein detected is unknown. The total protein kit is easy to use, but the color of the solution is often difficult to determine visually and the color is subjective when close to the detection limit. Today the results of the total protein swab may not correlate to FDA approved ELISA kits.[5]

Use of Micro Plate Assay for Total Protein Determination

Many of the disadvantages of the 3M™ Clean-Trace™ Surface Protein (Allergen) swab tests can be eliminated by converting the test to a spectrometric micro plate assay. The technology of the total protein swab is based on a common biochemical techniques used for protein determination, the biuret reaction. The biuret reaction will produce a purple color when proteins are treated with dilute copper sulfate at alkaline pH values. The purple color is created by the formation of a complex of a copper (II) ion with four nitrogen atoms, two from each of the two peptide chains.[15] Typically, the biuret reaction requires large amounts of protein (1-20mg).[15] The chemicals in 3M™ Clean-Trace™ Surface Protein (Allergen) swab tests have been modified to detect small quantities of protein using the principles of the biuret reaction. The solutions in the kit are separated by a double membrane containing some of the reagents for the reaction. When the membrane is punctured by the swab included in the kit, mixed and heated the user will determine a pass or fail results passed on the visual

color of the solution (Figure 1). The observation of a purple color is considered a positive result for the presence of protein. A gray colored solution is an inconclusive result and the manufacturer recommends re-cleaning the equipment surface and retesting.[15] The color of the solution is not always easy to determine visually when the amount of protein is low. The ability to convert the analysis of the swab test to a micro plate assay will allow the operator to read several tests within minutes, so the darkening of the solution with time will have minimal affect on the results. The problem with determining the color of the solution with the naked eye on low level samples would be eliminated. The amount of total protein detected on a surface could be quantified, translated to a concentration level, and a correlation can be made to the FDA approved ELISA kits.

Materials and Instruments

Materials used for analysis

98% Albumin from bovine serum (BSA) from Sigma, 1X PBS buffer pH 7.36, 3M™ Clean-Trace™ Surface Protein (Allergen) swab tests, Dry Egg White Solids from Sonstegard Food Co., Non-Fat Dry Milk Powder (NFDM) from Dairy Farmers of America, Soy Protein Isolate from Solae, sterile 200ul pipette tips from VWR, and micro plates from Costar.

Instruments used for analysis

VWR dry block heater with a 20 wells (13.9mm) block was provided by Sokol & Company. An Epoch Biotek uv-vis spectrophotometer micro plate reader with a range of 200-1000nm linked to Gen 2.0 software package was provided by GSU. The graphs and linear regression data analysis was performed using GraphPad Prism 5 software.

Method

Sensitivity Determination of the 3M™ Clean-Trace™ Surface Protein (Allergen) Swab Tests

Test solutions of BSA, egg white solids, NFDM and soy protein isolate were prepared at a concentration of 1mg/ml in 1X PBS buffer pH 7.36. The stick containing the surface swab was removed from the 3M™ Clean-Trace™ Surface

Protein (Allergen) swab tests. A series of quantities of the 1mg/ml test solutions were applied directly to the surface swab (2µg to 8 µg of BSA, 5µg to 25µg of egg whites, and 2µg to 15µg of NFDM and soy protein isolate). The swab was carefully reinserted into the housing tube. Protein is present everywhere in the environment including skin and hair, so basic sterile technique was used during the sample application steps of this procedure. The test kits were activated by pushing down on the sample stick handle (swab) until the top of the handle is level with the top of the device tube. The solutions in the activated test kits were combined by shaking the tubes rapidly from side-to-side for five seconds. The test kits were placed into a dry heat block preheated to 55^{o}C for 15 minutes. The color of the swab and solution was recorded and compared to the color key supplied on the test kit. To access the solution in the bottom of the test kit, the top portion of the kit (above the gray square on the color key) and swab were removed from the test kit. The solution from the test kit was pipette in a volume of 200µl into micro plate well and the absorbance at 560nm read on Epoch micro plate reader. The procedure was performed in triplicate for each test solution and blanks (negative controls) were prepared by following the same procedure minus the protein application step.

Results and Discussion

It is known that a person with a food allergy can have reaction if the target allergenic protein is present in an unsuspected food at concentrations of parts per million. The presence of food allergens in an unlabeled food can be the result of a

sanitation failure. The 3M™ Clean-Trace™ Surface Protein (Allergen) swab tests are used to determine the presence of protein on equipment surfaces. The swab tests are supplied with a color chart on every test (Figure 1). Any shade of purple confirms the presence of protein on the equipment surface tested. A visual observation of a gray colored solution is an inconclusive result and the manufacturer of the swab test suggests re-cleaning the equipment surface.[14] Unnecessary cleaning costs a food manufacturer time and money. Based on visual color determination the swab tests in this project, the swab test successfully detected the presence of 6µg of BSA, 10µg of egg white (~8.1µg protein), 15µg of non-fat dry milk (~5.0µg protein), and 15µg of soy isolate protein (~8.8µg protein). The visual color determination is often difficult in the presence of low quantities of protein. The color is not consistent or skewed by the presence of the swab. For example, figure 2 shows the color observation for BSA at 2, 4 and 6µg performed in triplicate. The color observed at 2µg was green, green and clear. The manufacturer does not provide directions on how to interrupt the results if a clear color is observed. The color observed at 4µg of BSA was gray color with a green tint, which can be confusing to the end user of the kit. The color results yielded from 6µg of BSA was a light purple with a gray tone. The difficulties in the interpretation of the color increased when allergenic foods were applied to the swab test kit. When 5µg of egg whites was applied to the test kit it produced a green colored solution with a purple colored swab (Figure 3). According to the manufacturer, the end user is to record the strongest color change in the solution or swab as the result.[14] The test kit was designed to be a simple tool to detect

presence of protein, but by having to determine the color of the swab and solution a layer of complexity is added and is the results are confusing to end user. The confusion in the color determination has been observed in real life situations on the production floor. The end user does not understand why the solution is green and the swab is purple and wants to use the green colored solution as the end result, which could open the manufacturer up to a possible allergenic contamination of the next production run. In this case, only one of the three tests at 5µg of egg yielded at purple colored swab, which shows the color of the swab can be unpredictable. At qualities of 10µg of egg whites one test kit produced a green color on top and purple color on the bottom (Figure 3). The manufacture does not provide directions as to whether the test kit should be shaken after the heat incubation at 55^{o}C. When the top of the tube and the swab was removed, the color observations caused by swab interference and two-toned layered results were eliminated (Figure 4). The same problems with visual color observation occurred with NFDM and soy protein isolate. At 10µg of NFDM the solution turned green and the swab purple and at 5µg of soy isolate protein the color was a mix between green and gray. The color observations for each component tested are listed in tables 1, 2, 3 and 4.

In order to avoid unnecessary re-cleaning of manufacturing equipment, it is necessary to know the amount of protein detected following sanitation of a target allergenic food. Protein quantification is possible by reading the absorbance of the solution at 560nm on a micro plate assay. The limit of detection (LOD) and limit of quantification (LOQ) were determined from the

standard deviation of five negative controls performed simultaneously and resulted in an absorbance of 0.048 for LOD and an absorbance of 0.160 for LOQ. In this study, the focus will be on detection of the lowest amount of protein or allergenic food above the LOD since no government standard has been set for how much of allergen needs to be removed in order to consider the equipment surfaces allergen "free". Many companies are using a value of 5ppm because that is the detection limit of the popular ELISA test kit. Future studies should focus on the LOQ once a standard of allergen "free" has been defined for equipment surfaces. The sensitivity of the test kit will vary depending on the protein detected since the kit is based on the biuret reaction, therefore; BSA was used as a baseline for comparison because it was the positive control chosen by the manufacturer during the verification of the test kit.[14] The lowest amount of protein detected above the detection limit in this study using the micro plate assay was 2µg of BSA, 5µg of egg white and or ~4.1µg of egg protein, 5µg of NFDM or ~1.7µg of milk protein and 5µg soy protein isolate or ~4.4µg of soy protein (Tables 1, 2, 3 and 4). In order to determine the LOD in micrograms, the absorbance of a series of low quantities of BSA and the food products were plotted and linear regression executed (Figures 5, 6, 7 and 8). The error bars on the graphs represent the standard deviation between the triplicate results. The slope and coefficient of determination (R^2) were determined from the linear regression from each graph. The R^2 values of the BSA and food products were 0.977 or higher which shows great linearity in swab test micro assay at low quantities of protein near the detection limit. The soy protein isolate yielded a

remarkable R^2 value of 0.993. The slope of the corresponding linear regression was used to calculate the LOD in micrograms for each test solution (Table 5). The LOD for BSA was 1.5µg, 2.3µg of egg white or ~1.5µg of egg protein, 5.0µg of NFDM or ~1.6µg of milk protein, and 2.8µg of soy protein isolate or ~2.5µg of soy protein. In order to compare the results from the micro plate assay of the 3M™ Clean-Trace™ Surface Protein (Allergen) swab tests to the popular ELISA kit, the volume of solution in the swab kit was quantified. The volume of solution in the swab tests varied. The lowest amount quantified was 620µl, this volume was used convert the results to part per million concentrations (ppm). The limit of detection in part per million concentrations for conversion of the 3M™ Clean-Trace™ Surface Protein (Allergen) swab tests to a micro plate assay was 2.4ppm for BSA, 3.1ppm for egg protein, 4.0ppm for soy protein, and 2.6ppm for milk protein (Table 5). All results were below the 5ppm limit of the ELISA detection kit.

Conclusions

The sensitivity of the 3M™ Clean-Trace™ Surface Protein (Allergen) swab tests can be improved by performing a micro plate assay on the test solution. In all cases, the detection protein was lower than the 5ppm standard set by the ELISA test kit. The advantage in using the 3M™ swab test for equipment surfaces is it will detect the amount of total protein on a surface and ELISA only detects the presence of a target protein, hence the difference in sensitivity between the 3M™ swab test micro assay and the commercially available ELISA kit is potentially greater than reported. The development of this micro plate assay will

provide the user a double duty purpose for the swabs tests. The user could perform a quick visual inspection for swab test that yield purple colored solution (high quantities of protein) and use a micro plate assay to determine the amount of protein present in the remaining swab test (low quantities of protein). The swab test kit could be redesigned with a removable base to provide easy access to the colored solution. Standards of target allergenic foods at specific protein concentration could be sold by the manufacturer to serve as positive low limit controls or for the use of protein determination. Future studies should included testing allergenic food product from all of the "big eight" food group and increasing the scale to include a variety of products within each allergenic food category. The goal with swabbing equipment surfaces and testing for allergenic protein is to ensure the equipment is protein "free". The conversion of the 3M™ Clean-Trace™ Surface Protein (Allergen) swab tests to a micro plate reader makes it is possible to reach sensitivities that are currently not available on a commercial scale.

Acknowledgement

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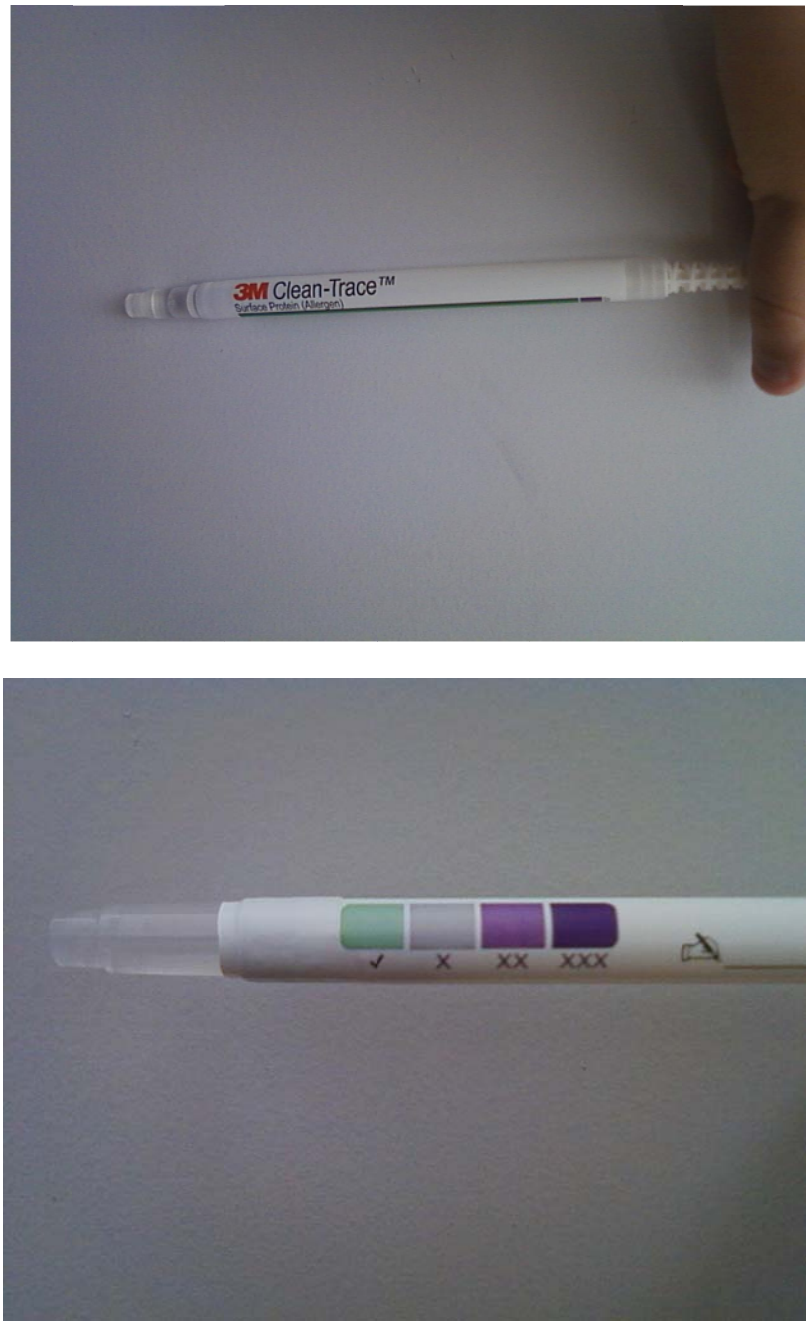

Figure 1: 3M™ Clean-Trace™ Surface Protein (Allergen) swab test

Top is a photo of a 3M™ Clean-Trace™ Surface Protein (Allergen) swab test. The hand in the picture is holding the swab portion of the kit. The bottom photo displays the color key for the color determination of the swab test kit results.

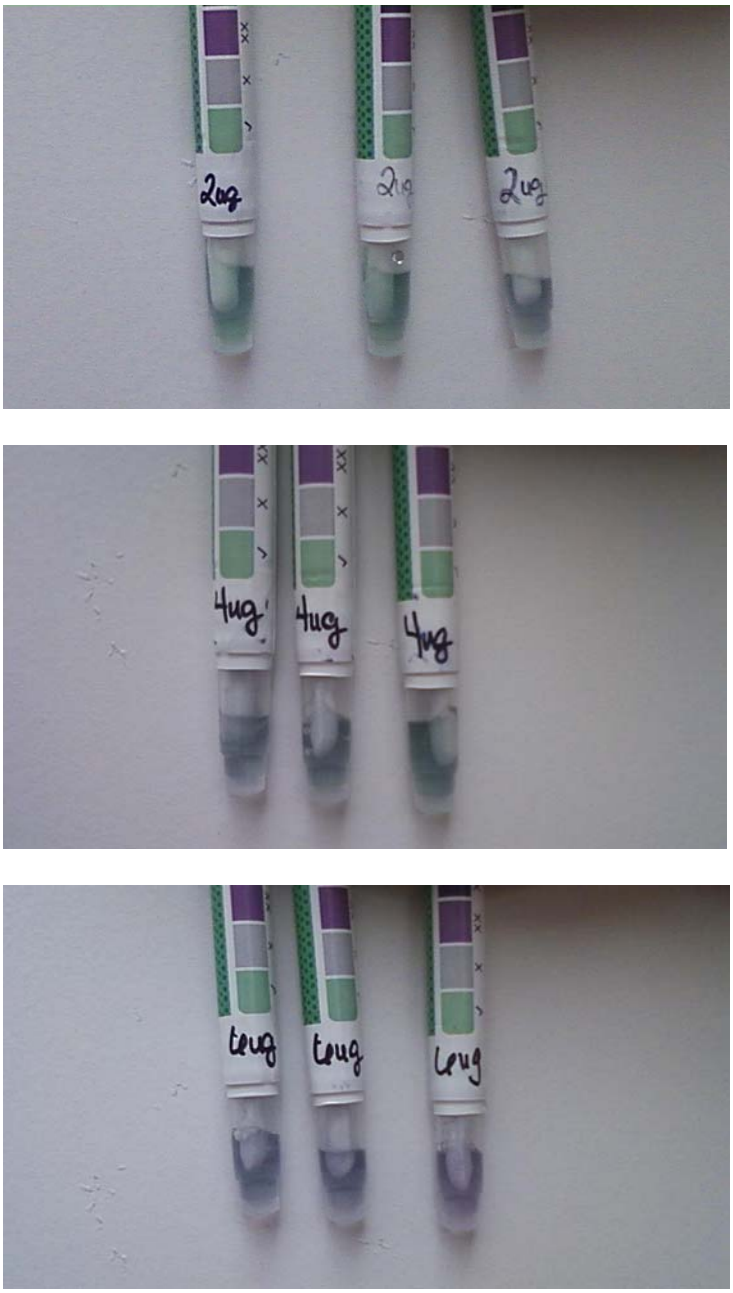

Figure 2: Photos of BSA Results BSA was applied in triplicate to swab tests in increments of 2, 4, 6, and 8µg. The photos display the colors observed at 2, 4, and 6µg of BSA. Top photo: the observed color for 2µg was green, green and clear. Middle photo: the color observed for 4µg of BSA was gray color with a green tint. Bottom photo: displays the color observed at 6µg were light purple with a gray tint.

Figure 3: Photos of Ambiguous Swab Tests Results

Often the color determination of the swab kit is ambiguous. The photo on the left is from the application of 5µg of egg whites to the swab kit. The result yielded a green colored solution with a purple colored swab. The photo on the right is from the application of 10µg of egg whites which resulted in solution with a green color on top and purple color on the bottom.

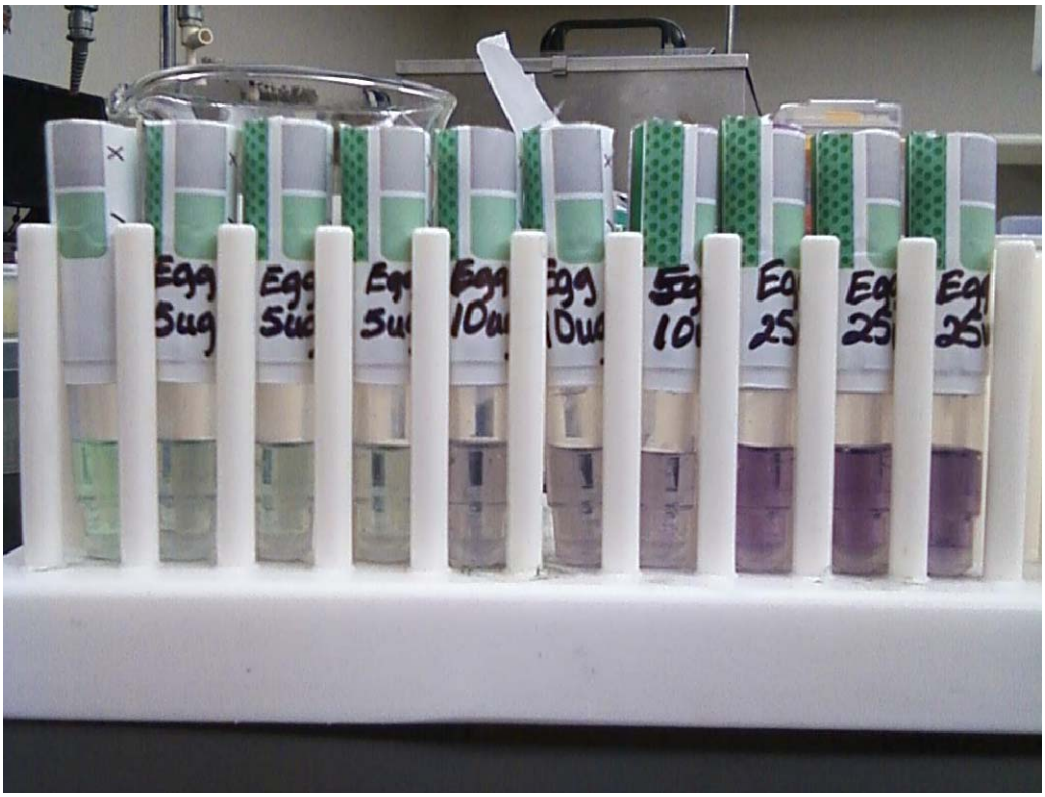

Figure 4: Photo of Egg White Swab Tests

The photo is swab test results from the application of 5, 10 and 25µg of egg white, after removal of the top portion of the test kit and the swab.

Bovine Serum Albumin (BSA)

BSA (µg)	BSA (ppm)	Average Absorbance 560nm	Standard Deviation	Observed Color
2	3	0.078	0.011	Clear/light green
4	6	0.136	0.001	Gray
6	10	0.192	0.010	Light purple
8	13	0.251	0.006	Light purple

Table 1: BSA Results

BSA solution (1mg/ml in PBS buffer pH 7.36) was applied to swab tests in quantities of 2, 4, 6, and 8µg. The color observed from the swab test and the absorbance at 560nm from a micro plate assay was recorded. The standard deviation was calculated from the average of three absorbance readings.

Egg Whites (81% Protein Content Per Sonstegard Food Company Nutritional Statement)

Egg (µg)	Egg (ppm)	Egg Protein (µg)	Egg Protein (ppm)	Average Absorbance 560nm	Standard Deviation	Observed Color
5	8	4.1	7	0.127	0.011	Greenish – gray
10	16	8.1	13	0.233	0.016	Light purple
25	40	20.3	33	0.502	0.041	Purple

Table 2: Egg White Results

Egg white solution (1mg/ml in PBS buffer pH 7.36) was applied to swab tests in quantities of 5, 10, and 25µg. The color observed from the swab test and the absorbance at 560nm from a micro plate assay was recorded. The standard deviation was calculated from the average of three absorbance readings.

Non-Fat Dry Milk (33% Average Protein Content Per Dairy Farmers of America Specification Sheet)

NFDM (µg)	NFDM (ppm)	NFDM Protein (µg)	NFDM Protein (ppm)	Average Absorbance 560nm	Standard Deviation	Observed Color
2	3	0.7	1	0.020	0.002	Light green
5	8	1.7	3	0.062	0.003	Light green/clear
10	16	3.3	5	0.093	0.018	Clear with purple spots on swab
15	24	5.0	8	0.146	0.010	Gray

Table 3: NFDM Results

Non-fat dry milk solution (1mg/ml in PBS buffer pH 7.36) was applied to swab tests in quantities of 2, 5, 10, and 15µg. The color observed from the swab test and the absorbance at 560nm from a micro plate assay was recorded. The standard deviation was calculated from the average of three absorbance readings.

Soy Protein Isolate (88% Protein Content Per Solae Nutritional Statement)

Soy (µg)	Soy (ppm)	Soy Protein (µg)	Soy Protein (ppm)	Average Absorbance 560nm	Standard Deviation	Observed Color
2	3	1.8	3	0.026	0.009	Green
5	8	4.4	7	0.096	0.019	Grayish-green
10	16	8.8	14	0.173	0.005	Gray
15	24	13.2	21	0.252	0.044	Light purple

Table 4: Soy Protein Isolate Results

Soy protein isolate solution (1mg/ml in PBS buffer pH 7.36) was applied to swab tests in quantities of 2, 5, 10, and 15µg. The color observed from the swab test and the absorbance at 560nm from a micro plate assay was recorded. The standard deviation was calculated from the average of three absorbance readings.

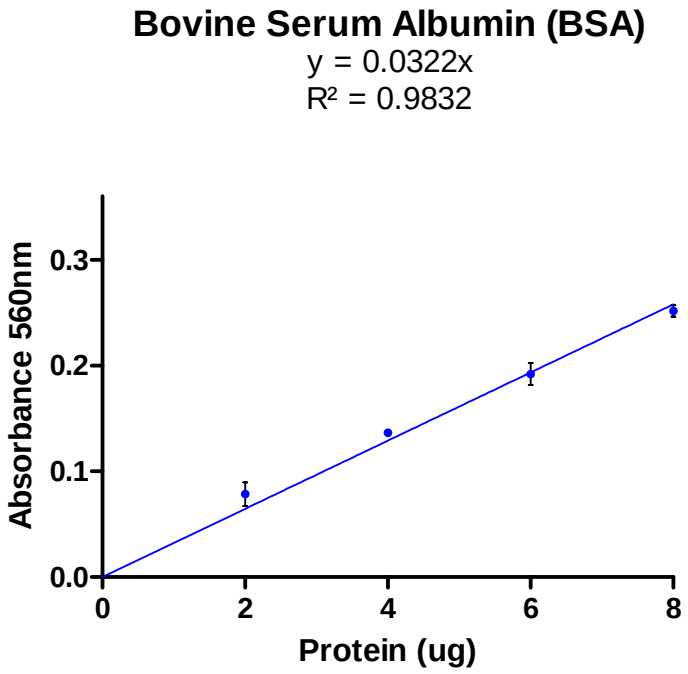

Figure 5: Linear Regression of BSA Results

Plot of the absorbance at 560nm verses the amount of BSA applied to the swab test in micrograms. Error bars on the graph represent the standard deviation of the triplicate results.

Figure 6: Linear Regression of Egg White Results

Plot of the absorbance at 560nm verses the amount of egg white applied to the swab test in microgram amounts. Error bars on the graph represent the standard deviation of the triplicate results.

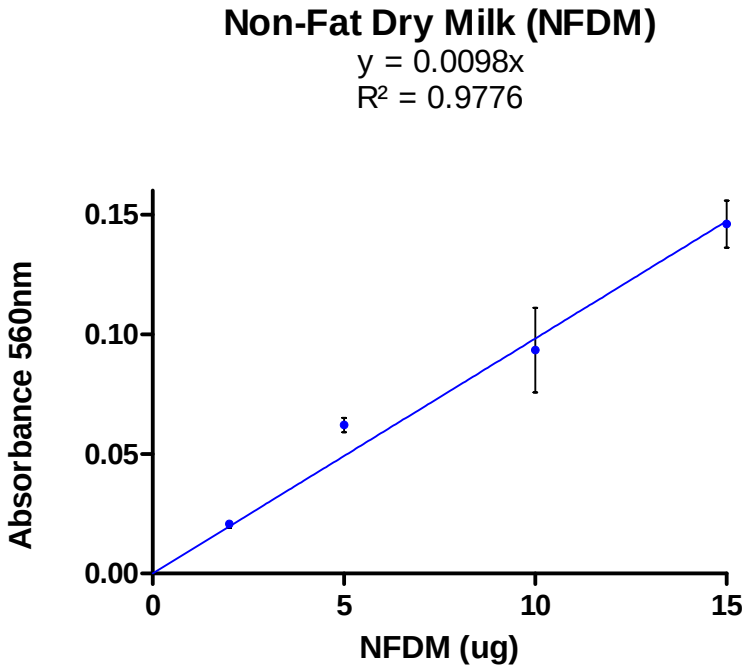

Figure 7: Linear Regression of NFDM Results

Plot of the absorbance at 560nm verses the amount of NFDM applied to the swab test in micrograms. Error bars on the graph represent the standard deviation of the triplicate results.

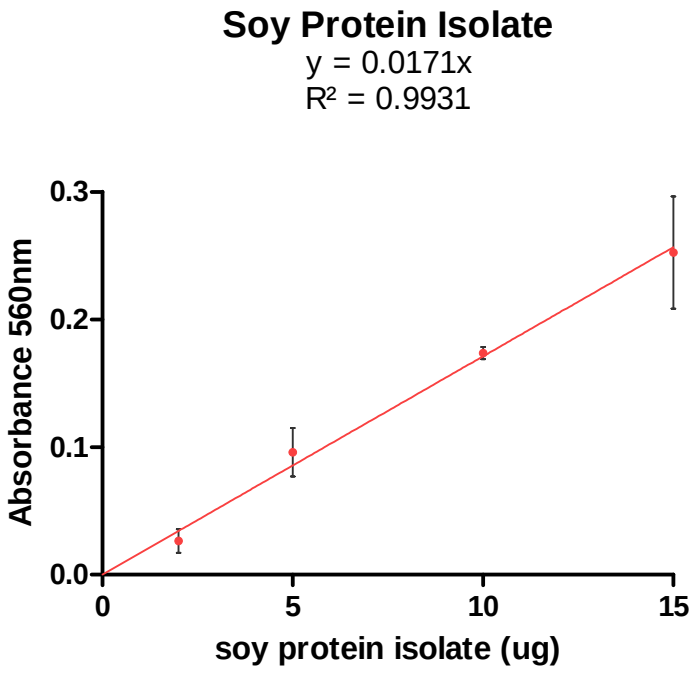

Figure 8: Linear Regression of Soy Isolate Results

Plot of the absorbance at 560nm verses the amount of soy isolate applied to the swab test in micrograms. Error bars on the graph represent the standard deviation of the triplicate results.

Graph	Slope of Linear Fit	R^2	LOD (µg)	LOQ (µg)	LOD Protein Only (µg)	LOD Protein only (ppm)
BSA	0.0322	0.9832	1.5089	5.0297	1.5089	2.4337
Egg Whites	0.0207	0.9804	2.3472	7.8240	1.9012	3.0664
Soy Isolate Protein	0.0171	0.9931	2.8413	9.4712	2.5004	4.0329
Non Fat Dry Milk	0.0098	0.9776	4.9579	16.526	1.6361	2.6389

Table 5: Summary of Limit of Detection Results

The slope and R^2 from linear regressions in figures 5, 6, 7, and 8 were tabulated. The LOD and LOQ for BSA and each food product were calculated from the respective slope value. The LOD protein only for each food product was determined using the estimated protein content stated on the suppliers nutritional and specification documents. Parts per million results were determined using the volume of solution in a swab test.