

Research Paper**Comparison of Detection Limits for Allergenic Foods between Total Adenylate (ATP+ADP+AMP) Hygiene Monitoring Test and Several Hygiene Monitoring Approaches****WATARU SAITO, KAZUKI SHIGA, AND MIKIO BAKKE****Kikkoman Biochemifa Company, Planning & Development Department, 376-2 Kamihanawa, Noda, Chiba 278-0033 Japan*

MS 20-017: Received 15 January 2020/Accepted 11 March 2020/Published Online 22 June 2020

ABSTRACT

Validation and verification of cleaning and inspection methods are essential to prevent the spread of allergens via cross-contact. Among the hygiene monitoring tests used on-site, the ATP test is rapid and provides quantifiable results. Nevertheless, because a wide variety of foods contain significant amount of ADP and/or AMP due to the degradation of ATP, the ATP+ADP+AMP (A3) test is preferred for detecting food debris. Hence, the A3 test may be valuable in screening food debris that may contain residual allergens. In this study, the detection limits of the A3 test for 40 foods that are regulated in several countries as allergenic were compared with those of the other hygiene monitoring tests used on-site: the conventional ATP test with similar sensitivity for ATP, the protein swab test that detects as little as 50 µg of protein, and the lateral flow immunoassay (LFI). The A3 test demonstrated lower detection limits than did the ATP test. The detection sensitivity of the A3 test was greater than that of the protein swab test except for its use on gelatin (extracted protein). The cleaning validation performance using a stainless steel model in fish and meat revealed that the A3 test is efficient in verifying the levels of remaining food debris. Although LFI displayed the best sensitivities for 10 of 14 foods, it is not commercially available for some specific allergens; however, the A3 test can detect such food debris. Moreover, the detection limits of the A3 test were preferable or comparable to those of LFI for crustacean shellfish and for processed grains, with the exception of wheat flour and buckwheat. A field study in a food processing plant demonstrated that the amount of both A3 and milk protein (enzyme-linked immunosorbent assay) considerably decreased as the cleaning steps progressed. Therefore, the A3 test is effective in detecting the risk for allergen cross-contact after inadequate cleaning.

HIGHLIGHTS

- ADP and/or AMP predominate over ATP in many allergenic foods.
- LFI showed better sensitivity than other nonspecific methods.
- The A3 test can detect foods for which LFI is not commercially available.
- The A3 test is effective in detecting the risk for allergen contamination.

Key words: Allergenic food residue detection; ATP+ADP+AMP; Detection limit; Hygiene monitoring; Rapid detection

At present, a remarkable increase has been observed in the numbers of individuals suffering from food allergies (7, 18). An allergic reaction occurs when a sensitive person's immune system recognizes the proteins in certain foods, leading to life-threatening body reactions. It is known that more than 160 foods can cause allergic reactions (11, 13). The U.S. Food and Drug Administration (FDA) lists eight common food items (milk, eggs, fish, crustacean shellfish, tree nuts, peanuts, wheat, and soybeans) that cause 90% of food-allergic reactions in the United States; these are known as the "Big 8."

Although there has been tremendous progress in the development of desensitization regimens and therapeutics,

strict avoidance of the offending food allergen is often the only manageable solution (9). As a result, sensitive consumers depend on accurate food labels to disclose the presence of allergenic ingredients. The Food Allergen Labeling and Consumer Protection Act of 2004 amended the Food, Drug, and Cosmetic Act to require that the manufacturers of all packaged food products sold in the United States list the presence of Big 8 ingredients when they are used in the product (10). In addition, mollusk, celery, mustard, sesame, lupin, and sulfur dioxide are listed as food allergens with mandatory disclosure in the European Union (1). Japanese food allergen labeling regulations include seven mandatory (egg, milk, wheat, buckwheat, peanut, shrimp, and crab) and 21 recommended (almond, abalone, squid, salmon roe, orange, cashew nuts, kiwi fruit, beef, walnut, sesame, salmon, mackerel, soybean, chicken,

* Author for correspondence. Tel: +81-4-7123-5827; Fax: +81-4-7123-5813; E-mail: mbakke@mail.kikkoman.co.jp.

banana, pork, matsutake, peach, yam, apple, and gelatin) allergenic ingredients (1, 17). Moreover, tomato is recognized as an allergen food in Korea (1). Japanese food regulations set the mandated threshold for allergen labeling at more than 10 µg of soluble allergen protein per g of food (1, 17). To counter celiac disease, in which immunological responses damage the small intestine, the Food Allergen Labeling and Consumer Protection Act permits the voluntary use of the term “gluten-free” on food labels if the product contains less than 20 ppm of gluten (20). As a result, allergen control is becoming an increasingly important component of the overall food safety programs for the food industry.

Recently, the FDA’s Food Safety Modernization Act enhanced its authority to recall foods contaminated with allergens (6, 21). Moreover, the act also added requirements that the FDA issue regulations that require food facilities to implement allergen controls where necessary. One example of control is prevention of cross-contact that can occur via the transfer of allergens during processing or handling, that is, when the allergen-containing and nonallergen-containing foods or ingredients are produced in the same facility or on the same processing line and when the allergen controls are not appropriately implemented (6, 13). Because cross-contact can occur due to incomplete cleaning of the food contact surfaces, validation and verification of cleaning and inspection methods are essential (5, 13).

The cleaning level can be generally verified by visual inspection. Although immediate visual assessment is an easy method to determine whether the equipment is clean, accessibility, lighting, surface, and visual acuity of inspectors are important (13). Therefore, even in instances where the equipment surfaces appear to be visibly clean, analytical methods are recommended to verify the absence of allergenic food residues. Several analytical approaches have emerged (5, 13), including enzyme-linked immunosorbent assay (ELISA) and lateral flow immunoassay (LFI) for specific allergen residues (5, 13), ATP detection (13, 22), general protein detection (13), PCR to detect DNA fragments from the allergic food (12), and mass spectrometry methods to detect specific target peptides from the allergenic sources (9). ELISA, PCR, and mass spectrometry are known as highly sensitive and specific methods. However, they can be difficult to use on-site in the production area because they require expensive, specialized equipment and laboratories, along with highly trained personnel to conduct the laborious pipetting procedure, perform calculations using standard curves (for ELISA), and analyze large quantity of data (for mass spectrometry) (5). Because rapid methods can be easily used in a production area, rather than only in the laboratory, their use has increased, and LFI, protein swab tests, and ATP tests have been generally used.

ATP is the universal energy source found in all living beings. Because ATP is not a protein, it is not feasible to assay the allergen protein directly or specify the allergen using ATP tests. The reason for using the ATP test for allergen verification is that, when the concentration of the surface-bound ATP from food ingredients is reduced below a defined value, the surface can be declared as clean and

free of allergens. The ATP test is preferred as it is rapid (as short as 30 s) and easy to use for relatively unskilled personnel. Nevertheless, conventional ATP test systems have a limitation, wherein ATP is degraded to ADP and AMP by heat, acid or alkali, and enzymes. Because conventional ATP tests cannot detect these degradation products, they can fail to indicate insufficient cleaning (3).

Recently, a novel hygiene monitoring system was developed to measure total adenylate (ATP+ADP+AMP; A3) (3, 4). The A3 test for the stainless steel surfaces was approved for AOAC Research Institute Performance Tested Methods certification no. 051901 (2). In a previous study, the amount of A3 was remarkably greater than that of ATP in a variety of ingredients and processed foods including allergens (3). It is also shown that ADP became the predominate adenylate in raw animal tissue (19). Accordingly, the A3 assay is presumed to be highly suitable for determining cleaning efficacy. Nevertheless, no comparison data of the A3 swab test and other rapid tests is available, for example, comparison with the protein swab test and LFI for the detection of foods that are known as allergens.

In this study, the detection limits of the A3 test for allergenic foods were compared with those of the conventional ATP test, the protein swab test, and LFI. A field test for monitoring cleanliness of the facilities in a food processing plant was also carried out to evaluate the correlation of the data between the A3 test and ELISA.

MATERIALS AND METHODS

Preparation of food samples. All foods and beverages used in this study are commercially available. The foods assayed in this study include fish (canned tuna, raw mackerel, and raw salmon), crustaceans (raw shrimp and boiled crab meat), dairy (milk, yogurt, cream cheese, Camembert cheese, and ice cream), whole egg, raw meat (chicken, pork, and beef), mollusk (abalone and squid), salmon roe, gelatin (powder, boiled), buckwheat (boiled noodle), peanuts (roasted), soybean, wheat flour (boiled), bread, beer, almond (roasted), hazelnut (roasted), cashew (roasted), walnut, mustard (seed), sesame (roasted), matsutake mushroom, coconut milk, celery, tomato, orange, kiwi fruit, banana, peach, apple, and yam. Edible portions of solid foods (10 g) were homogenized with nuclease-free water (90 mL) in a blender, resulting in 10-fold diluted samples. Serial 10-fold dilutions were performed using nuclease-free water for the ATP and A3 test, protein swab tests, and the determination of the protein concentration. The sample preparations for LFI are described below.

Food detection via A3 and ATP tests. The A3 assay was performed using a commercially available test kit, LuciPac A3 Surface/Lumitester PD-30 (Kikkoman Biochemifa, Tokyo, Japan) (3, 4). Sample solutions (100 µL) were pipetted onto dry swabs. After completely inserting the swab stick into the main tube, the samples, extraction solution, and reagents were thoroughly mixed. The device was then inserted immediately into the luminometer, and the resulting luminescence was measured (measurement time: 10 s). All measurements were carried out at 23°C, and the data were electronically recorded. The measurement output was relative light units (RLU; measurement range: 0 to 999,999 RLU). Based on the calibration curves studied in the previous report, it was verified that the A3 assay afforded equivalent linear calibration curves between RLU and the amounts of ATP, ADP,

and AMP in logarithmic scales (2, 3). To compare the sensitivity of food detection between the ATP and A3 assays, the LuciPac II/Lumitester C-110 (Kikkoman Biochemifa), which is a conventional ATP test based on the luciferin-luciferase assay, was also evaluated (3, 4). The measurements were repeated three times, and the mean values were reported.

Food detection via protein swab test. The protein swab test used for this study was Clean-Trace Surface Protein Plus Test Swab (3M, St. Paul, MN). The detection limit that is described in the manufacturer's statement is 50 µg of protein. Samples (100 µL) were applied to the dried swab supplied with the kits, and the assay was performed according to the manufacturer's instruction. After completely inserting the swab stick into the main tube, the samples and reagents were thoroughly mixed for 5 s. After leaving the swab upright at 23°C for 10 min, the result was interpreted by observing the color: light green, negative (not detected); gray, suspicious; purple, positive (detected) result.

Food detection via LFI. Monoclonal antibody technology-based Allergeneye Quick (for peanuts [Ara h 1], buckwheat [BW24KD], wheat [gliadin], milk [α 1-casein], egg [ovalbumin], and crustacean [Tropomyosin]) from Prima Meat Packers (Tokyo, Japan) and Allergeneye Immunochromatography (for soybean [7S globulin] and sesame [11S globulin]) from Kikkoman Biochemifa were used for the evaluation of detection limits of LFI. Each detection limit that is described in the manufacturer's statement is 0.2 ppm (200 ng/mL) of allergenic protein in food for Allergeneye Quick and 0.1 ppm (100 ng/mL) of allergenic protein in foods for Allergeneye Immunochromatography. The dairy products (milk, yogurt, cream cheese, Camembert cheese, and ice cream) were assayed using LFI for milk. Shrimp and crab were assayed using LFI for crustaceans. Wheat flour and bread were assayed using LFI for wheat. The assays were performed according to the manufacturer's instruction. The samples for Allergeneye Quick were prepared by serial 10-fold dilutions of blended foods (10-fold) as above with phosphate buffered saline (PBS). Regarding the samples for Allergeneye Immunochromatography, blended foods (10-fold) as above were additionally diluted with nuclease-free water by 5-fold and then 20-fold dilution with attached extraction buffer (total 1,000-fold). Successively, the samples were heated in the boiling bath for 10 min and serially diluted with the extraction buffer solution (extraction buffer:nuclease free water = 19:1) by 10-fold. After dipping the strips in the samples for 3 s, taking them out, and leaving them at 23°C for 10 min (Allergeneye Quick) or dipping the strips in the samples at 23°C for 20 min (Allergeneye Immunochromatography), the result was interpreted by observing the bands: single band, negative (not detected); one faint band and clear single band, suspicious; two bands, positive (detected) result.

Determination of protein concentration in foods. The protein in each sample was determined using Pierce Coomassie (Bradford) protein assay kit (Thermo Fisher Scientific, Waltham, MA). A series of standard solutions (50, 25, 12.5, 6.25, and 3.13 µg/mL bovine serum albumin [BSA] standard; Thermo Fisher Scientific, Waltham, MA) was prepared for the calibration curve. Each standard or unknown sample (150 µL) was pipetted into the microplate wells (AS ONE Corporation, Osaka, Japan). Next, the Coomassie reagent (150 µL) was added to each well and mixed for 30 s. After incubating the plate for 10 min at 23°C, absorbance at 595 nm was measured on the plate reader (TriStar²S LB 492, Berthold Technologies, Bad Wildbad, Germany). Assays were performed in duplicate, and the average measurement for the

blank was subtracted from the measurements of all other individual standard and sample replicates. Successively, the mean values for the standard solutions and unknown samples were obtained. The standard curve was prepared by plotting the average measurement for each BSA standard versus its concentration in micrograms per milliliters. Using the standard curve, the protein concentration estimate was determined for each unknown sample.

Interpretation of the detection limit of each assay. Recent validation study demonstrated that the limit of detection of the A3 test is ca. 10 RLU (2). The manufacturer recommends the typical benchmark value indicating a clean stainless steel surface for their ATP and A3 tests as 200 RLU. According to the calibration curves that have been shown in the previous study (2, 3), 200 RLU corresponds to ca. 100 fmol per assay for adenylates. For the stainless steel surface, the dilution factors required to achieve a theoretical 200 RLU were calculated according to the actual dilution factors and the measurement values (RLU). In this study, they were considered as the detection limits for each device for convenience.

For the protein swab and LFI, if the results were clearly judged, the detection limit can be concluded as being between the lowest detectable concentration and the highest nondetectable concentration. Because the samples were serially diluted by 10-fold, the detection limit was considered to be one-third of the lowest detectable concentration for convenience. If the result was "suspicious," the concentration of the sample was considered to be the detection limit.

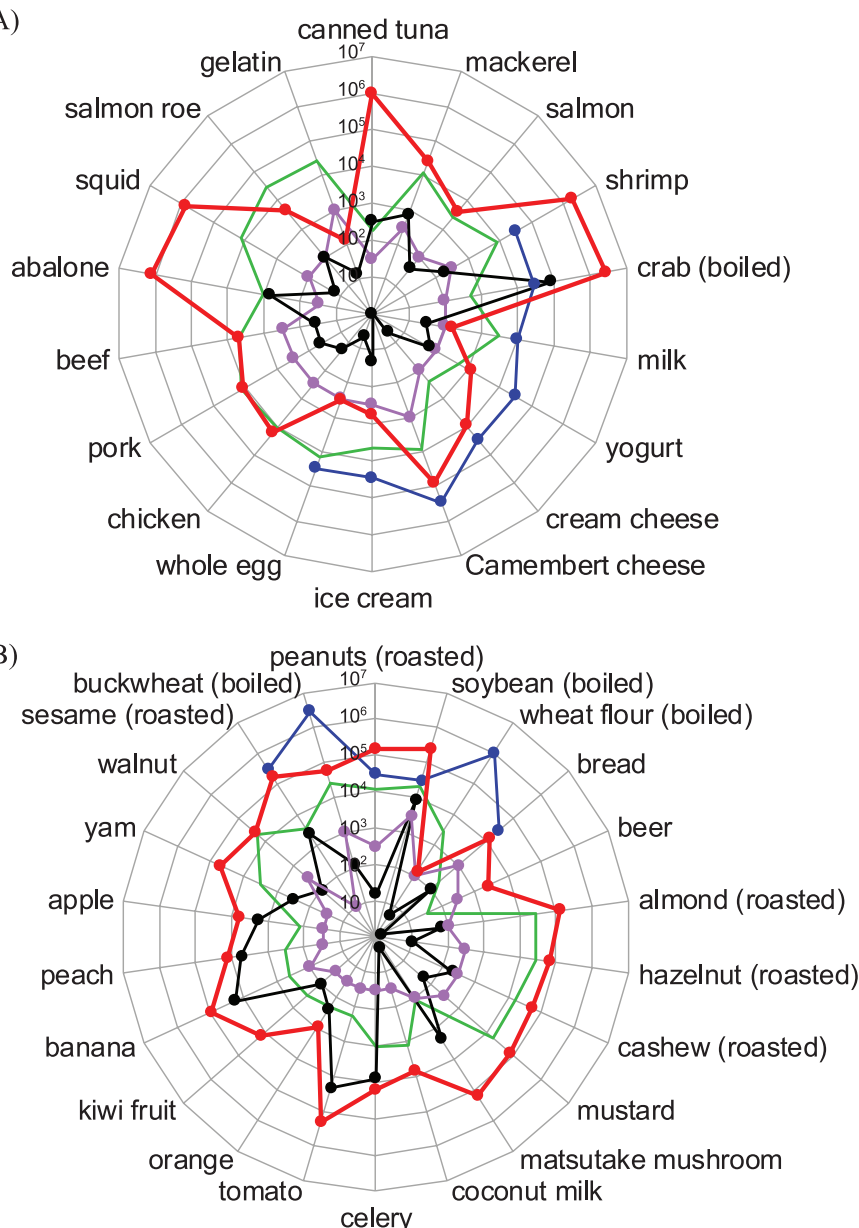
This study is based on the assumption that sanitation verification is performed by swabbing. The ATP and A3 tests and the protein swab test can employ a swabbed sample as given. In contrast, LFI required the swabbed sample to be captured in a solution. Therefore, the sensitivity of LFI was underestimated by one digit for convenience.

As the reference, the theoretical dilution factor that can include 10 ppm of total protein in each food was calculated using the protein determination results because Japanese food regulations set the mandatory threshold for allergen labeling at more than 10 µg of allergen protein per g of food (1, 17).

Sanitation monitoring of stainless steel via the A3 and protein swab tests. Stainless steel coupons (10 by 10 cm) were exposed to commercially available raw salmon and chicken meat by scrubbing for three test repeats. These plates were washed thrice. First, they were rinsed with tap water (20°C) for 30 s; second, they were rinsed with hot tap water (55°C) for 30 s; and third, they were cleaned using a sponge with dishwashing detergent and were further rinsed with tap water (20°C). Two swabs for the A3 and protein swab tests were premoistened with running tap water (same as used in the washing processes). After each wash, the square surfaces (10 by 10 cm) were simultaneously swiped 10 times vertically and horizontally with the two swabs while holding with one hand, and then the A3 and protein swab tests were carried out as aforementioned. The means for the A3 test for each sample were reported.

Correlation study based on A3 and milk allergen protein in the food production facility. The field study was carried out in a facility that produces seasoning mixes for ready-to-cook meals. Products using milk as a raw material were selected as the test subjects. The nine sampling points that were tested included the spatula (silicon) and wire mesh at raw material inlet, the insertion slot for raw materials and its nearby surface, the inner wall, and the impeller of the mixing tank, the hopper, the nozzle, and the

FIGURE 1. Detection limits of rapid hygiene monitoring test kits for allergenic foods. The numbers on the scales indicate dilution factors. The 10^5 -fold dilutions are equivalent to 10 ppm of food sample. Green line is equivalent to 10 ppm of protein (Bradford assay, standard: BSA) for each food. The A3 test (red), the conventional ATP test (black), the protein swab test (detection limit: 50 μ g of protein, purple), and lateral flow immunoassay (LFI; blue). The ATP and A3 tests are products of the same manufacturer, and they show similar sensitivity for ATP. The detection limits of the ATP and A3 tests are determined by the dilution factors to achieve a theoretical 200 RLU because their typical benchmark value at a clean stainless steel surface is 200 RLU. Because LFI required the swabbed sample to be captured in a solution, its sensitivity was underestimated by one digit for convenience. (A) Animal foods, (B) plant foods.



saucer of filling machine (stainless steel). The assessment was carried out in three steps: before cleaning, after a rinse with hot water (75°C), and after complete washing using an alkaline cleaner (90°C) followed by a rinse with hot water (75°C). The surfaces were swiped using two swabs; the swab of LuciPac A3 Surface moistened with tap water and the premoistened swab attached with Pro-media ST-25 PBS (Elmex, Tokyo, Japan). Successively, the A3 test was performed using the swabs of LuciPac A3 Surface as aforementioned. The swabs of Pro-media ST-25 PBS were washed in 5 mL of PBS, following which the samples were analyzed with the ELISA kit for milk (detection limit, 0.78 ng/mL; FASTKIT ELISA Ver.III Milk, NH Foods, Osaka, Japan). To detect milk at the lowest possible concentration, each sample (100 μ L) was applied into the wells without further 400-fold dilution using extraction buffer and dilution buffer. The subsequent assays were carried out according to the manufacturer's instruction. Swiped milk allergen (micrograms) was calculated by multiplying the measurement values (nonograms per milliliter) and PBS volume (5 mL) and then dividing them by 1,000. The detection limits of the A3 test, ATP test, and the protein

swab test for the final product were also assessed according to the aforementioned method.

RESULTS

Comparison of the detection limits of the A3 test, ATP test, protein swab test, and LFI for various allergenic foods. The assays for various allergenic foods using each device (Supplemental Table S1) presented the radar charts of the detection limit (Fig. 1). For example, the sample of 10^5 -fold dilution is equivalent to 10 ppm of food, and this indicates that the amount of the food sample for the swabbing assays is 1 μ g of food per assay because 100 μ L of sample was applied on these swabs. If the red dots that indicate the detection limits of the A3 test fall outside the axis of 10^5 -fold dilution, for example, canned tuna, shrimp, crab, abalone, squid, peanuts, soybean, almond, matsutake mushroom, tomato, banana, and, sesame, then 1 μ g of food residue can presumably be detected by the A3 test. If the

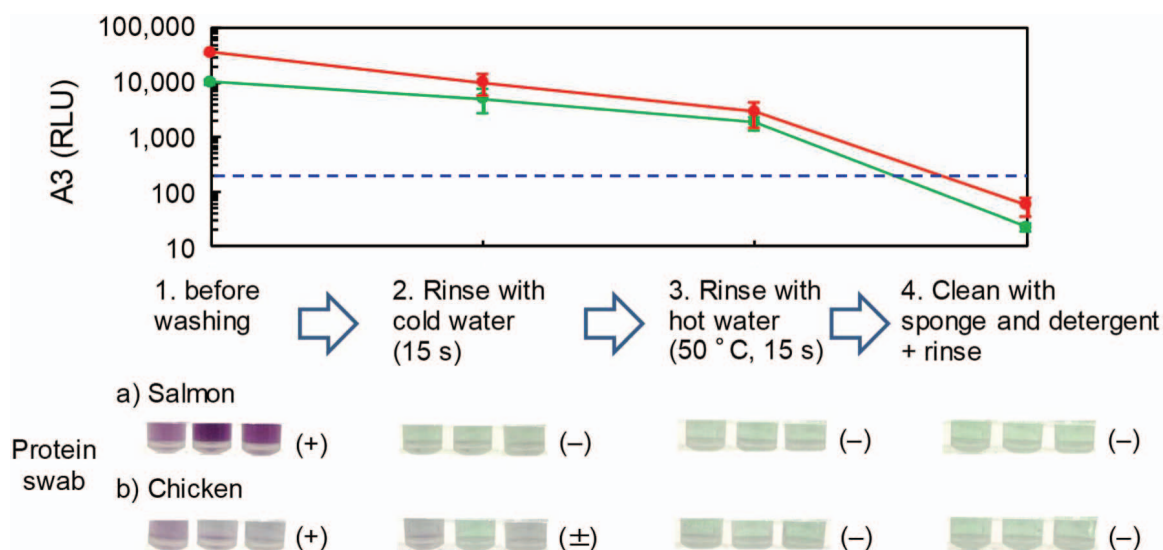


FIGURE 2. Sanitation monitoring of stainless steel exposed to raw fish or meat using the A3 test and the protein swab test. The protein swab test can detect as little as 50 μg of protein. (Graph) The results of the A3 test for raw salmon (red) and raw chicken (green). The typical benchmark value (200 RLU) is presented by the blue dotted line. Each step of washing is presented at the bottom. (Picture) The results of the protein swab test for raw salmon (top) and raw chicken (bottom). The result was interpreted by observing the color: light green, negative (not detected, -); gray, suspicious ($\pm$); purple, positive (detected, +). Each step of washing is demonstrated at the top.

protein level is the focus, the chart of 10 ppm of protein (green) provides a suitable reference. As aforementioned, the results of this detection level using the swabbing assays indicate that they may be able to screen food debris with 1 μg of protein per assay. The chart demonstrates that the A3 test can detect total adenylates, assumed to mix approximately 1 μg of protein in several food residues, for example, meat, fish, crustacean shellfish, processed dairy (except for ice cream), and plant-based foods (except for wheat flour). Compared with the ATP test (black), the detection limit of the A3 test was superior. Moreover, the detection limits of the A3 test were also superior to those of the protein swab test (purple) for many foods, although the results of these two tests were comparable for milk, ice cream, whole egg, and wheat. The protein swab test performed better than the A3 test only for gelatin, which is an extracted animal protein. Note that the sensitivity of LFI (blue) (Fig. 1 and Table S1) was underestimated by one digit because LFI required the swabbed sample to be captured in a solution, and it can be seen as 10 times dilution for convenience. (That is, actual detection limits of LFI for homogenized food solution were one digit better than the results in Fig. 1 and Table S1.) The detection limits of LFI were superior to those of the A3 test for 10 of 14 foods, although the detection limit of the A3 test was preferable or comparable to that of LFI for crustacean shellfish and for processed grains, with the exception of wheat flour and buckwheat.

Comparison of cleaning validation performance of A3 test and protein swab test. The cleaning verification performance with stainless steel for practical use was successively compared between the A3 test and protein swab test. Stainless steel coupons were exposed to meat (raw chicken) and fish (raw salmon), which are recom-

mended to be labeled as allergenic ingredients in Japan (1, 17). They were washed three times, and square areas were swabbed with the A3 test and the protein swab test simultaneously at each step, after which the subsequent assays were carried out. As presented in Figure 2, the protein swab test that can detect as little as 50 μg of protein successfully indicated a positive (purple) result before washing; however, after an ineffective washing step (i.e., just a rinse with cold and hot water), it indicated that the surface was clean enough (green), although the washing was not complete. This result was in accordance with the data showing that the protein swab test hardly responded to raw chicken and salmon (Fig. 1A). In contrast, for the A3 test (presented in the graph in Fig. 2), the 200-RLU level, which is a typical benchmark value indicating a clean surface, was not achieved until complete washing with detergent and rinsing was performed.

Comparison of the assay data of the A3 test and ELISA for milk protein in the food production facility.

Successively, a field study in a food manufacturing plant was carried out to evaluate the practical performance of the A3 test. Namely, detection of milk protein by ELISA and A3 was examined after each cleaning step in the production facility for the seasoning mix that contained processed milk (e.g., cheese). The final product revealed 86 and 13 RLU after 10^5 -fold and 10^2 -fold dilutions by the A3 and ATP tests, respectively. The product was judged to be suspicious after 10^2 -fold dilution by the protein swab test. Thus, the A3 test exhibited several orders of magnitude higher detection sensitivity for the seasoning mix than did the ATP test and the protein swab test. Figure 3 shows that milk protein decreased through the washing steps; it was reduced from 10 to 40 μg before washing and was reduced to 0.1 μg or

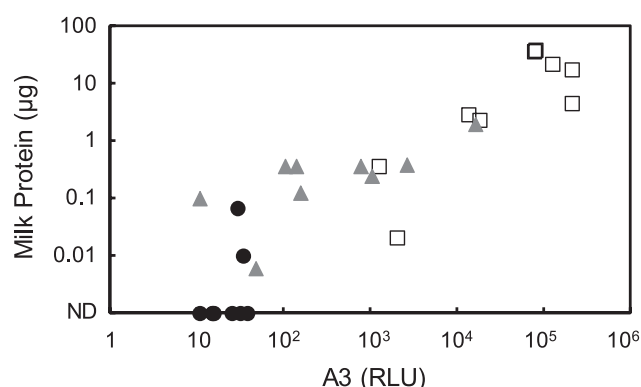


FIGURE 3. The correlation analysis between the A3 test and ELISA (for milk protein) in the food production facility. Before washing (open square), after a rinse with hot water (closed triangle), after complete washing using an alkaline cleaner and a rinse with hot water (closed circle).

less after the final wash. Thereafter, the A3 test indicated 10^3 to 2×10^5 RLU before washing and less than 100 RLU after the final wash. Accordingly, the A3 test worked effectively as an allergen monitoring tool after washing in the commercial food facilities.

DISCUSSION

In this study, a conventional ATP test from the same manufacturer was chosen to compare the sensitivity of food detection between the ATP and A3 assays because it has been demonstrated that the same amount of ATP resulted in similar RLU measurements (3). Figure 1 (red and black) demonstrated that ADP and/or AMP are the predominant adenylates over ATP in many allergenic foods. To be sure, there are various ATP swab tests from different manufacturers that can present higher RLU values for the same amount of ATP (3). However, because the concentrations of the A3 in many allergenic foods were higher by several orders of magnitude than that of the ATP, the ATP test needs to exhibit orders of magnitude higher sensitivity to become competitive to the performance of the A3 test. The protocol of the evaluation of the detection limit in this study seems appropriate for checking the performance of other ATP tests. Note that information about the typical benchmark value for each device and validation of the linear calibration curves for pure ATP at low levels around the benchmark values are likely essential (2).

From an operating perspective, the protein swab test was easy to use because it did not require a measurement instrument; it was preferred for screening allergens because they are proteins. On the other hand, note that it takes ca. 10 min for judging, and the data collected are of limited use because it is a qualitative test. Figure 1 (purple) and Figure 2 suggested that the detection sensitivity of a swab test that can detect as little as 50 µg of protein seems insufficient. Many commercial total protein swab tests are available, and some of them are able to detect more than 10-fold less protein. Therefore, such highly sensitive protein swabs may show better sensitivity than the A3 test not only for gelatin but also for some dairy, egg, and wheat products.

LFI is known as the most specific and often the most sensitive method among the allergen monitoring tests used on-site (13). Because detection by LFI is achieved by binding of target proteins and antibodies, any changes in the binding properties of the target proteins will influence the assay results (13). Therefore, in general, the hydrolysis or denaturation of allergenic proteins through metabolism, thermal processing, or exposure to chemical agents may affect the accuracy of LFI (8, 13–15). LFI that was used in this study showed stable results for both raw materials and processed foods, especially for dairy (Fig. 1, blue). Because the A3 test can detect not only ATP but also its decomposition products (ADP+AMP) simultaneously, it seems to be effective in detecting debris from both ingredients and processed foods with high sensitivity (3, 19). In fact, the detection limits of the A3 test for yogurt, cheese, ice cream, and bread were better than that for milk and wheat flour, presumably due to the increase in the ADP and/or AMP via processing or the additions of other ingredients. This test also has other benefits. Because LFI cannot be used for several common allergenic foods, such as meat and fish (5), the A3 test is useful for screening such food debris. Accordingly, the A3 test looks suitable for the verification of the sanitation standard operating procedure (SSOP) for almost all allergenic foods. The stainless steel model tests in the previous study (3) and this study (Fig. 2) also support this conclusion.

However, because the A3 test does not directly detect allergen protein, it needs to be judiciously applied for practical allergen monitoring in food manufacturing facilities. According to the Food Allergy Research and Resource Program (FARRP) at the University of Nebraska, LFI, often used in combination with quantitative ELISA testing of finished products, is recommended to validate allergen control approaches (5). Then, SSOP for each processing line and each formulation should be developed and validated by employing qualitative ELISA or the coupling of LFI with visual inspection. FARRP also advocates that visual inspections, the ATP test, or the protein swab test can be used to verify the appropriate application of SSOP each time once it is validated (5). The correlation study between the A3 test and quantitative ELISA for a food manufacturing facility (Fig. 3) is an example of determination of the benchmark value for the A3 test to judge whether the SSOP was applied appropriately. Figure 3 suggests that 100 RLU seems appropriate as the benchmark value in this facility because it could be achieved after complete washing, although it was hardly achievable after incomplete washing. As a result, the cleaning level of 0.1 µg of milk protein contamination in a surface (10 by 10 cm) can be monitored there. This result supported that the A3 was an effective marker in verifying the SSOP in this facility. If ELISA or LFI tests for target allergen are not commercially available, a general quantitative protein assay, such as the Bradford assay, may be applied for validating the A3 test. Note these two points: First, denaturation of proteins through thermal processing, or exposure to chemical agents, may affect the solubility of protein and the accuracy of the protein assay. Second, the standard (BSA) is not necessarily correlated with the protein in the target samples. Preferably, the

benchmark value for each manufacturing line should be set individually through validation because the state of the surfaces being tested varies (including type of material, age, moisture, scratches, etc.) and the formulation containing allergens is different.

Although validation and verification of cleaning is essential for the prevention of allergen cross-contact, the amount of protein that should be targeted in sanitation verification has not been clear. On the other hand, for example, Japanese food regulations set mandated allergen labeling thresholds at more than 10 µg of soluble allergen protein per g in finished product (1, 17). Note that detectable allergens may not always be found in finished product, whereas positive test results may be found on equipment areas that are difficult to clean (5). Large volumes of product flow down a manufacturing line in several processing facilities. The residues remaining on the equipment would be considerably diluted by the subsequent product. Therefore, finished product testing can often reveal that occasional positive swab test results on the equipment surfaces are still tolerable. Undoubtedly, more attention should be given to the risk of unexpected contamination of particulates containing allergens. Accordingly, monitoring allergens in finished products also seems to be important to validate the allergen control approaches as recommended by FARRP. Note that the A3 test is not applicable to test allergens in the finished products because it is not specific for allergens.

In the food industry, residual food debris is also recognized as a hazard that may cause foodborne illness. Food residues on surfaces are the source of nutrients for microorganisms. Moreover, it is known that organic matter can interfere with the antimicrobial activity of disinfectants in at least two ways (16). In general, this interference occurs due to a chemical reaction between the germicide and the organic matter, resulting in a complex that is less germicidal, reducing the active germicide available for disinfection and sterilization or totally eliminating germicidal activity. Alternatively, the organic material can protect microorganisms from the germicide by acting as a physical barrier. Bacteria including opportunistic pathogens can adhere to, be protected by, proliferate, and form biofilms within any residual food debris. Therefore, the A3 test, which can indicate inadequate cleaning, is useful for monitoring risks for allergen cross-contact and foodborne illness.

In summary, the amounts of A3 in many allergenic foods were shown to be larger by several orders of magnitude than those of ATP. In this study, the detection limits for allergenic food using the A3 and ATP tests, the protein swab test, and LFI were indicated. In this study, LFI showed better sensitivities than the A3 test for many foods; on the other hand, the A3 test is faster and less expensive. It is easier to record A3 test results, and the A3 test can monitor food allergens for which the LFI method is not commercially available. Therefore, the A3 test is useful to validate and verify the effectiveness of cleaning programs for the control of allergen cross-contact.

ACKNOWLEDGMENT

We appreciate Saitama Kikkoman Company for their great cooperation in providing field study data by allowing the use of their manufacturing facilities for this research.

SUPPLEMENTAL MATERIAL

Supplemental material associated with this article can be found online at: <https://doi.org/10.4315/JFP-20-017.s1>

REFERENCES

- Allen, K. J., P. J. Turner, R. Pawankar, S. Taylor, S. Sicherer, G. Lack, N. Rosario, M. Ebisawa, G. Wong, E. N. C. Mills, K. Beyer, A. Fiocchi, and H. A. Sampson. 2014. Precautionary labelling of foods for allergen content: are we ready for a global framework? *World Allergy Organ. J.* 7:10.
- Bakke, M., K. Sakurai, N. Tanaka, W. Saito, and K. Nishimoto. Validation study of LuciPac™ A3 surface for hygiene monitoring through detection of ATP, ADP, and AMP from stainless steel surfaces: AOAC Performance Tested MethodSM 051901. *J. AOAC Int.* in press.
- Bakke, M., and S. Suzuki. 2018. Development of a novel hygiene monitoring system based on the detection of total adenylate (ATP+ADP+AMP). *J. Food Prot.* 81:729–737.
- Bakke, M., S. Suzuki, E. Kiriara, and S. Mikami. 2019. Evaluation of the total adenylate (ATP+ADP+AMP) test for cleaning verification in healthcare settings. *J. Prev. Med. Hyg.* 60:E140–E146.
- Baumert, J. L., and S. L. Taylor. 2013. Best practices with allergen swabbing. *Food Saf. Mag.* Available at: <https://www.foodsafetymagazine.com/magazine-archive1/junejuly-2013/best-practices-with-allergen-swabbing/>. Accessed 6 January 2020.
- Bedford, B., Y. Yu, X. Wang, E. A. Garber, and L. S. Jackson. 2017. A limited survey of dark chocolate bars obtained in the United States for undeclared milk and peanut allergens. *J. Food Prot.* 80:692–702.
- Berin, M. C., and H. A. Sampson. 2013. Food allergy: an enigmatic epidemic. *Trends Immunol.* 34:390–397.
- Cao, W., D. Watson, M. Bakke, R. Panda, B. Bedford, P. S. Kande, L. S. Jackson, and E. A. E. Garber. 2017. Detection of gluten during the fermentation process to produce soy sauce. *J. Food Prot.* 80:799–808.
- Croote, D., and S. R. Quake. 2016. Food allergen detection by mass spectrometry: the role of systems biology. *NPJ Syst. Biol. Appl.* 2:16022.
- Gendel, S. M., N. Khan, and M. Yajnik. 2013. A survey of food allergen control practices in the U.S. food industry. *J. Food Prot.* 76:302–306.
- Helfe, S. L., J. Nordlee, and S. L. Taylor. 1996. Allergenic food. *Crit. Rev. Food Sci. Nutr.* 36:69–89.
- Holzhauser, T. 2018. Protein or no protein? Opportunities for DNA-based detection of allergenic foods. *J. Agric. Food Chem.* 66:9889–9894.
- Jackson, L. S., F. M. Al-Taher, M. Moorman, J. W. DeVries, R. Tippet, K. M. Swanson, T. J. Fu, R. Salter, G. Dunaif, S. Estes, S. Albillos, and S. M. Gendel. 2008. Cleaning and other control and validation strategies to prevent allergen cross-contact in food-processing operations. *J. Food Prot.* 71:445–458.
- Panda, R., H. F. Zoerb, C. Y. Cho, L. S. Jackson, and E. A. E. Garber. 2015. Detection and quantification of gluten during the brewing and fermentation of beer using antibody-based technologies. *J. Food Prot.* 78:1167–1177.
- Parker, C. H., S. E. Khuda, M. Pereira, M. M. Ross, T. J. Fu, X. Fan, Y. Wu, K. M. Williams, J. DeVries, B. Pulvermacher, B. Bedford, X. Zhang, and L. S. Jackson. 2015. Multi-allergen quantitation and the impact of thermal treatment in industry-processed baked goods by ELISA and liquid chromatography-tandem mass spectrometry. *J. Agric. Food Chem.* 63:10669–10680.
- Rutala, W. A., and D. J. Weber. 2016. Monitoring and improving the effectiveness of surface cleaning and disinfection. *Am. J. Infect. Control* 44:e69–76.

17. Shoji, M., R. Adachi, and H. Akiyama. 2018. Japanese food allergen labeling regulation: an update. *J. AOAC Int.* 101:8–13.
18. Sicherer, S. H., and H. A. Sampson. 2018. Food allergy: a review and update on epidemiology, pathogenesis, diagnosis, prevention, and management. *J. Allergy Clin. Immunol.* 141:41–58.
19. Smith, N. W., J. J. Sindelar, and S. A. Rankin. 2019. Quantities of adenylate homologues (ATP+ADP+AMP) change over time in prokaryotic and eukaryotic cells. *J. Food Prot.* 82:2088–2093.
20. U.S. Food and Drug Administration. 2013. Food labeling; gluten-free labeling of foods. 21 CFR Part 101, docket FDA-2005-N-0404, RIN 0910-AG84. *Fed. Regist.* 78:47154–47179. Available at: <http://www.gpo.gov/fdsys/pkg/FR-2013-08-05/pdf/2013-18813.pdf>. Accessed 6 January 2020.
21. U.S. Food and Drug Administration. 2018. FSMA final rule for preventive controls for human food. Available at: <https://www.fda.gov/food/guidanceregulation/fsma/ucm334115.htm>. Accessed 6 January 2020.
22. Wang, X., O. A. Young, and D. P. Karl. 2010. Evaluation of cleaning procedures for allergen control in a food industry environment. *J. Food Sci.* 75:T149–T155.