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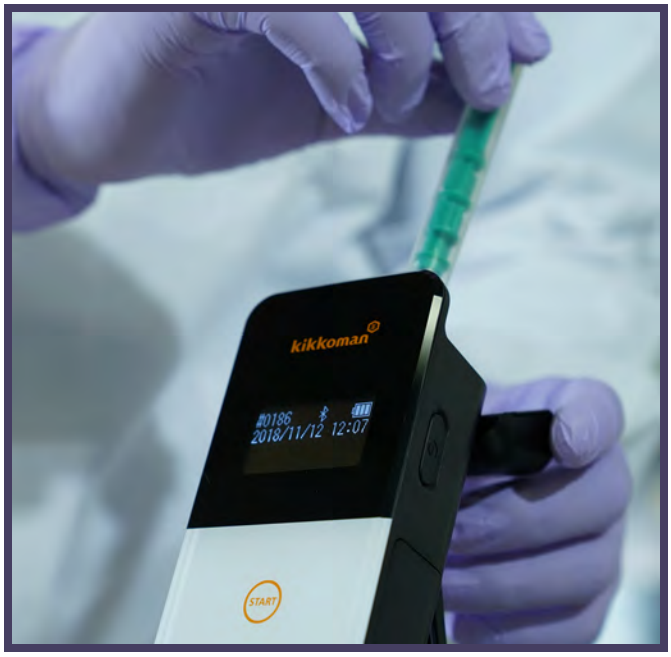

Sanitation Monitoring of Stainless Steel Surfaces with a Test for Total Adenylates

ABSTRACT

ATP rapid hygiene monitoring tests are useful for the implementation of hazard analysis critical control point systems and hazard analysis risk-based preventive controls programs. The removal of food residues on surfaces after washing is essential because these residues can promote microbial growth and present a risk for contamination of foods with allergens. However, conventional ATP tests may fail to detect food residues on surfaces because of degradation of ATP to ADP and AMP. Recently, an ATP-ADP-AMP (A3) test has been used as a powerful tool for revealing inadequate cleaning processes and the subsequent presence of contamination. In this study, the A3 test and three commercially available ATP tests were evaluated in a simulated sanitation monitoring situation with stainless steel coupons. For ham (1,000-fold dilution), the A3 test results were 157,389 relative light units (RLU) and the other commercially available ATP tests results were 0 to 62 RLU. The results of other foods are as follows: raw chicken (100-fold dilution), 15,872 RLU (A3) and 20 to 173 RLU (ATP); beer (10-fold dilution), 10,777 RLU (A3) and 0 to 200 RLU (ATP); yogurt (100-fold dilution), 18,371 RLU (A3) and 911 to 3,104 RLU (ATP); and orange juice (1,000-fold dilution), 4,568 RLU (A3) and 615 to 1,995 RLU (ATP). Therefore, the A3 test is a more accurate tool for the verification of hygiene levels.

INTRODUCTION

Cleanliness of food contact surfaces is fundamental for food safety. In particular, accumulation of food residues due to inadequate cleaning is a known risk factor for foodborne illnesses. Food residues can contain pathogens *(9)*, become a source of nutrients for microbial growth *(10)*, and interfere with effective sanitation *(11, 15)*. Food residues derived from foods known to cause allergies may cross-contaminate nonallergenic foods or ingredients produced in the same facility or on the same processing line *(6)*. Therefore, interest in the assessment of cleanliness includes a concern for bacterial contamination and organic debris.

For the implementation of hazard analysis critical control point systems and hazard analysis risk-based preventive

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control programs, hygiene monitoring to provide rapid and timely results is required so corrective actions can be taken immediately. Visual assessment alone is not accurate and reliable even though it is economical *(5, 6)*. Traditional bacterial cultures are often used for sanitation evaluation. However, culture incubation takes a few days, precluding immediate feedback and timely action. Protein swab tests and lateral flow immunoassays are quick and simple methods for identification of food debris and allergens. The swab-based protein test can be used to detect food residues and a broad range of allergens, but these tests are qualitative and nonspecific and may not be effective with low levels of residual protein contamination *(7)*. Lateral flow immunoassays can be used to detect allergenic foods qualitatively and selectively with high sensitivity and thus are suitable for the control of allergens but are not appropriate for monitoring organic debris that does not contain the target proteins *(6)*. Hygiene monitoring tests based on the presence of ATP have also been widely used because of their speed and simplicity. ATP is the energy molecule found in animals, plants, and microorganisms. Although ATP tests are nonspecific, they can reveal the presence of food debris and bacteria, indicating a risk of bacterial proliferation and cross-contact with allergens. ATP measurements is based on the firefly luciferase reaction in which light is produced from luciferin and ATP. The amount of light produced is proportional to the amount of ATP in a sample and can be quantified by measuring the light with a luminometer and is expressed as relative light units (RLU). Benchmark values are set to determine baseline cleanliness in a facility, and these benchmark values are important guidelines for the evaluation of cleanliness. Cleanliness is designated as "pass" when the measured RLU fall below the benchmark value and as "fail" when the RLU are above the benchmark. When the test indicates a failing result, the recommendation is to reclean the test site, revise the cleaning procedures, or replace the failed equipment.

However, conventional ATP test systems can be inefficient because ATP is degraded to ADP and AMP by heat, acids, or alkalis, and enzymes *(3)*. Raw meat and fish contain mainly ADP, regardless of species *(3)*, and ADP is the predominant adenylate in bovine, porcine, and poultry carcasses *(14)*. In contrast, raw whole eggs, shellfish (shrimp, oysters, scallops, and abalone), and processed meats and seafood (sausage, bacon, beef jerky, canned fish, and dried fish) contain large amounts of AMP *(3)*. AMP is also found abundantly in many plant seeds and nuts, which are widely known as allergens *(3)*. Hence, conventional ATP tests that indicate only ATP concentrations might fail to detect other food residues and would fail to identify inadequately cleaned surfaces.

A novel ATP-ADP-AMP (A3) test has been developed based on a luciferin-luciferase assay with advanced enzyme chemistry *(3, 4, 12)*. In the luciferase reaction, ATP is degraded into AMP, which then inhibits luciferase, resulting in a decrease in light production. To detect AMP simultaneously and maintain light production, ATP is regenerated from AMP with the pyruvate orthophosphate dikinase reaction *(3)*. ADP is then converted to ATP by a pyruvate kinase reaction. A device to simultaneously detect ATP, ADP, and AMP has been successfully developed. The limit of detection for each adenylate is ca. 2.5 fmol *(2)*. This A3 test has been validated for the detection of ATP, ADP, and AMP on stainless steel surfaces and has been approved by the Association of Official Analytical Collaboration with a performance tested method certification *(2)*. This A3 test is expected to provide for a more sensitive and reliable indicator of cleanliness than the conventional ATP test.

The protocol of an ATP test consists of sample collection by swabbing, extraction of the swab material, a bioluminescence reaction, and an instrument-based detection of light. In our previous study comparing conventional ATP tests and the A3 test, diluted food samples were pipetted onto each swab *(3, 12)*. Further comparisons that include swabbing efficacy are also important because this essential component of the sampling phase is also affected by the variability of the performance of the swabbing devices and moistening agents. In the present study, swabbing assays of food residues on stainless steel coupons were carried out to compare the sensitivity of the A3 test and conventional ATP tests.

FIGURE 1. The measurement of swabbing pressure on the scale pan of the analytical balance using the A3 test swab. When the swab shaft slightly bent, the balance showed approximately 70–100 g.

MATERIALS AND METHODS

A3 and ATP tests

The A3 test was performed with a commercially available test kit (LuciPac A3 Surface/Lumitester PD-30, Kikkoman Biochemifa, Tokyo, Japan) according to the manufacturer's directions. The three ATP tests used in this study were also commercially available and were performed in accordance with manufacturers' instructions. The measurements in all tests were recorded in RLU.

TABLE 1. Luminescence intensity for pure adenylates obtained with the A3 test and three conventional ATP tests[a]

Test	ATP			ADP			AMP		
	Mean (RLU)	SD_r (RLU)	RSD_r (%)	Mean (RLU)	SD_r (RLU)	RSD_r (%)	Mean (RLU)	SD_r (RLU)	RSD_r (%)
A3	1,615	143	8.9	1,699	95	5.8	1,633	158	7.6
ATP-A	907	73	8	0	0		0	0	0
ATP-B	5,209	1,408	27	0	0		0	0	3
ATP-C	6,198	1,633	26.3	16	3	15.9	15	3	14

[a]ATP, ADP, and AMP dilutions were 10^{-12} mol per assay. Mean values were obtained from 10 measurements. SD_r, standard deviation of repeatability; RSD_r, relative standard deviation of repeatability.

Luminescence intensity for pure adenylates

ATP·2Na, ADP·K, and AMP·2Na were purchased as analytical grade chemicals from Oriental Yeast (Tokyo, Japan). According to the certificates of analysis that accompanied these products, the purity of ATP, ADP, and AMP were 99.9, 96.1, and 99.9%, respectively. The concentrations of ATP, ADP, and AMP excluding salt and hydrated water (A) were 86.3, 84.2, and 82.7%, respectively. Formula molecular weight (MW) of ATP, ADP and AMP as anhydrous free acid were 507.2, 427.2, and 347.2, respectively. The amounts of ATP, ADP, and AMP powder required to prepare 10 mL of 10^{-2} M solutions were calculated with the following formula: 10 × A/MW. Hence, 58.8 mg of ATP·2Na, 50.7 mg of ADP·K, and 42.0 mg of AMP·2Na were dissolved in 10 mL of nuclease-free water to prepare each 10^{-2} M adenylate solution. ATP, ADP, and AMP solutions (1×10^{-7} M and 1×10^{-8} M) were then prepared by dilution with nuclease-free water.

ATP, ADP, and AMP solutions (1×10^{-12} mol per assay) or nuclease-free water (control) were pipetted onto swabs of the A3 test and the ATP tests. For the A3 test, 100 µL of 1×10^{-8} M solutions or water were pipetted onto the dry swabs. For the ATP tests, 10 µL of 1×10^{-7} M solutions or water were also pipetted onto the premoistened swabs included with the kits. Measurements were repeated 10 times for each aliquot and test. The mean, standard deviation of repeatability (SD_r), and relative standard deviation of repeatability (RSD_r) were reported, where

$$SD_r = \sqrt{\frac{\sum_{t-1}^{x}(X_t - X)^2}{n-1}}$$

$$RSD_r = \frac{SD_r}{\text{mean}} \times 100$$

Swabbing assays with stainless steel coupons

Raw chicken, ham, yogurt, beer, and orange juice samples used for the tests were purchased from a market. Solid foods were blended with nine parts of water. Successive serial 10-fold dilutions were made for all samples. Each diluted solution (250 µL) was spread over a square stainless steel (SUS304) coupon (10 by 10 cm) and dried at 30°C for 30 min. Twelve replicate coupons with each food sample were prepared for three repeated assays of the four tests. The swabs of the A3 test were moistened with nuclease-free water before use. The premoistened packaged swabs were used for the other ATP tests. The stainless coupons were swabbed with suitable pressure bending the swab shaft slightly *(Fig. 1)*. Each coupon was swabbed 10 times vertically and horizontally, and the RLU results were reported as the mean, SD_r, and RSD_r.

RESULTS AND DISCUSSION

Luminescence intensity for pure adenylates

The various brands of ATP tests have produced different results even with the same amount of ATP *(3)*. In the present study, the RLU results of the A3 test and the three ATP tests (ATP-A, ATP-B, and ATP-C) for nuclease-free water and ATP were compared *(Table 1)*. Almost no signal for water was detected by any of the devices as expected. For 1×10^{-12} mol ATP, the three ATP tests produced readings of 907, 5,209, and 6,198 RLU, respectively, and the A3 test produced 1,615 RLU. The differences in the readings were likely due to differences in the reagents, light detection systems, and/or scale units used by the different manufacturers for presenting the results *(1, 8)*.

ADP and AMP were also analyzed with the A3 test and the three ATP tests *(Table 1)*. The A3 test produced 1,699 and 1,633 RLU for 1×10^{-12} mol dilutions of ADP and AMP, respectively. Thus, the A3 test detected all three adenylates at equal concentrations, based on the almost equal RLU readings. In contrast, the conventional ATP tests produced readings

TABLE 2. Evaluation of swabbing efficacy for swabbing tests through measurements by application of foods onto swabs or swabbing foods on stainless coupons

Analyte	Assay method	Amount (µg)		A3[a]	ATP-A[b]	ATP-B[b]	ATP-C[b]
Ham	Pipetting[d]	10	Mean RLU[f]	8990	0	0	32
(1000-fold)[c]	Swabbing[e]	250	Mean RLU[g]	157389	0	13	62
			s_r[h]	3099	0	18	9
			RSD_r, %[i]	2.0	-	141.4	14.9
			Recovery, %[j]	70	-[k]	-[k]	8
Raw chicken	Pipetting	100	Mean RLU	101	1	10	37
(100-fold)	Swabbing	2500	Mean RLU	15872	20	40	173
			s_r	987	4	15	84
			RSD_r, %	6.2	18.0	38.3	48.6
			Recovery, %	632[l]	160[l]	16	19
Beer	Pipetting	1000	Mean RLU	737	0	0	32
(10-fold)	Swabbing	25000	Mean RLU	10777	0	200	29
			s_r	1306	0	3	10
			RSD_r, %	12.1	-	1.3	35.0
			Recovery, %	59	-[k]	-[k]	4
Yogurt	Pipetting	100	Mean RLU	465	379	18	608
(100-fold)	Swabbing	2500	Mean RLU	18371	911	1829	3104
			s_r	1344	316	1329	912
			RSD_r, %	7.3	34.7	72.6	29.4
			Recovery, %	158[l]	10	418[l]	20
Orange juice	Pipetting	10	Mean RLU	572	191	205	687
(1000-fold)	Swabbing	250	Mean RLU	4568	615	862	1995
			s_r	1201	153	795	1136
			RSD_r, %	26.3	24.8	92.3	56.9
			Recovery, %	32	13	17	12

[a]The A3 test.

[b]Three commercially available ATP tests.

[c]Dilution factors.

[d]Aliquots of 10 µL of each food were applied onto the swabs by pipetting and measurements were carried out.

[e]Aliquots of 250 µL of each food were spread over a square stainless steel coupon with each side measuring 10 cm and dried completely. Then, each area was swabbed 10 times vertically and horizontally and measurements were carried out.

[f]Measurements were repeated twice.

[g]Measurements were repeated 3 times.

[h]Standard deviation of repeatability.

[i]Relative standard deviation of repeatability.

[j]Swabbing recovery rates (%) were calculated using the formula: Swabbing Mean RLU / (Pipetting Mean RLU × 25) × 100.

[k]These swabbing recovery rates could not be calculated because the measurement values in pipetting assays were 0 RLU.

[l]These swabbing recovery rates were over 100% probably because additional AMP was generated by degradation of RNA through the dryness on the stainless steel coupon in swabbing assays *(13)*. Another reason may be that cells were injured by drying, and more adenylates were extracted.

of 0 to 16 RLU for ADP and AMP, which were almost the same as those for nuclease-free water. Hence, this comparison confirmed that the A3 test detected all three adenylates whereas the conventional ATP tests detected only ATP. These results were expected because the conventional ATP tests were based on the firefly luciferase reaction, which utilizes only ATP; however, the A3 test combined this reaction with enzymatic conversions of ADP and AMP to ATP.

Swabbing assays with stainless steel coupons

To evaluate the performance of the A3 test and the three ATP tests, stainless steel coupons with fixed amounts of dried food were prepared, and swabbing assays were conducted with all four tests *(Table 2)*. For ham (1,000-fold dilution), the A3 test result was 157,389 RLU and the commercially available ATP test results were 0 to 62 RLU; thus, these tests could not sensitively detect ham residues. For raw chicken (100-fold dilution), the A3 test result was 15,872 RLU and the other ATP test results were 20 to 173 RLU. The results seem reasonable because in previous studies the dominant adenylates were ADP in raw meat and AMP in many processed meats *(3, 14)*. For beer (10-fold dilution), the A3 test result was 10,777 RLU and the other ATP test results were 0 to 200 RLU, which can be explained by the fact that the dominant adenylate present in beer is AMP *(3)*. These data suggest that the A3 test is more sensitive for detecting food residues, and the conventional ATP tests failed to detect these residues that remained after inadequate washing. These results indicate that the A3 test could be applied for detection of other food residues in which the dominant adenylates are ADP and/or AMP, such as seafood, eggs, and plant seeds, which are known allergenic foods *(3)*.

The ATP tests were better able to detect residues of yogurt (100-fold dilution) and orange juice (1,000-fold dilution) because these foods contain more ATP *(3)* *(Table 2)*. However, the A3 test was still the most sensitive tests for detecting these food residues.

Food residues on surfaces can be a source of pathogens, food spoilage organisms, and allergens. However, conventional ATP tests used for verification of cleaning procedures may falsely indicate that a surface is clean despite the presence of food debris. In our study, the A3 test detected food residues on stainless steel surfaces that the conventional ATP tests failed to detect. Because a wide variety of foods contain significantly more ADP and/or AMP than ATP, the A3 test is a better method than conventional ATP tests for rapid monitoring of the hygiene of food contact surfaces.

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