Use of a Sensitive Adenosine Triphosphate Method to Quickly Verify Wet Cleaning Effectiveness at Removing Food Soils and Allergens from Food Contact Surfaces

Robert S. Salter[1], Sue Hefle[2], Lauren S. Jackson[3], Amy McBroom[4] and Katherine M. J. Swanson[4]

1. Charm Sciences, Inc., 2. Food Allergy Research and Resource Program (FARRP), University of Nebraska, 3. Food and Drug Administration- National Center for Food Safety and Technology (NCFST), 4. Ecolab, Inc.

Introduction

Rapid methods are needed to verify that food soils are removed during equipment cleaning to prevent cross contact of foods with allergens. The objectives of this work were to evaluate and compare a sensitive adenosine triphosphate (ATP) swab (AllerGiene®) and a conventional ATP swab (Pocketswab®) to the results obtained with protein-specific allergen (ELISA) kits to determine if the ATP systems could be used as an indicator test for verifying the effectiveness of wet cleaning operations for removing allergenic milk proteins. Three types of milk products were evaluated in this preliminary study to determine feasibility. The study design was divided into three parts:

1. Evaluate the sensitivity of ATP tests and allergen-specific tests with three different milk solutions.
2. Measure the ability of ATP and ELISA kits to detect dried milk residues on dirty, partially cleaned and cleaned stainless steel plates.
3. Determine the ability of ATP tests and allergen-specific tests to detect milk soils in a dairy processing (ice cream) operation.

Materials and Methods

Pocketswab® (conventional ATP test), AllerGiene® (sensitive ATP test) and LumGiene™ analyzer were made by Charm Sciences, Inc, Lawrence, MA 01843. Allergen protein-specific tests included Neogen Alert® Total Milk Protein (Lansing, MI), ELISA Systems qualitative casein™ (Queensland, Australia), and FARRP in-house pecan and walnut ELISA tests.

Part 1. ATP Solutions: ATP (Sigma, code FLAAS, St. Louis, MO) solutions were prepared with ATP-free water (Charm Sciences Inc.) in autoclaved glassware. The 10 ppm ATP solution concentration was verified at OD259nm. Femtomoles reported were per 35ul solution absorbed in swab.

Milk Solutions: Solutions were made with ATP-free water in 15 mL plastic conical tubes:

- NIST SRM 1549 skim milk powder diluted to concentrations of 1-1000 ppm
- ZCS powder whole milk (Charm Sciences, Inc.)- reconstituted 12.5g/100ml, then further diluted to concentrations of 1- 1000 ppm.
- Locally purchased whole pasteurized milk was frozen for use throughout the study by all labs. The thawed milk was diluted to 1-1000 ppm.

Part 2. Experiments of milk soils on stainless steel plates (6˝ x 6˝) were done with whole pasteurized milk diluted in ATP-free water. The milk solutions (0.75 mL) were pipetted on the surface and dried at 88ºC for 1.5 h. Spots were swabbed simultaneously with conventional and sensitive ATP swabs and with a polyester swab moistened in PBS buffer. The PBS swab was placed in sterile tube containing 1.1 mL of water. The swab solution was split for ELISA analyses according to kit instructions. In other experiments, undiluted whole milk (0.75 mL) was pipetted onto the surface of the stainless steel plates and dried at 88ºC for 1.5 h. The plates were washed in a 100 gallon COP (clean out of place) tank with 60 gallons water or chlorinated alkali detergent (Exxcelerate, Ecolab, Inc.) for different times/temperatures. After washing, plates were rinsed with room temperature city water, then ATP-free water prior to swabbing. Plates were scored visually using scale ranging from 0 (no milk residue remaining) to 5 (no residue removed).

Part 3. An ice cream plant was evaluated and three cleaning processes were identified for testing:

- The freezer was hand cleaned between ice cream flavors using Alkaline Detergent Powder (A&L Formula 22, A&L Laboratories, Inc)
- The pasteurizer was semi-CIP cleaned using chlorinated alkaline cleaner (Exxelerate, Ecolab Inc.), blended acid detergent (AC-55-5 Red, Ecolab Inc.) and low foaming acid sanitizer (Mandate, Ecolab Inc.) solutions at recommended use concentrations.
- The hold tank was CIP cleaned with chlorinated alkaline cleaner (Exxelerate).

The cleaning processes were evaluated at each step of the cleaning process. Equipment locations were simultaneously swabbed with sensitive ATP, conventional ATP and PBS moistened swabs for protein-specific allergen tests. Subsequent cleaning was performed until sensitive ATP test results were 0 RLU (Relative Light Units) or met background ATP levels of COP cleaning. PBS swabs used in ELISA tests were frozen for subsequent analysis.

Results and Discussion

Part 1. Detection limits of ATP tests and ELISA assays

1. ATP RLU were converted to a decision positive or negative based on RLU exceeding a limit. The most sensitive "zero" limit was applied for sensitivity determination.
2. The detection limits for ATP in solution using the sensitive ATP test and conventional ATP test were 0.05-0.1 femtomoles (fmol) and 2 fmol, respectively (data not shown). At 0.1 fmol, average RLU was 102500 with a CV of 125% as determined in two independent measurements (n=10).
3. The sensitive ATP test detected the three milk products at concentrations that caused positive results with both ELISA kits and there were no positive ELISA results at concentrations tested negative with the sensitive ATP test (Table 1).
4. The conventional ATP test was not able to detect the milk products at concentrations that caused positive results with the ELISA kits (Table 1).
5. The ATP tests had variable results with CVs of 300% at concentrations near and below limits of detection (LOD), defined as the lowest concentration with 100% positive (Table 1). At these concentrations ATP detected were very close to instrument background causing large variation. Typical CVs above the LOD were 20 to 40%.
6. The sensitive ATP test was able to detect reconstituted ZCS milk solutions at lower concentrations (2 and 4 ppm) than solutions of fluid whole milk (Table 1). The reason for this higher sensitivity was not determined and may be an anomaly.
7. It is important to note that the sensitive ATP test is not a direct measure of the presence of allergenic milk proteins. It may not detect the presence of some milk-based products that contain low ATP levels, e.g. fat powder ingredient containing casein. Thus, it is important to determine if a specific food contains detectable levels of ATP when implementing an ATP cleaning verification program.

Table 1: Sensitivity of ATP and Specific Allergen Tests for Milk Products in Solution.

Sample	Concentration (ppm)	sensitive ATP mean RLU (CV%)	sensitive ATP #pos./#tested	conventional ATP mean RLU (CV%)	conventional ATP #pos./#tested	Milk Protein ELISA (ppm)	Casein ELISA (ppm)
NIST	1000			27597 (30%)	**10/10**		
Skim	100	4930886 (26%)	**3/3**	5315 (15%)	**10/10**	**>10ppm**	**>5ppm**
Milk	80			3400 (15%)	**10/10**		
Powder	60			2617 (46%)	9/10		
(ppm=ug/L)	40			1087 (79%)	**10/10**		
	20			55 (292%)	3/10		
	10	341184 (29%)	**10/10**	0 (0%)	0/10	**>10ppm**	**>5ppm**
	8	193334 (36%)	**10/10**			**>5ppm**	**>5ppm**
	6	871510 (249%)	**10/10**			**>5ppm**	**>5ppm**
	4	99602 (60%)	**10/10**			<5ppm	**>1ppm**
	2	66732 (41%)	**10/10**			<5ppm	**>1ppm**
	1	20208 (282%)	3/10			<5ppm	<1ppm
Reconstituted	1000			7684 (50%)	**10/10**		
ZCS	500			2721 (56%)	**10/10**		
Milk *	250			84 (366%)	1/10		
(ppm=uL/L)	100	458927 (9%)	**3/3**	0 (0%)	0/10	**>5ppm**	**>5ppm**
	10	149532 (302%)	6/10			<5ppm	<1ppm
	8	651553 (306%)	6/10			<5ppm	<1ppm
	6	3151 (195%)	3/10			<5ppm	<1ppm
	4	125870 (173%)	**10/10**			<5ppm	<1ppm
	2	29853 (79%)	9/10			<5ppm	<1ppm
	1	0 (0%)	0/10			<5ppm	<1ppm
Whole	1000			5579 (40%)	**10/10**		
Pasteurized	500			1121 (82%)	**10/10**		
Milk	250			229 (61%)	5/10		
(ppm=uL/L)	100	189512 (22%)	**3/3**	0 (0%)	0/10	**>5ppm**	**>5ppm**
	10	4087 (152%)	6/10			<5ppm	<1ppm
	8	582 (316%)	1/10			<5ppm	<1ppm
	6	511 (316%)	1/10			<5ppm	<1ppm
	4	39281 (309%)	3/10			<5ppm	<1ppm
	2	14763 (316%)	1/10			<5ppm	<1ppm
	1	8519 (238%)	2/10			<5ppm	<1ppm

* Reconstituted ZCS milk liquid ppm may be converted to powder ppm by multiplying concentration by factor 0.11.
Positive results are in bold. Positive ATP determinations based on RLU > 0. Allergen tests are based on triplicate results.

Part 2. Detection of the presence of milk soils on a stainless steel surface

1. Sensitive ATP test detected dried milk solutions on stainless steel surfaces at lower concentrations than ELISA kits (Table 2).
2. Conventional ATP test detected dried milk on stainless steel surfaces at the same concentrations as ELISA kits (Tables 2 & 3).
3. Both ATP tests detected dried whole milk on surfaces similar to their liquid solution sensitivity while detection limits for dried milk solutions with ELISA kits were higher (400 ppm; Table 2) than for liquid milk solutions (100 ppm; Table 1). Possible explanations could be reduced ELISA detection of milk proteins during heating/drying or reduced recovery of proteins from dried soils with the swabs.
4. Cleaning stainless steel plates with hot water (73.8˚C) or with chlorinated alkaline detergent solution (62.8˚C) for less than 3 min resulted in visual detection of milk soil and detection with ATP and ELISA tests (Table 3).
5. Background ATP levels, potentially caused by water quality or chemical detergent interference, resulted in sensitive ATP backgrounds as high as 878,000 RLU (Table 3). More research is needed to examine ATP sensitivity in the presence of non-food ATP background.

Table 2: Detection of whole pasteurized milk solutions dried on the surface of stainless steel plates with ATP and ELISA milk protein kits.

Frozen Whole Milk	Sensitive ATP (RLU)	Conventional ATP (RLU)	Milk Protein ELISA (ppm)	Casein ELISA (ppm)
25ppm	**76190**	0	<5 ppm	<1 ppm
50ppm	**124381**	0	<5 ppm	<1 ppm
100ppm	**165127**	0	<5 ppm	<1 ppm
200ppm	**633015**	0	<5 ppm	<1 ppm
400ppm	**1512897**	**1193**	**>5ppm**	**~1 ppm**
600ppm	**2405703**	**1879**	NT	NT
800ppm	**2759645**	**5505**	**>10 ppm**	**>1 ppm**

NT = not tested.
Positive results are in bold.
ATP tests are done in duplicate and therefore CV not shown. ELISA tests n=2 swabs tested in duplicate.

Table 3: ATP and Allergen-Specific Testing Following Surface Cleaning of Stainless Steel Plates.

Soil	Cleaning Solution	Temp. (°C)	Clean Time (min)	Sensitive ATP mean RLU (CV%)	Conventional ATP mean RLU (CV%)	Milk Protein ELISA (ppm)	Casein ELISA (ppm)	Visual Score
Whole Milk	none	-	0	**222255705 (14%)**	**370070 (20%)**	**>10ppm**	**>5ppm**	5
None	Cl Alk Det	62.8	5	207691 (163%)	0	< 5ppm	<1ppm	0
Whole Milk	Cl Alk Det	62.8	20	208329 (150%)	0	< 5ppm	<1ppm	0
Whole Milk	Cl Alk Det	62.8	5	192224 (44%)	0	NT	NT	0
Whole Milk	Cl Alk Det	62.8	3	1923168 (109%)*	7857 (222%)*	<5ppm	<1ppm	0
Whole Milk	Cl Alk Det	62.8	3**	**3160514**	**431**	**~10ppm**	**>5ppm**	1
Whole Milk	Cl Alk Det	62.8	1	**27005674 (58%)**	**10301 (59%)**	**>10ppm**	**>5ppm**	1-2
Whole Milk	Water	72.8	30	**20551349 (46%)**	**45273 (39%)**	**>10ppm**	**>5ppm**	2

* At 3 minutes there were 3 additional sensitive ATP positive and 2 conventional ATP positives with negative ELISA results.
** A replicate at 3 minutes with visible soil separately reported.
Positive results are in bold. ATP results are the average of 6 determinations. Maximum sensitive ATP RLU of cleaned plates was 878820. ATP RLUs were considered positive when they exceeded this background level.

Part 3. Evaluation of ATP vs. ELISA kits in food industry settings.

1. The speed of ATP testing (30 seconds), simplicity of use, and immediate feedback prompted additional cleaning efforts in the ice cream plant.
2. Visually clean equipment surfaces after butter pecan ice cream production have positive ATP results and below LOD results for ELISA tests (Table 4). Results after visual cleaning from maple walnut ice cream production were positive ATP and ELISA tests.
3. Following a standard cleaning procedure achieved 0 RLU with conventional ATP, but took three additional manual scrubbings to achieve 0 RLU by sensitive ATP test (Table 4).
4. Positive detection (2 out of 3 Total Milk Protein tests) in the final cleaning of the butter pecan ice cream run was not consistent with results obtained by the casein test (<1 ppm) or the prior negative results from 2 previous cleanings (Table 4). The reason for the differences in the results is not known, but might be due to different standards, antibodies or detection limits of the tests.
5. After manual and COP cleaning of freezer (Table 4) and CIP of the plate pasteurizer (data not shown), baseline sensitive ATP RLU values of <60,000 were obtained with negative results in ELISA tests. Further research is needed to determine if these RLU baseline data could be used as a benchmark (pass/fail limit) to establish the adequacy of the cleaning procedure in this production area.
6. A hot water rinse was not sufficient to remove soil in the hold tank as measured by the ATP and ELISA tests (Table 5).
7. After complete hold tank cleaning, sensitive ATP RLU was 0 when the wall was sampled and milk proteins were at concentrations below the detection limit of the ELISA tests (Table 5). The importance of selecting an appropriate sampling site after cleaning is exemplified by the wall (0 RLU) versus drain (positive with both ATP tests). The cleaning system would require additional study to determine if the drain is the most appropriate site to sample after cleaning.

Table 4: ATP and Allergen-Specific Tests for Ice Cream Freezer Cleaning.

Ice Cream Type	Cleaning Type	Sample site	Conventional ATP (RLU)	Sensitive ATP (RLU)	Milk Protein ELISA (ppm)	Casein ELISA (ppm)
Butter	Rinse VC S1	baffles	**1999**	**678413**	< 5 ppm	< 1 ppm
Pecan	Rinse VC S2	dasher	**1630**	**793149**	< 5 ppm	< 1 ppm
	Light Cleaning S1	connector	**32332**	**15235517**	< 5 ppm	**> 5 ppm**
	Light Cleaning S2	dasher	0	**340255**	< 5 ppm	< 1 ppm
	Complete Clean #1 S1	baffles	**7867**	**73667**	< 5 ppm	< 1 ppm
	Complete Clean #1 S2	chamber	0	14914	< 5 ppm	< 1 ppm
	Complete Clean #2 S1	baffles	0	**253459**	< 5 ppm	< 1 ppm
	Complete Clean #2 S2	connector	0	5396	< 5 ppm	< 1 ppm
	Complete Clean #3 S1	baffles	0	25304	**> 10 ppm***	< 1 ppm
	Complete Clean #3 S2	dasher	0	56332	< 5 ppm	< 1 ppm
	Complete Clean #4 S1	dasher	0	0	**> 10 ppm***	< 1 ppm
	Complete Clean #4 S2	baffles	0	0	< 5 ppm	< 1 ppm
Maple	Rinse VC S1	baffles	**479437**	**87935764**	**~ 5 ppm**	< 1 ppm
Walnut	Rinse VC S2	dasher	**689**	**61490**	< 5 ppm	< 1 ppm
	Light Cleaning S1	baffles	0	**135311**	< 5 ppm	< 1 ppm
	Light Cleaning S2	dasher	0	**152449**	< 5 ppm	< 1 ppm
	Final Manual Clean	chamber	0	0	< 5 ppm	< 1 ppm
	Final COP tank #1 S1	baffles	0	3550	< 5 ppm	< 1 ppm
	Final COP tank #1 S2	dasher	0	52872	< 5 ppm	< 1 ppm

VC= Visually Clean. BLD=Below Limit Detection.
* Two replicates of sample were >10ppm and one replicate was <5ppm.
Positive results are in bold. Two sampling sites were tested after each cleaning condition. Swabs for each allergen test were extracted and tested n=3. Sensitive ATP RLU < 60000 were not bolded representing a non-zero limit application based on collected data.

Table 5: ATP and Specific Allergen Testing of Hold Tank during CIP.

CIP Cycle	Conventional ATP (RLU)	Sensitive ATP (RLU)	Milk Protein ELISA (ppm)	Casein ELISA (ppm)
Water Rinse	**511450**	**283464732**	**>5-10 ppm**	**> 5 ppm**
Chlor. Alk cleaner	0	**10686**	< 5 ppm	< 1 ppm
After CIP Cycle from drain	**728**	**493804**	< 5 ppm	< 1 ppm
After CIP Cycle from Wall	0	0	< 5 ppm	< 1 ppm

Positive results are in bold. Sampling sites were the tank wall and the drain. ATP results are the average of 2 determinations while ELISA results are the average of two swabs tested in triplicate.

Conclusions

1. The sensitive ATP test detected milk solutions that tested positive with ELISA tests.
2. The conventional ATP test did not detect milk solutions that tested positive with ELISA tests.
3. The sensitive ATP test detected milk residues on wet cleaned surfaces at levels detected by milk specific ELISA tests.
4. ATP detection is not specific to allergens, but is an indication of soil presence.
5. The sensitivity, speed (30 seconds) and simplicity of the sensitive ATP test might make it a useful tool in a cleaning verification program in wet-cleaned systems.
6. Further research and experience with practical application of sensitive ATP in food plants is needed. Non-zero RLU limits may be required to accommodate test variation and background ATP levels. Soils detected by these non-zero limits should be demonstrated to meet ELISA test sensitivity in equipment cleaning experiments.
7. Work is in progress to study the effects of detergent residues on the sensitivity of ATP and ELISA tests.
8. Work is needed to study the use of ATP and ELISA kits for detecting other food soils (peanut, soy, egg) on wet-cleaned food-contact surfaces.
9. More research is needed to determine the recovery and detection of ATP and proteins from dried food soils.

Acknowledgements

Authors wish to express gratitude for the contributions of;
Debra M. Lambrecht of FARRP,
Fadwa Al-Taher of NCSFT,
and Stephen Holmes of Charm Sciences, Inc.