MYCOSCIENCE INC.

Specialists in Microbiology & Regulatory Affairs

TERSANO C/O SCIREG INC.
EFFICACY STUDY OF LOTUS SANITIZING SYSTEM FOR USE AS A SANITIZER FOR FOOD CONTACT SURFACES

CLIENT: Tersano c/o SciReg, Inc.
12733 Directors Loop
Woodbridge, VA 22192

TEST#: 06-1029 SCI

STUDY INITIATION DATE: 12/22/06

REPORT TO: Felicia L. Sellers

STUDY COMPLETION DATE: 3/20/07

SAMPLE DESCRIPTION:

LOTUS® Sanitizing System w/ Pour Spout Attachment, Part # LBU100K, Received 11/20/06
Active Ingredient: Ozone (O_3)

TESTS REQUESTED:

Sanitizer for Food Contact Surfaces

TEST PURPOSE:

To determine the sanitizing activity of the Lotus® Sanitizing System's generated test agent (O_3 - Ozone), at a 1 minute contact time.

TEST PROCEDURE:

Reference Food Contact Protocol 12/12/06 – SCIREG INC. EFFICACY STUDY OF LOTUS SANITIZING SYSTEM FOR USE AS A SANITIZER FOR FOOD – CONTACT SURFACES (See Attached)

1.0 Preparation of Test Cultures

The *S. aureus* and *E. coli* cultures were maintained at 2-8°C and transferred to Nutrient Agar plates with incubation at 35 - 37°C for 24 +/- 2 hours. A minimum of (3) consecutive daily transfers were made. The final transfer was made by swabbing the culture with a pre-moistened P.B. H_2O swab to the surface of (3) Nutrient Agar plates. The plates were incubated for 18 - 24 hours at 35 - 37°C. The bacterial cultures were harvested from the surface of Nutrient Agar plates using 5mL of P.B. H_2O per plate and sterile spreading sticks. The harvested suspensions were pooled in a sterile 50cc. centrifuge tube with sterile glass beads. The tube was vortexed for 1 minute and each suspension was filtered through Whatman # 2 paper and collected in another sterile 50 cc. centrifuge tube. Suspensions were diluted further as necessary with additional sterile P.B. H_2O and standardized with a spectrophotometer so that the final suspension concentrations were ~ 1.0×10^{10}/mL.

1.1 Test Article Preparation

The Lotus® Sanitizing System was prepared for use by inserting the Booster Cartridge in the system per the manual instructions. Two generations of the Lotus® Sanitizing System generated test agent (O_3) were tested in duplicate by exposure to suspensions of the test microorganisms. For each generation of test agent, 300mL of sterile tap water at 16-18°C were added to the Lotus® pour spout attachment. The pour spout attachment was placed on the Lotus® Sanitizing System and activated per the manual instructions. The first cycle of generated O_3 was a conditioning cycle and was discarded. Subsequent test cycles of O_3 were generated according to the manual instructions and the total system generation time was documented for each cycle.

1.2 Testing

Immediately (≤ 30 seconds) after each generation cycle was complete, a 9.9mL aliquot of the test agent, was gently pipetted to a sterile tube in duplicate for each challenge microorganism. A 0.1mL aliquot of the test organism suspension was added to each tube and they were vortexed for not more than 3 seconds. The tubes were then allowed to sit for the 1 minute contact time. Immediately following the contact time serial dilutions (10^{-1}, 10^{-2}, and 10^{-3}) were performed in 9mL AOAC Neutralizing Fluid tubes and were plated (pour plate) in duplicate to molten/tempered Tryptone Glucose Extact Agar (TGEA) plates containing neutralizers. The plates were incubated for 48 +/- 2 hours at 35 - 37°C. After the incubation the plates were enumerated and recorded. Temperature readings were performed on the sterile tap water on removed 10mL aliquots pre and post test agent generation for each test cycle, and recorded. Room temperature was also recorded.

1.3 Controls

1.3.1 Parallel Numbers Control

A parallel numbers control count was performed using sterile 16 - 18°C tap water for each test microorganism. A single 9.9mL aliquot of water was subjected to the identical conditions as in 2.3 above for each test microorganism. After the 1 minute contact time, appropriate serial dilutions (10^{-8}, 10^{-9}) were plated, incubated, enumerated, and recorded as in 1.3 above.

1.3.2 Inoculum Counts

Inoculum suspension counts were performed via serial dilution in P.B. H_2O tubes and were plated, incubated, enumerated, and recorded as above.

1.3.3 Sterility Controls

Sterility controls were run on all test media. 1mL of each dilution fluid was plated to TGEA w/ neutralizers and were incubated with the test plates. The sterility controls exhibited no growth.

1.4 Neutralizer Effectiveness

To demonstrate the absence of residual antimicrobial effect in the neutralizer medium, <200 CFU of the test microorganism were inoculated to a tube containing 9mL of AOAC neutralizing broth and 1mL of freshly generated test agent. A control tube containing 10mL of AOAC Neutralizing Fluid only was inoculated for comparison. The tubes were vortexed and 1mL aliquots was plated in duplicate via pour plate to TGEA containing neutralizers, and were incubated for 48 +/- 2 hours at 35 - 37°C. Comparable growth on these plates after incubation confirmed neutralizer effectiveness.

1.5 Microorganism Confirmation

All plates were examined for purity. The challenge microorganisms were confirmed to be *E. coli* and *S. aureus* based on gram stain and colony morphology. The sterility controls exhibited no growth.

RESULTS:

Tersano Lotus Sanitizing System – 1 Minute Contact *Escherichia coli* - ATCC #11229				
O_3 Generation #	**Plated Dilution**	**CFU / Plate**	**CFU / mL**	**% Reduction**
1) Rep. #1	-1	7 / 5	6.0×10^1	>99.999%
	-2	0 / 2		
	-3	0 / 0		
1) Rep. #2	-1	16 / 14	1.5×10^2	>99.999%
	-2	2 / 0		
	-3	0 / 0		
2) Rep #1	-1	27 / 11	1.9×10^2	>99.999%
	-2	1 / 0		
	-3	0 / 0		
2) Rep. #2	-1	0 / 0	$<1.0 \times 10^1$	>99.999%
	-2	0 / 0		
	-3	0 / 0		
Parallel #'s Control	-6	112 / 115	1.1×10^8	NA
	-7	12 / 14		
Inoculum Count	-8	122/113	1.2×10^{10}	NA

Tersano Lotus Sanitizing System – 1 Minute Contact *Staphylococcus aureus* - ATCC #6538				
O_3 Generation #	**Plated Dilution**	**CFU / Plate**	**CFU / mL**	**% Reduction**
1) Rep. #1	-1	1 / 0	1.0×10^1	>99.999%
	-2	0 / 0		
	-3	0 / 0		
1) Rep. #2	-1	39 / 37	3.8×10^2	>99.999%
	-2	2 / 8		
	-3	0 / 0		
2) Rep #1	-1	7 / 5	6.0×10^1	>99.999%
	-2	1 / 0		
	-3	0 / 0		
2).Rep. #2	-1	0 / 0	$<1.0 \times 10^1$	>99.999%
	-2	0 / 0		
	-3	0 / 0		
Parallel #'s Control	-6	86 / 84	8.5×10^7	NA
	-7	15 / 10		
Inoculum Count	-8	91/87	8.9×10^9	NA

Experimental Start Date: 12/23/06 **Experimental End Date: 12/27/06**

Sterile Tap Water Pre O_3 Generation Temperature:	17°C
Sterile Tap Water Post O_3 Generation Temperature:	20°C
Room Temperature:	21°C
O_3 Generation Time:	2 Minutes, 18 Seconds per Cycle
Media Sterility Controls:	Negative (No Growth)

Neutralizer Effectiveness:

	Neutralizer (9 ml) + Test Agent (1 ml)	Neutralizer (10 ml) Control
E. coli	15 / 13 CFU*	11 / 16 CFU
S. aureus	10 / 8 CFU	7 /9 CFU

* Duplicate plate counts for the test microorganism listed in CFU (Colony Forming Units).

Equivalent growth was seen in the sample and control plates confirming neutralization of the sample active (O_3).

Calculations

Percent reductions were calculated using the following equation:

$$\frac{\text{Parallel Control (CFU/mL)} - \text{Test Result (CFU/mL)}}{\text{Parallel Control (CFU/mL)}} \times 100$$

CONCLUSIONS:

Under the conditions of the test, the Lotus Sanitizing System was effective at reducing the initial microbial populations by >99.999% in a 1 minute contact time when tested against *Escherichia coli* ATCC # 11229, and *Staphylococcus aureus* ATCC # 6538. All of the controls met the criteria for a valid test. These conclusions are based on observed data.

Analyst/s: [signature] Date: 3/20/07

Reviewed By: R. Whalen Date: 3/20/07

SCIREG INC.
EFFICACY STUDY OF LOTUS® SANITIZING SYSTEM
FOR USE AS A SANITIZER FOR FOOD-CONTACT SURFACES

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PURPOSE OF THE STUDY

To determine the sanitizing activity of the Lotus® Sanitizing System's generated test agent (O_3 - Ozone), at a 1 minute contact time.

TEST SYSTEM AND JUSTIFICATION

The test article will be tested per a modification of the AOAC Official Method 960.09 Germicidal and Detergent Sanitizing Action of Disinfectants test to meet the efficacy data requirements in the US EPA DIS/TSS-4: Sanitizing Rinses (for previously cleaned food-contact surfaces). The test microorganisms to be used in this study will be ***Escherichia coli*** **ATCC # 11229, and** ***Staphylococcus aureus*** **ATCC # 6538.**

TEST ARTICLES

(1) Tersano Lotus® Sanitizing System with Attachments
(2) Sterile Tap Water

TEST SUBSTANCE CHARACTERIZATION

The identity, strength, purity, stability, solubility, and chemical composition of the test material are the responsibility of the sponsor.

METHODS

The study will be a modification of the AOAC Official Method 960.09 Germicidal and Detergent Sanitizing Action of Disinfectants test with a 1 minute contact time.

MEDIA/MATERIALS

1.0 Nutrient Agar Petri Plates
1.1 Tryptone Glucose Extract Agar (TGEA) w/ AOAC Neutralizer
1.2 9mL AOAC Neutralizing Fluid Dilution Tubes
1.3 Neutralizers: Polysorbate 80, Lecithin
1.4 9mL Phosphate Buffered Water Dilution Tubes (P. B. H_2O)
1.5 Sterile Tap Water (500mL Bottles)
1.6 Sterile 50cc centrifuge tubes
1.7 Spectrophotometer w/ sterile tubes
1.8 Sterile Test Tubes
1.9 Sterile Swabs
1.10 Water Bath
1.11 Calibrated Thermometer, Thermocouple
1.12 Laminar Flow Hood/Workstation
1.13 Sterile spreading sticks
1.14 35 - 37°C incubator
1.15 Sterile glass beads
1.16 Vortex mixer
1.17 Bacterial Colony Counter
1.18 1mL, 10mL sterile pipets
1.19 Automatic pipette
1.20 Micropipette w/ sterile 10 – 100uL tips
1.21 Glass Filtration Flask
1.22 Vacuum Pump
1.23 Sterile Filter Funnels
1.24 Whatman #2 paper (Sterile 47mm discs)
1.25 Timer

PROCEDURE

2.0 Preparation of Test Cultures

The *S. aureus* and *E. coli* cultures are maintained at 2-8°C and are transferred to Nutrient Agar plates with incubation at 35 - 37°C for 24 +/- 2 hours. A minimum of (3) consecutive daily transfers are made. The final transfer is made by swabbing the culture with a pre-moistened P.B. H_2O swab to the surface of (3) Nutrient Agar plates. The plates are incubated for 18 - 24 hours at 35 - 37°C.

2.1 The bacterial cultures are harvested from the surface of Nutrient Agar plates using 5mL of P.B. H_2O per plate and sterile spreading sticks. The harvested suspensions are pooled in a sterile 50cc. centrifuge tube with sterile glass beads. The tube is vortexed for 1 minute and each suspension is filtered through Whatman # 2 paper and is collected in another sterile 50 cc. centrifuge tube. Suspensions will be diluted further as necessary with additional sterile P.B. H_2O and standardize with a spectrophotometer so that the final suspension concentrations are ~ 1.0×10^{10}/mL. Suspensions are prepared daily and are refrigerated when not in use.

2.2 Test Article Preparation

Prepare the Lotus® Sanitizing System for use by inserting the Booster Cartridge in the system per the manual instructions. Two generations of the Lotus® Sanitizing System generated test agent (O_3) will be tested in duplicate by exposure to suspensions of the test microorganisms. For each generation of test agent, 300mL of sterile tap water at 16 - 18°C will be added to the Lotus® pour spout attachment. The pour spout attachment will be placed on the Lotus® Sanitizing System and activated per the manual instructions. The first cycle of generated O_3 will be a conditioning cycle and will be discarded. Subsequent test cycles of O_3 will be generated according to the manual instructions and the total system generation time will be documented for each cycle. The system will prompt the user when the cycle is complete.

2.3 Test

Immediately (≤ 30 seconds) after each generation cycle is complete, a 9.9mL aliquot of the test agent, will be gently pipetted to a sterile tube in duplicate for each challenge microorganism. A 0.1mL aliquot of the test organism suspension will be added to each tube and they will be vortexed for not more than 3 seconds. The tubes will then be allowed to sit for the 1 minute contact time. Immediately following the contact time serial dilutions (10^{-1}, 10^{-2}, and 10^{-3}) will be performed in 9mL AOAC Neutralizing Fluid tubes and will be plated (pour plate) in duplicate to molten/tempered Tryptone Glucose Extact Agar (TGEA) plates containing neutralizers. The plates will be incubated for 48 +/- 2 hours at 35 - 37°C. After the incubation the plates will be enumerated and recorded.

2.4 Temperature readings will be performed on the sterile tap water on removed 10mL aliquots pre and post test agent generation for each test cycle, and will be recorded. Room temperature will also be recorded.

2.5 Controls

Parallel Numbers Control

A parallel numbers control count will be performed using sterile 16 - 18°C tap water for each test microorganism. A single 9.9mL aliquot of water will be subjected to the identical conditions as in 2.3 above for each test microorganism. After the 1 minute contact time, appropriate serial dilutions (10^{-8}, 10^{-9}) will be plated, incubated, enumerated, and recorded as in 2.3 above.

Inoculum Counts

Inoculum suspension counts will be performed via serial dilution in P.B. II_2O tubes and will be plated, incubated, enumerated, and recorded as above.

Sterility Controls

Sterility controls will be run on all test media. 1mL of each dilution fluid will be plated to TGEA w/ neutralizers and will be incubated with the test plates. Negative controls will be run on the agar media.

2.6 Neutralizer Effectiveness

To demonstrate the absence of residual antimicrobial effect in the neutralizer medium, <200 CFU of the test microorganism will be inoculated to a tube containing 9mL of AOAC neutralizing broth and 1mL of freshly generated test agent. A control tube containing 10mL of AOAC Neutralizing Fluid only will be inoculated for comparison. The tubes will be vortexed and 1mL aliquots will be plated in duplicate via pour plate to TGEA containing neutralizers, and will be incubated for 48 +/- 2 hours at 35 - 37°C. Comparable growth on these plates after incubation will confirm neutralizer effectiveness.

2.7 Microorganism Confirmation

Surviving organisms will be confirmed as *E. coli* or *S. aureus* by microscopic (Gram stain) and macroscopic examination.

RESULTS

To be considered valid, results must meet standard effectiveness: at least a 99.999% reduction in the number of test microorganisms over that of the parallel numbers control in the 1 minute contact time. Results will be reported according to actual count and % reduction over the parallel control count.

REPORT

The final report will include an identification of all test articles, summary of the methods used, any modifications to the study, results, summary, and any other pertinent information.

RECORDS

All documentation, data, and final reports derived from this study will be retained in the archives at Mycoscience Laboratories, 25 Village Hill Rd., Willington, CT, 06279.

REFERENCES

1) Official Methods of Analysis of AOAC International, 17th Edition, 2000, Section 6.3.03.
2) EPA DIS/TSS-4: Efficacy Data Requirements, Sanitizing Rinses (for previously cleaned food-contact surfaces).

APPROVALS

Sponsor Approval: Patricia Fallon Date: 12/12/06

Mycoscience Labs: R. Arsenault Date: 12/22/06

*typo TLB 12/13/06