Haley & Aldrich, Inc.
70 Blanchard Road
Suite 204
Burlington, MA 01803
617.886.7400

09 February 2016
File No. 41741-002

Corrugated Packaging Alliance
500 Park Blvd., Suite 985
Itasca, IL 60143

Attention: Mr. Dennis Colley
Executive Director

Subject: Effectiveness of the Time and Temperature Profile of Corrugation to Eliminate Microbial Loads

Dear Mr. Colley:

As corrugated containers are commonly used to store and transport fresh produce from farm to table, the Corrugated Packaging Association's (CPA) member companies have historically monitored the microbial cleanliness of corrugated containers. However, due to recent information citing the potential of reusable plastic containers (RPCs) to harbor excessive microbial loads, the CPA sponsored two recent studies conducted by NSF International (NSF). The first study was conducted to verify the microbial cleanliness of corrugated containers when manufactured using the typical time/temperature of the corrugation process; the second study evaluated lower temperatures that may be used as the industry works to reduce the temperature of corrugation and their environmental footprint through a reduction in the associated energy usage. The results of the CPA sponsored studies, which demonstrate the sanitizing effects of the corrugation time/temperature profiles and the corresponding log reduction of thermotolerant microorganisms on corrugated coupons, are summarized herein.[1]

## Background Information

### CORRUGATED DATA

The corrugated industry has historically evaluated the microbial cleanliness of corrugated containers via multiple pathways. Each of these efforts detailed below has provided information that supports the industries' position on the microbial cleanliness of corrugated containers:

- High temperature short time (HTST) and higher heat short time (HHST) curves, commonly used by the dairy industry to assess temperatures that result in the destruction of pathogens were reviewed against the time/temperature profile of a typical corrugation process. HHST and HTST

[1] Sanitization as defined by the U.S. Environmental Protection Agency (USEPA) requires a 5-log reduction of organisms after the application of a sanitizer under standardized laboratory conditions.

curves indicate that a temperature of 191°F for 1.0 second will result in a 99.999% (5-log) reduction (IDFA, 2014).

In a typical corrugation process, the containerboard attains a temperature of 190°F +/- 10 °F for approximately 8-9 seconds. Taking into consideration differences between the dairy matrix and the corrugated material, the time/temperature profile of the corrugation process was assessed to be sufficient to effectively eliminate microbial contamination (Sanders, 2011).[2]

- Routine microbial testing of finished products by container manufacturers confirmed the microbial cleanliness of corrugated containers. Finished product testing for overall aerobic organisms as well as pathogenic microbes verified that the microbial loads present on the corrugated containers were below those considered by scientific experts to be acceptable, even after storage at the production facility for up to two months (Sanders, 2014a).

  As no specific regulatory limits are available for containers used for food transport, the number of microorganisms detected was evaluated against those quoted by Dr. Keith Warriner of the University of Guelph to evaluate the cleanliness of containers used in the transport of fresh produce. Per Dr. Warriner, acceptable levels of organisms include up to 10,000 total organisms/container and no more than 1,000 pathogenic indicator organisms/container (Warriner, 2013). These levels are consistent with European regulatory guidelines (New South Wales Food Authority, 2013; European Commission, 2011).

- An industry-wide field test of corrugated containers also affirmed the cleanliness of the containers at various distribution facilities across multiple geographies.

  The field testing, following a protocol established by Dr. Trevor Suslow of the University of California - Davis, included sampling of over 360 different containers from 12 unique shipments and multiple corrugated manufacturers at five customer locations in three states, demonstrated that all containers evaluated in the field study met the sanitation standards defined by Dr. Keith Warriner. Specifically, all containers sampled had microbial loads of less than 10 microorganisms per container (Sanders, 2015a).

## FOOD-BORNE ILLNESS AND PACKAGING

The Centers for Disease Control and Prevention (CDC) has indicated that fresh produce is a potential source of contamination that may lead to food-borne illness (CDC, 2015). In fact, produce has been estimated to have contributed 46% of domestically-acquired illnesses and 23% of the deaths between 1998 and 2008 (Painter et al, 2013). Despite the fact that there is no documented evidence for transport containers to be the source of food-borne illness, there is evidence that microorganism loads can reach over 10,000,000 organisms per container and that the transfer of organisms from containers to fresh produce can occur (Sanders, 2014b; Danyluk, 2012). Based on these findings, as well as observations showing dirty, wet RPCs arriving for use at the field, confidence that shipping and transport containers will not serve to contribute to potential microbial loads has been somewhat eroded. The potential for RPCs to harbor significant levels of microorganisms has recently been detailed in the press (Zuraw, 2015; Williams, 2015; Andrews, 2014). Multiple field studies conducted to determine the potential microbial

[2] The HHST/HTST curves were evaluated taking into consideration not only the published HHST/HTST values, but also the difference between the corrugation medium and dairy products where it is commonly used.

loads on RPCs showed that up to 49% of those containers failed to meet the identified sanitation standards (Warriner, 2013; Warriner, 2014; Sanders, 2015a). Further, bench scale testing conducted to evaluate the ability of organisms to establish biofilms, which resist sanitization by common antimicrobial substances used by the industry, indicated that organisms can readily adhere to the RPC surfaces and that those organisms are not readily removed (Clayborn, 2015; Sanders, 2015b). Understanding the potential for produce shipping containers to harbor microorganisms is critical for growers, distributors, retailers and food service companies as they try to meet the intent of the U.S. Food and Drug Administration's (FDA) Food Safety Modernization Act (FSMA), which focuses on preventing potential food safety risks rather than reacting to issues after they occur (FDA, undated).

Specifically, the microbial hazards and the potential for cross-contamination associated with inanimate objects, including totes and bins have been recognized by the U.S. Food and Drug Administration (FDA, 1998). Internationally, a United Nations Food and Agriculture Organization technical document, *Management of reusable plastic crates in fresh produce supply chains* (Rapusas and Rolle, 2009) highlights the need for special attention on transport containers so that they do not contribute to product decay or spoilage, and/or human foodborne illness. These recent regulatory efforts should serve to elevate grower, shipper, and affiliated industries awareness of the need for science-based programs to manage these risks.

## Study 1: Evaluation of typical corrugation time/temperature profile

### GOAL/PURPOSE

The study was conducted to confirm that the time/temperature profile of a typical corrugation process is sufficient to mitigate microbial contamination and effectively sanitize the coupons. To test the hypothesis, an organism inoculum was applied to corrugated container board and heated; the level of organisms before and after heating was assessed and the log reduction was calculated. If the testing resulted in a 5-log reduction in organisms, the test result was deemed acceptable.

A time/temperature profile consistent with that found in a typical corrugating manufacturing process, where container board top liners reach temperatures of 190°F +/- 10 °F for 8-9 seconds, was employed in the study (O'Banion, 2015). This top liner typically represents the food contact surface of the container.

The testing entailed replicating the corrugation process from the single-facer through the hot plates, excluding the bridge. In the manufacturing process, the top liner is joined with the medium at the single-facer at a temperature of approximately 200°F though a web distance of 22 feet. The single-face board is combined with the bottom liner at the double-backer at a top liner temperature between 190 – 207°F. The length through the double-backer is 24 feet. The combined board then travels 66 feet through the hot plate section where the top liner temperature reaches between 190-200°F. The exposure time of 8.4 seconds is calculated using an average run speed of 800 feet per minute (fpm over a total distance of 112 feet. To mimic the corrugation process, the laboratory sandwiched corrugated coupons between two, 1" thick aluminum plates pre-heated to 215°F for 22 seconds. Under these conditions, the liner board reached the desired temperatures (180 - 200°F) for 9 seconds (See Figure 1).

To evaluate the effects of the corrugation process on the viability of microbes, organisms were selected for the study that (a) may be found on the fresh produce, (b) are recognized human pathogens that have resulted in illness attributed to fresh produce, and/or (c) have been assessed to be equally or more

thermotolerant than the organisms identified in (a) or (b). Based on this selection process, a cocktail of *Escherichia coli* (ATCC 25922), two strains of *Escherichia coli* (O157:H7) (ATCC 51657 & ATCC 43890), and *Salmonella enterica* subsp. *enterica* serovar *Enteritidis* (ATCC 13076) was used to inoculate the corrugated coupons in the study.[3]

The microbial reductions attained following exposure of the inoculated corrugated coupons to the time/temperature conditions of the corrugation process were then compared to EPA requirements for chemical sanitizers (5-log reduction) to confirm that the process would be sufficient to mitigate the presence of pathogenic organisms on corrugated containers.

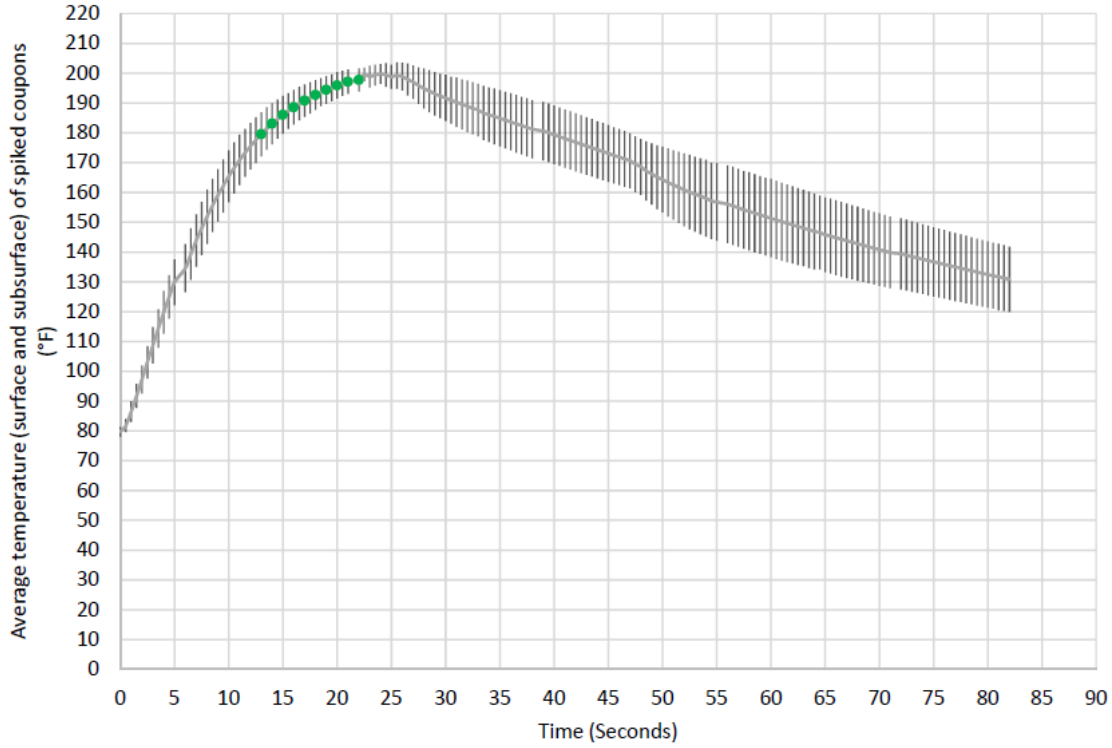
## TEST PROTOCOL

A brief synopsis of the procedure used by NSF to evaluate the effectiveness of the corrugation process to mitigate the presence of microorganisms follows. For more details, please see Attachment 1.

1. NSF International received two lots of corrugated material from two different corrugated manufacturers for testing. The corrugated material was, upon receipt, cut into 4" square sections (coupons) for the testing. 22 coupons/lot were assigned to 1 of 3 groups as follows:
   - 2 coupons/lot (blanks);
   - 10 unheated coupons/lot; and
   - 10 heated coupons/lot.
2. Coupon blanks were evaluated to confirm that the pretest sanitization protocol (UV radiation) was sufficient to generate a baseline showing the absence of organisms on the coupons.
3. 0.5 mL of a cocktail containing four different organisms was spread across the surface of both the heated and unheated coupon subsets. The organisms included in the cocktail include *Escherichia coli* (ATCC 25922), two strains of *Escherichia coli* (O157:H7) (ATCC 51657 & ATCC 43890), and *Salmonella enterica* subsp. *enterica* serovar *Enteritidis* (ATCC 13076).
4. The number of organisms present in the cocktail was established so that the final level of recoverable organisms from unheated coupons would meet or exceed a 5-log/coupon. After inoculation, each coupon was allowed to air dry for approximately 10 minutes before processing.
   a. Coupons designated for "heating" were placed between two, 1" thick aluminum plates for 22 seconds allowing the top liner of the board to reach temperatures between 180° and 200 °F for 9 seconds. To confirm the temperature profile of the coupons, thermocouples were placed in contact with the surface and subsurface of the inoculated liner. Figure 1 represents the time/temperature curve employed in the study.

[3] The two E. coli (O157:H7) strains and the Salmonella strain used are known human pathogens, the other E. coli strain used was used as a surrogate to model the heat resistance of the Salmonella Montevideo and Poona (Eblen, 2005).

**Figure 1: Study 1 Corrugated Coupon Time/Temperature Curve**




b. After heating, each coupon was placed into 100 mL of Letheen broth within 1 minute for processing.

c. Concurrently, a paired unheated coupon was processed alongside a corresponding heated coupon; each unheated coupon was also placed into a separate 100 mL of Letheen broth for processing.

d. Viable organisms were then eluted from the coupons via stomaching.

e. Dilutions from each coupon eluent were plated on selective media (Petrifilm® and XLD agar) to determine the residual level of *E. coli* and *Salmonella* spp., respectively.

f. Microbial levels before and after heating were assessed to determine the log reductions for each matched pair of coupons from each lot.

## RESULTS

The results of Study 1 show that the time/temperature profile of a typical corrugator resulted in a 5-log reduction of a cocktail of two *E. coli* O-157 strains, *Salmonella enteridis,* and an *E.coli* strain with similar thermotolerance to heat-labile *Salmonella* Montevideo and *Salmonella* Poona, when heating to 180 – 200°F for 9 seconds.

- The log reductions for both *E.coli* and *Salmonella* attained using a time/temperature profile consistent with the time/temperature of the corrugation process met or exceeded the EPA's requirement for chemical sanitizers (5-log reduction).
- None of the heated samples exhibited any microbial growth.
- The blanks (pretest coupons) indicate that the process employed prior to inoculation and heat treatment was sufficient to sanitize the coupons, eliminating confounding organism contamination.
- The results displayed no difference between the two different lots of corrugated material evaluated.

Tables 1 and 2 provide a summary of the study data. Table 1 provides logarithmic values as well as a comparison to EPA sanitizer efficacy requirements, while Table 2 provides information in arithmetic terms.

**Table 1: Study 1 Results (Log basis) with comparison to EPA Chemical Sanitization Requirements**

| Sample | Organism | Blank Coupon Avg. (Log CFU/ml) | Unheated Coupon Avg. (Log CFU/ml) | Heated Coupon Avg. (Log CFU/ml) | Avg. Log Reduction | Meets EPA Sanitizer Log Reduction Requirement (5-Log) |
|---|---|---|---|---|---|---|
| Lot 1 | *E. coli* | <0.1-Log | 6.33-Log | <0.1-Log | 6.03-Log | Yes |
| | *Salmonella* | <0.1-Log | 5.59-Log[4] | <0.1-Log | 5.49-Log | Yes |
| Lot 2 | *E. coli* | <0.1-Log | 6.42-Log | <0.1-Log | 6.12-Log | Yes |
| | *Salmonella* | <0.1-Log | 5.31-Log[4] | <0.1-Log | 5.21-Log | Yes |

[4] The data shows that the two Salmonella spp. used in the study were likely more susceptible to desiccation which occurred during the 10 minutes between organism inoculation and organism elution.

**Table 2: Study 1 Results (Arithmetic basis)**

| Sample | Organism | Blank Coupon Avg. (CFU/ml) | Unheated Coupon Avg. (CFU/ml) | Heated Coupon Avg. (CFU/ml) |
|---|---|---|---|---|
| Lot 1 | *E. coli* | <1 | 2,140,000 | <1 |
| | *Salmonella* | <1 | 390,000 | <1 |
| Lot 2 | *E. coli* | <1 | 1,260,000 | <1 |
| | *Salmonella* | <1 | 204,000 | <1 |

## Study 2: Evaluation of the effectiveness of lower temperature profiles

### GOAL/PURPOSE

This screening study was conducted to evaluate the ability of lower corrugation temperatures to result in a 5-log reduction of organisms. The test protocol was developed based on the methodology of Study 1. These reduced temperature profiles were chosen as the corrugated industry moves to new technologies, and aims to reduce its energy usage and overall environmental footprint. The time/temperature profiles evaluated in this study included: (1) 150°F (+/- 10°F) for 8-9 seconds, (2) 160°F (+/- 10°F) for 8-9 seconds and (3) 170°F (+/- 10°F) for 8-9 seconds.[5] Details of the study can be found in Attachment 2: Corrugator Effect on Microbial Contamination, NSF International, January 15, 2016.

### TEST PROTOCOL

1. A single lot of corrugated material was used for the testing. Upon receipt, the corrugated material was cut into 4" square sections (coupons) for the testing. A total of 15 coupons were used in the study:
    a. Two coupons were used to represent uninoculated blanks (background organisms).
    b. Four coupons were assigned to the unheated test group.
    c. Nine coupons (three coupons per time/temperature profile) were assigned to the heated test groups.
2. 0.5 mL of a cocktail containing four different organisms was spread across the surface of both the heated and unheated coupon subsets. The organisms used in the cocktail included *Escherichia coli* (ATCC 25922), two strains of *Escherichia coli* (O157:H7) (ATCC 51657 & ATCC 43890), and *Salmonella enterica* subsp. *enterica* serovar *Enteritidis* (ATCC 13076).

    The number of organisms present in the cocktail was established so that the final level of recoverable organisms from the unheated coupons would meet or exceed a 5-log/coupon. After inoculation, each coupon was allowed to air dry for approximately 10 minutes before processing.
3. Coupons designated for "heating" were placed between two, 1" thick aluminum plates for up to 19 seconds allowing the top liner of the board to reach the desired temperatures. To confirm the temperature profile of the coupons, thermocouples were placed in contact with the surface and

[5] These temperature profiles will be noted in the remainder of this document simply as 150°F, 160°F and 170°F.

subsurface of the inoculated liner. Figures 2 - 4 represents the time/temperature curves employed in the study.

**Figure 2: Study 2 – 150°F +/- 10°F Time/Temperature Curve**

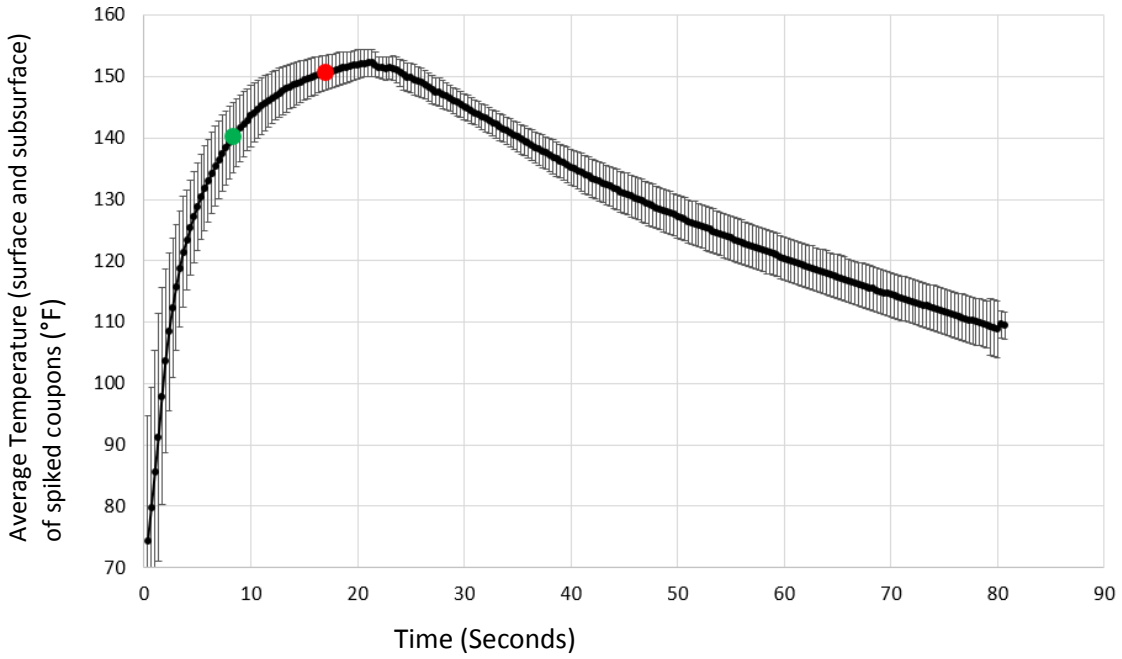



**Figure 3: Study 2 – 160°F +/- 10°F Time/Temperature Curve**

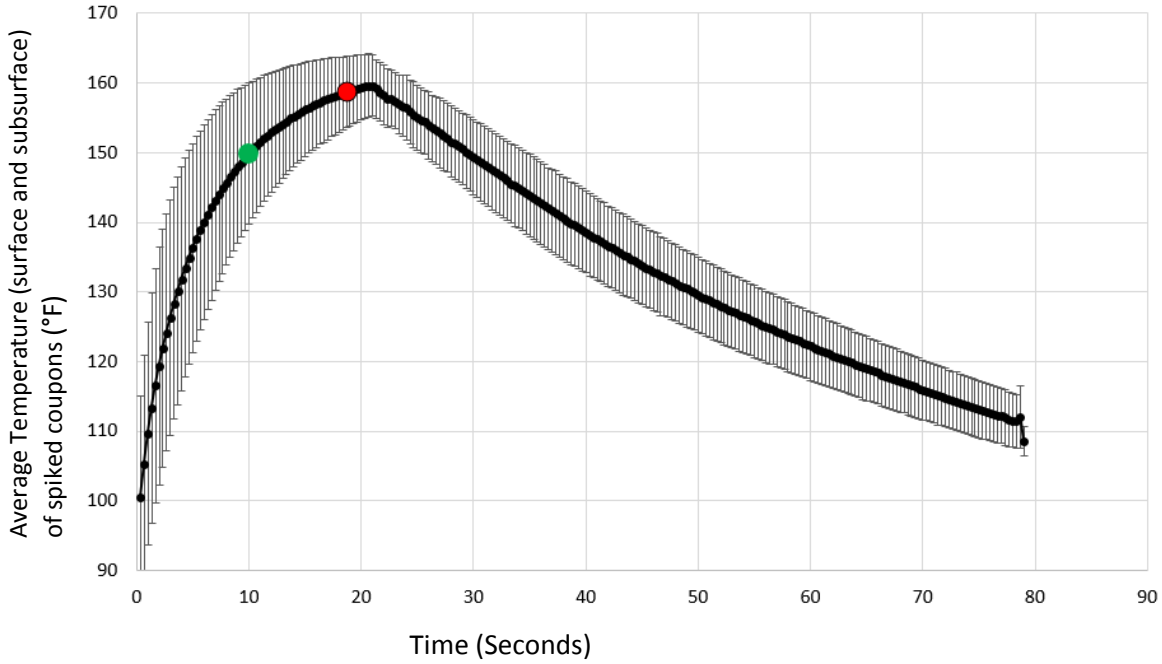
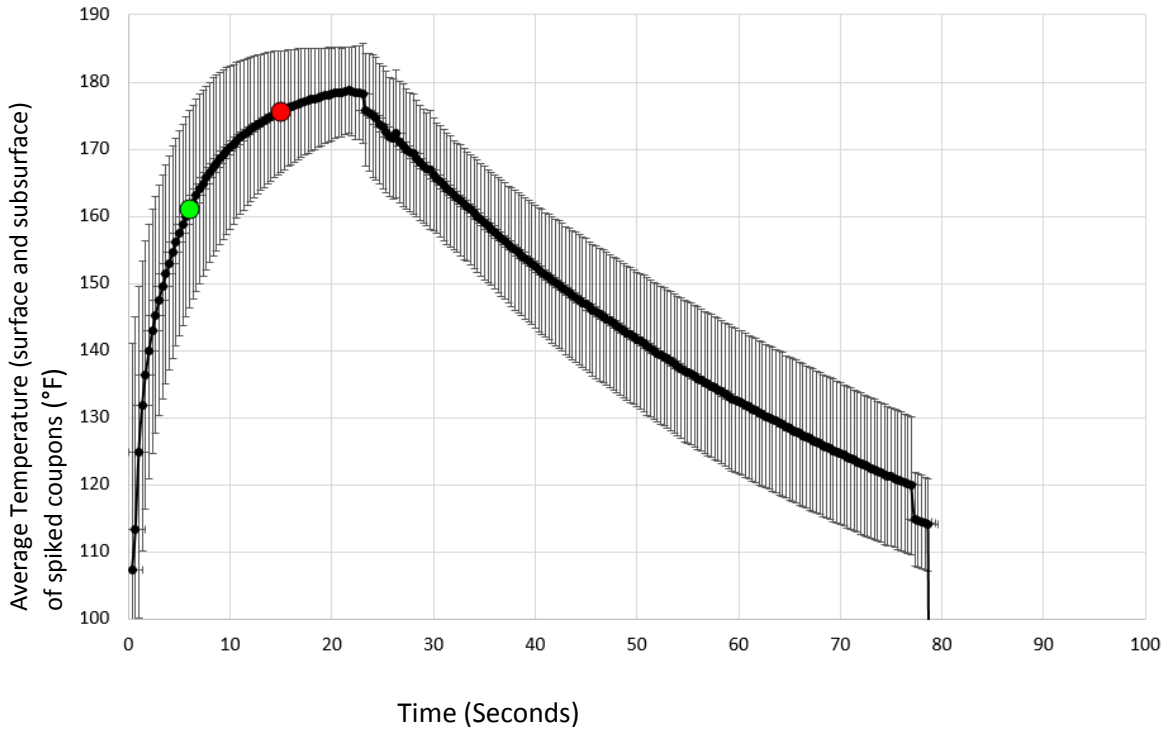


**Figure 4: Study 2 – 170°F +/- 10°F Time/Temperature Curve**




4. After heating, coupons were placed into aliquots of 100 mL of Letheen broth within 1 minute.
5. Unheated coupons were similarly placed into 100 mL of Letheen broth for processing.
6. Paired heated and unheated coupons were processed according to the order or operation detailed in Table 3 to minimize effects of desiccation on microorganism viability.

**Table 3: Study 2 - Order of operation**

| Coupon | Spike | Target Temperature (+/- 10°F) | Operational Order | | |
|---|---|---|---|---|---|
| 1 | Uninoculated | Unheated | 1 | | |
| 2 | Uninoculated | Unheated | 1 | | |
| 3 | Inoculated | Unheated | 1 | | |
| 4 | Inoculated | 150°F | 1 | | |
| 5 | Inoculated | 150°F | 1 | | |
| 6 | Inoculated | 150°F | 1 | | |
| 7 | Inoculated | Unheated | 1 | 2 | |
| 8 | Inoculated | 160°F | | 2 | |
| 9 | Inoculated | 160°F | | 2 | |
| 10 | Inoculated | 160°F | | 2 | |
| 11 | Inoculated | Unheated | | 2 | 3 |
| 12 | Inoculated | 170°F | | | 3 |
| 13 | Inoculated | 170°F | | | 3 |
| 14 | Inoculated | 170°F | | | 3 |
| 15 | Inoculated | Unheated | | | 3 |

7. Viable organisms were eluted from the coupons into the Letheen broth via stomaching.
8. Dilutions from each eluent were plated on selective media (Petrifilm® and XLD agar) to determine the residual level of *E. coli* and *Salmonella* spp., respectively.
9. Microbial levels before and after heating were used to determine the percent and log reductions realized for the total microbial load as well as each individual organism genus at each time/ temperature evaluated.

## RESULTS

The results of Study 2 show that temperatures at or above 160°F for 9 seconds result in a 5-log reduction of a cocktail of the various organisms evaluated.

- Coupons exposed to 150°F for 9 seconds result in a 4.34-log reduction of total organisms.
- The log reductions obtained for *E.coli* and *Salmonella spp.* at time/temperature profiles of 160°F and 170°F for 9 seconds met or exceeded the reduction specified by the EPA's for chemical sanitizers (5-log reduction).
- Time/temperature profiles of 150-170°F for 8-9 seconds were sufficient to mitigate the presence of viable *Salmonella* from all test samples, with an overall microorganism reduction of ≥5-log.
- None of the samples exposed to temperatures at or above 160°F had residual organisms above the acceptable microbial limits for pathogenic indicator organisms of 1000 organisms/ container cited by Dr. Warriner (Warriner 2013).

Tables 3 - 5 provide a summary of the data from Study 2. Table 3 provides data on the overall microbial load, while Tables 4 and 5 provide data on the individual microbial groups evaluated (*E.coli* or *Salmonella spp*.).

**Table 3: Study 2 – Microbial Efficacy Results (*E. coli* and *Salmonella combined*)**

| Target Temperature | Blank Coupons* | Unheated Inoculated Coupons* | Heated Inoculated Coupons* | Percent Reduction | Log Reduction | Meets EPA Chemical Sanitizer Requirements |
|---|---|---|---|---|---|---|
| 150°F | <1 | 7.80 +/- 0.31 | 3.46 +/- 0.7 | 99.995 | 4.34 | No |
| 160°F | | | 2.21 +/-0.88 | >99.999 | >5 | Yes |
| 170°F | | | <2 +/- 0.00 | >99.999 | >5 | Yes |

* (Avg. Log CFU/coupon +/- Std. Dev)

**Table 4: Study 2 – Microbial Efficacy Results - *E.coli* only**

| Target Temperature | Blank Coupons* | Unheated Inoculated Coupons* | Heated Inoculated Coupons* | Percent Reduction | Log Reduction | Meets EPA Chemical Sanitizer Requirements |
|---|---|---|---|---|---|---|
| 150°F | <1 | 7.68 +/- 0.30 | 3.46 +/- 0.7 | 99.994 | 4.22 | No |
| 160°F | | | 2.21 +/-0.88 | >99.999 | 5 | Yes |
| 170°F | | | <1.70 +/- 0.00 | >99.999 | >5 | Yes |

* (Avg. Log CFU/coupon +/- Std. Dev)

**Table 5: Study 2 - Microbial Efficacy Results – *Salmonella spp.* only**

| Target Temperature | Blank Coupons* | Unheated Inoculated Coupons* | Heated Inoculated Coupons* | Percent Reduction | Log Reduction | Meets EPA Chemical Sanitizer Requirements |
|---|---|---|---|---|---|---|
| 150°F | <1 | 7.16 +/- 0.38 | <2.00 +/- 0.00 | >99.999 | >5 | Yes |
| 160°F | | | <2.00 +/- 0.00 | >99.999 | >5 | Yes |
| 170°F | | | <2.00 +/- 0.00 | >99.999 | >5 | Yes |

***** (Avg. Log CFU/coupon +/- Std. Dev)

## DISCUSSION

The time temperature curves observed during the 8-9 second evaluation showed that:

- The maximum temperature attained during the evaluation of the effects of 150°F +/- 10°F was approximately 152°F with temperatures of 150°F or greater only attained for 2 seconds.
- The maximum temperature attained during the evaluation of the effects of 160°F +/- 10°F was approximately 160°F, with that temperature just being reached at the end of the evaluation period.
- The maximum temperature attained during the evaluation of the effects of 170°F +/- 10°F was approximately 175°F with temperatures of 170°F or greater attained for approximately 5 seconds.

When corrugated coupons were inoculated with thermotolerant organisms and subsequently exposed to temperature profiles of 160°F or 170°F for 8-9 seconds, a >5-log reduction in microorganisms was realized, effectively sanitizing the combined board.

Exposure of the inoculated coupons to 150°F for 8-9 seconds resulted in a 5-log reduction of *Salmonella spp*., but only a 4.22-log reduction in *E.coli.* However, as previously noted, when the 150°F temperature profile curve (Figure 2) is more closely evaluated, a temperature of 150°F was only reached for approximately 2 seconds. Further, this bench study only evaluated the portion of the corrugation process from the single-facer through the hot plates. It did not incorporate effects from the residence time/temperatures from other components of the process (i.e., the bridge), the desiccation of components both pre- and post-corrugation, or the effects of antimicrobials that may be incorporated into the starch (which serves as the glue in the corrugation process).

Despite the exclusion of these other factors that would likely have additional antimicrobial effects as well as the minimal amount of exposure time at 150°F and above, a 5-log reduction of *Salmonella spp*. and a 4.22-log reduction in *E.coli* was still observed. Should the industry wish to pursue the use of a corrugation temperature of 150°F, additional studies should be conducted.

## Conclusions

Based on the results of both studies, the time/temperature profiles of 160, 170 and 190°F +/- 10°F for 8-9 seconds were shown to effectively mitigate the presence of microorganisms on corrugated container coupons. Each profile met the EPA chemical sanitizer requirements, which specify that treatment must result in a 5-log reduction of microorganisms. Exposure of the corrugated coupons to 150°F for 8-9 seconds resulted in a log reduction for *Salmonella spp*. of ≥5-log, but only a 4.22-log reduction was realized for *E.coli.*

These results support the historical data generated by the corrugated industry, which shows that the current process for manufacturing single-use corrugated is sufficient to mitigate the significant presence of pathogenic organisms on corrugated materials, thereby mitigating the potential for the introduction of organisms into food. The data indicate that both current practices and potential future efforts to decrease environmental footprints (through the reduction of the heat of corrugation to as low as 160 +/- 10°F) will not adversely affect efforts to provide clean corrugated packaging to the produce industry.

Sincerely yours,
HALEY & ALDRICH, INC.

Mark Jackson
Senior Toxicologist
Regulatory Compliance Specialist

Maryann Sanders
Senior Regulatory Compliance Specialist
Microbiologist

Attachments:

Attachment 1: Corrugator Effect on Microbial Contamination, NSF International, November 4, 2015
Attachment 2: Corrugator Effect on Microbial Contamination, NSF International, January 15, 2016

## REFERENCES

1. Andrews, J. 2014. Studies Find Reusable Produce Containers Often Contaminated. Food Safety News. November 20.

2. Center for Disease Control (CDC). 2015. Foodborne Outbreaks: List of Selected Multistate Foodborne Outbreak Investigations. http://www.cdc.gov/foodsafety/outbreaks/multistate-outbreaks/outbreaks-list.html. Accessed October, 2015.

3. Clayborn, J. Adams, J. Baker, C. Ricke, S. 2015. Assessment of Salmonella spp. Attachment to Reusable Plastic Containers Based on Scanning Electron Microscopy and BAX® PCR. Journal of Food Research. Vol. 4, No. 2, 2015.

4. Danyluk M. and Schneider K. 2012. Pathogen transfer risks associated with specific tomato harvest and packing operations. Center for Produce Safety.

5. Eblen, D. Bassam, A. Sapers, G. 2005. Studies to Select Appropriate Nonpathogenic Surrogate Escherichia coli Strains for Potential use in Place of *Escherichia coli* O157:H7 and *Salmonella* in Pilot Plant Studies. Journal of Food Protection, Vol. 68, No. 2, Pages 282-291.

6. European Commission, 2011. Commission Decision of 8 June 2001 laying down rules for the regular checks on the general hygiene carried out by the operators in establishments according to Directive 64/433/EEC on health conditions for the production and marketing of fresh meat and Directive 71/118/EEC on health problems affecting the production and placing on the market of fresh poultry meat. June 8. http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2001:165:0048:0053:EN:PDF. Accessed November 1, 2015.

7. European Corrugated Packaging Association (FEFCO). 2011. EHEC bacteria cannot survive in corrugated packaging. June 9.

8. Food and Drug Administration (FDA). 2014. Code of Federal Regulations (CFR) Title 21. Section 1240.61 Mandatory Pasteurization for all milk and milk products in final package form intended for direct human consumption. Available at: http://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/CFRSearch.cfm?fr=1240.61. Revised April 1.

9. Food and Drug Administration (FDA). 1998. Guidance for Industry - Guide to Minimize Microbial Food Safety Hazards for Fresh Fruits and Vegetables.

10. Food and Drug Administration (FDA). Undated. Food Safety Modernization Act. www.fda.gov/Food/GuidanceRegulation/FSMA/. Accessed November 9, 2015.

11. Food Safety Authority of Ireland. 2006. 3rd Trimester National Microbiological Survey 2006 (06NS3): Examination of the microbiological status of food preparation surfaces. https://www.fsai.ie/uploadedfiles/food_prep_surfaces.pdf. Accessed October 25, 2015.

12. International Dairy Foods Association (IDFA). 2014. High-Temperature Shorter Time and Higher-Heat Shorter Time chart for destroying pathogens in food. http://www.idfa.org/news-views/media-kits/milk/pasteurization. Accessed November 12, 2015.

13. New South Wales Food Authority. 2013. Environmental Swabbing: A guide to method selection and consistent technique. FI170/1303.

http://www.foodauthority.nsw.gov.au/_Documents/science/environmental_swabbing.pdf. Accessed February 4, 2015.

14. O'Banion, B. 2015. Personal Communication – Re: Update on corrugated cardboard thermal profile study. October 22.

15. Painter, J. Hoekstra, R. Ayers, T. Tause, R. Braden, C. Angulo, F. Griffin, P. 2013. Attribution of Foodborne Illnesses, Hospitalizations, and Deaths to Food Commodities by using Outbreak Data, United States, 1998–2008. Emerging Infectious Diseases. Vol. 19, No. 3. March.

16. Rapusas, R, Rolle, R. 2009. Management of Reusable Plastic Crates in fresh produce supply chains – a technical guide. Food and Agriculture Organization of the United Nations.

17. Sanders, M. 2014a. Microbiological Status of Finished Paper/Corrugated Boxes. November.

18. Sanders, M. 2014b. Assessing the Potential of Reusable Plastic Containers (RPC) to Harbor and Transfer Microbial Loads. October.

19. Sanders, M. 2015a. Field Study to Assess the Microbiological Status of Corrugated Containers and Oher Produce Storage and Shipping Containers upon Delivery to the Customer Location. February.

20. Sanders, M. 2015b. A Review of Sanitizer Efficacy on *Salmonella* Biofilms present on Reusable Plastic Containers. July 26.

21. Sanders, M. 2011. High temperature short time (HTST), and Higher heat short time (HHST) microbial risk reduction review. Internal International Paper report. July 11.

22. Warriner, K. 2013. Microbiological standards for Reusable Plastic Containers within Produce Grower Facilities. University of Guelph, Department of food Science, June.

23. Warriner, K. 2014. Microbiological standards for Reusable Plastic Containers within Produce Grower Facilities within Ontario and Quebec. University of Guelph, Department of Food Science, October.

24. Williams, C. 2015. Study: Storage for Food Oft Soiled – UA says bacteria sticks after wash. Arkansas Democrat Gazette. November 7.

25. Zuraw, L. 2015. Reusable Plastic Containers are Difficult to Clean. Food Safety News. October 22.

# ATTACHMENT 1

# Corrugator Effect on Microbial Contamination
# NSF International
# November 4, 2015

**NSF International – Applied Research Center**

789 N. Dixboro Rd. Ann Arbor, MI 48015, USA
1-800.NSF.MARK | +1-734.769.8010 | www.nsf.org

# TEST REPORT

**Send to:** Fibre Box Association
25 Northwest Point Boulevard, Suite 510
Elk Grove Village, Illinois 600007

**Result: COMPLETE** **Report Date: 04-November-2015**

| | |
|---|---|
| Customer Name: | Fibre Box Association |
| Location of Testing: | NSF Ann Arbor |
| Description: | Corrugator Effect on Microbial Contamination |
| Test Type: | Test Only |
| Job Number: | J-00184630 |
| Project Number: | 10014843 |
| NSF Corporate: | C0262787 |
| Project Manager: | J. Vantine |

**Executive Summary**:
Fibre Box Association contracted the Applied Research Center at NSF International to determine if the corrugation process is sufficient to mitigate microbial contamination on the container board that occurs prior to corrugation.
The surface of 2 sample lots of containerboard material were inoculated with a microbial challenge population of thermotolerant bacteria. This inoculated containerboard was then exposed to heat at a timed interval to simulate the corrugation process. The exposure of 185 ± 5 °F for 8-9 seconds was sufficient to eliminate microbial contamination.

**Thank you for working with the Applied Research Center! We hope to collaborate again with you soon!**

Please contact your Project Manager if you have any questions or concerns pertaining to this report.

**Report Authorization:** Robert Donofrio
Digitally signed by Dr. Robert Donofrio /jv
DN: cn=Dr. Robert Donofrio /jv, o=NSF International, ou=Director - Applied Research Center, email=Donofrio@NSF.ORG, c=US
Date: 2015.11.04 09:06:52 -05'00'

Robert Donofrio – Director, Applied Research Center

**Scope of Test Report**

The objective of the study was to understand if the process of corrugation eliminates microbial contamination on the containerboard material. A selection of thermotolerant organisms representative of foodborne pathogens that are of concern in the produce industry were selected.

Organism cocktail:
*Escherichia coli* ATCC 25922*
*Escherichia coli* ATCC 43890 (O157:H7)
*Escherichia coli* ATCC 51657 (O157:H7)
*Salmonella enterica* subsp. *enterica* serovar Enteritidis ATCC 13076
* According to Eblen et al. (J. Food Prot., 2005). *E. coli* ATCC 25922 is, relative to tested pathogenic *E. coli* strains, heat-labile and may be used as a surrogate to model the heat resistance of the heat-labile *Salmonella* strains like *Salmonella* Montevideo G4639 and *Salmonella* Poona RM 2350

The corrugation process itself was reviewed and the exposure of 185 ± 5 °F for 8-9 seconds was selected to replicate the hot plate processing segment of the corrugation process. In the manufacturing process the component materials are pre-heated and brought together for a final pressing. In representative facilities this pressing has been measured to be between 193 °F (Top Liner) and 240 °F (Bottom Liner) for an average of 66 feet at a rate of 700 fpm. In our simulation the containerboard material was not be pre-heated.

In order to replicate the desired temperature and time exposure, NSF conducted method verification (See Figure 1) to determine the correct placement and removal times to achieve the 185 ± 5 °F for 8-9 seconds.
One inch thick aluminum plaques were heated in an oven at various temperatures, ranging from 180F to 220F until the desired effect on the containerboard was achieved.

The organism cocktail was applied to the 4" × 4" area of containerboard material and allowed to dry for 10 minutes. The coupons were then carefully wrapped in foil and exposed to the simulated corrugation process. At the completion of the exposure the coupons were allowed to cool for 1 minute to replicate the material moving along the production line and then placed into a stomacher bag containing buffer. After simulated processing the containerboard plaques and foil press material were stomached to determine the remaining population of challenge organisms.

Proposed Sampling

- 2 lots of container board were evaluated; each tested in 22 locations (4 × 4 inch cut-outs; "coupons").
    - 20 spiked (10 sampled without heating, 10 sampled after heating)
    - 2 unspiked (1 sampled without heating, 1 sampled after heating)

The level of the inoculated surrogate organisms on the container board before and after the simulated corrugation process was used to demonstrate the efficacy of the corrugation process to eliminate organisms.

**Methodology**

**Methods:**

1. Thermocouple Temperature Profile Study
    a. 4" × 4". cardboard coupons were spiked on the outer liner surface (rough material side) with 500 µL of sterile BNaClPT (per liter: FLUKA Peptone Hy-Soy® T, 1 g; Tween 80, 1 mL; KH2PO4, 3.6 g; Na2HPO4, 7.2 g; NaCl, 4.3 g; pH 7.0 +/- 0.2) containing 50 mM Trehalose. 50 mM Trehalose was included to protect the inoculum against dessication (die-off) during the drying process (S.B. Leslie et al. 1995 Appl. Environ. Microbiol.). The solution was immediately spread across the liner using a T-spreader and then allowed to dry for 10 minutes.

    b. A thermocouple was affixed to the coupons in order to monitor and document the temperature profile of the cardboard coupons during the heating process and one minute of cooling.

    c. Note: After affixing the thermocouple, the coupon surface was topped with a 4" x 4" segment of heavy duty aluminum foil and then wrapped in heavy duty aluminum foil.

    d. Phase 1: the thermocouple was affixed to the outer portion of the upper cardboard liner. This phase was deemed complete when an oven temperature was identified which quickly heated three consecutive replicate coupons to 185 ± 5 °F and maintained that temperature for 8-9 seconds. The selected oven temperature was used in Phase 2.

    e. Phase 2: the thermocouple was affixed to the inner portion of the upper cardboard liner and the heating process repeated on new spiked coupons.

    f. The aim of these two phases was to capture the temperature profile of the outer liner from both of its sides.

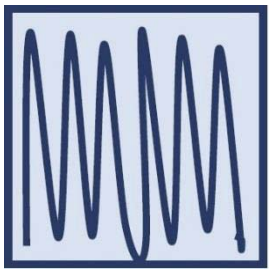
    g. As long as the temperature profile observed in phase 2 was within 10°F of that observed within phase 1, the oven temperature observed in phase 1 was used in the full study plan. If the observed phase 2 temperature profiles were more than 10°F different than that of phase 1, NSF and the client discussed the results and decided on how to proceed.

2. Full Study
    1. Each of the following samples was UV-sterilized for ten minutes:
        a. One side of a 10" X 6" piece of heavy duty aluminum foil (enough for all coupons)
        b. Both sides of a 4" x 4" piece of Heavy Duty Aluminum foil (enough for all coupons)
        c. Both sides of a 4" x 4" cardboard coupon from each lot (11 of each type for unheated sampling and heated sampling)
            i. This totaled 44 coupons (22 of each type)

    2. A master spike suspension mixture of the following organisms in BNaClPT + 50 mM Trehalose was created:
        a. a. *Escherichia coli* ATCC 25922
        b. b. *Escherichia coli* ATCC 43890 (O157:H7)
        c. c. *Escherichia coli* ATCC 51657 (O157:H7)
        d. d. *Salmonella enterica* ATCC 13076

    3. All organisms from step 2 were made to target densities of 1 x $10^9$ CFU/mL in BNaClPT + 50 mM Trehalose. Equal parts of the cell suspensions were mixed together. This master suspension was used to inoculate all 44 coupons.

    4. Using a calibrated pipette, the inoculum was added onto the surface of the cardboard coupon following the pattern shown below and immediately spread using a T-spreader to inoculate the coupon.



    5. Only two coupons of each type and treatment were inoculated at a time to minimize variability in drying and processing time (i.e., only 2 coupons of each lot for each treatment type; 4 total per round of testing).

    6. Coupons were allowed to dry for ten minutes and were then covered with the 4" X 4" piece of corresponding aluminum foil.

7. The large piece of aluminum foil was folded to cover the samples that were to be heated:

    a. Covered coupons were placed between two pre–heated aluminum blocks held at 215 ºF
    b. After 22 seconds the coupons were removed, allowed to rest for 1 minute and then processed using the same procedure in step 8 (below)

8. The unheated coupons along with the 4" × 4" piece of corresponding aluminum foil was placed in a pre-labeled stomacher bag that contained 100 mL of Letheen broth and stomached for 30 seconds.

9. Unheated samples were diluted to $10^{-3}$, $10^{-4}$, and $10^{-5}$ and plated on 3M™ Petrifilm *E.coli*/Coliform Count Plates for detection and quantification of *E. coli* and to XLD agar for detection and quantification of *Salmonella.*

10. Heated samples were diluted to "neat (zero)", $10^{-1}$, and $10^{-2}$ and plated on 3M™ Petrifilm *E.coli*/Coliform Count Plates for detection and quantification of *E. coli* and to XLD agar for detection and quantification of *Salmonella.*

11. 3M™ Petrifilm *E.coli*/Coliform Count Plates and XLD agar plates were incubated for 48 ± 4 hours at 35 ± 1 ºC.

**Results and Discussion**

Thermocouple Temperature Profile Study:

With an aluminum block temperature of 215 °F, the average temperature of the upper cardboard liner (measured from above and below the liner surface) reached 180 – 200 °F in 13 seconds and was maintained in that temperature range for 8-9 seconds (Figure 1). These results were communicated to the client and this exposure protocol (22 second heated-plaque exposure) was deemed appropriate for the full study.

Full Study:

As can be seen from the data shown in Table 1 in Appendix A, the reduction of total cells from the cardboard coupons under the conditions tested is between 6.41 and 6.46 $Log_{10}$, which equates to a ~99.9999% reduction (6 $Log_{10}$) in viable cells on the substrate. In fact, each sample tested no challenge organism could be detected as can be seen in Table 3 in Appendix A.

Additional raw data are provided in Appendix A, including the temperature profile for the test as well as raw data for each sample tested.

**Scope of Work Revisions**

- Scope of work authorized: September 9th, 2015
- Version: 10014843 1 10012015 authorized 10/01/2015 contained the following revisions:
    - Amended from Proposal Letter format to Attachment/Annex format to track changes.
    - Updated **Methodology** (2) to include details clarifying the use of 'T" spreader.
    - Updated **Methodology** (3) to decrease the time span from inoculation to sampling to under 10minutes.
    - Updated **Methodology** to clarify that plating will be completed in duplicate.
    - Updated **Methodology** (6) to remove reference to swabbing and update with stomaching.
    - Deleted **Figure 2** – which detailed swabbing approach.
    - Updated **Sample Processing** section to accurately detail methods. (Remove references to swabbing.)
    - Updated footer to include page numbering new (project) version number 10014843 1

- Version: 10014843 2 10192015 authorized 10/19/2015 contained the following revisions:
    - Changes Made: Addition of new header and details to Method Development and Verification section
      Revision of costs to accommodate additional method verification and laboratory services.
    - Updated footer to include new (project) version number 10014843 2

## Appendix A

## Result Tables and Figures

**Table 1: Reduction in viable organisms from the heating process.**
The following table displays the number of organisms present after heat treatment and for coupons that received no heat treatment. Reduction percentages are shown as a calculation of heated organisms remaining to unheated.

| | | | Total cells (E. coli and Salmonella) (Log CFU/mL in eluent) (Avg. ± SD) | | | E. coli (Log CFU/mL in eluent) (Avg. ± SD) | | | Salmonella (Log CFU/mL in eluent) (Avg. ± SD) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Lot | Received Date | Description | Unheated (*n* = 10) | Heated (*n* = 10) | Reduction % | Unheated (*n* = 10) | Heated (*n* = 10) | Reduction % | Unheated (*n* = 10) | Heated (*n* = 10) | Reduction % |
| 1 | 9/9/2015 | 23 PC'S OF 12"X12" CARDBOARD | 6.41 ± 0.23 | <1[a,b] | 99.9999 | 6.33 ± 0.21 | <1[a] | 99.9999 | 5.59 ± 0.34 | <1[b] | 99.999 |
| 2 | 9/15/2015 | 15 PC'S OF 24"X24" CARDBOARD | 6.46 ± 0.25 | <1[a,b] | 99.9999 | 6.42 ± 0.25 | <1[a] | 99.9999 | 5.31 ± 0.39 | <1[b] | 99.999 |

[a]The limit of detection for *E. coli* was 50 CFU (i.e., 50 CFU of viable challenge organism could have survived the simulated corrugation procedure and the enumeration protocol was not sensitive enough to detected them).
[b]The limit of detection for *Salmonella* was 100 CFU (i.e., 100 CFU of viable challenge organism could have survived the simulated corrugation procedure and the enumeration protocol was not sensitive enough to detected them).

789 N. Dixboro Rd. Ann Arbor, MI 48015, USA
1-800.NSF.MARK | +1-734.769.8010 | www.nsf.org

# TEST REPORT

**Table 2: Organism numbers on unheated lots (Raw Data).**
The following table displays the number of organisms present for coupons that received no heat treatment.

| Total Log CFU/mL (all organisms) | | | | | Total Log CFU/mL (all organisms) | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Sample | Replicate | *E. coli* | *Salmonella* | Sum | Sample | Replicate | *E. coli* | *Salmonella* | Sum |
| Lot 1 | 1 | 6.60 | 5.68 | 6.65 | Lot 2 | 1 | 6.39 | 5.17 | 6.42 |
| | | | | | | | | | |
| | 2 | 6.37 | 5.69 | 6.45 | | 2 | 6.49 | 5.10 | 6.51 |
| | | | | | | | | | |
| | 3 | 6.22 | 5.65 | 6.32 | | 3 | 6.41 | 5.60 | 6.48 |
| | | | | | | | | | |
| | 4 | 6.63 | 6.12 | 6.75 | | 4 | 6.28 | 5.33 | 6.32 |
| | | | | | | | | | |
| | 5 | 6.08 | 5.13 | 6.13 | | 5 | 6.66 | 5.14 | 6.67 |
| | | | | | | | | | |
| | 6 | 6.26 | 5.46 | 6.32 | | 6 | 6.28 | 5.30 | 6.32 |
| | | | | | | | | | |
| | 7 | 6.57 | 5.94 | 6.66 | | 7 | 6.58 | 5.60 | 6.63 |
| | | | | | | | | | |
| | 8 | 6.21 | 5.58 | 6.30 | | 8 | 5.85 | 4.41 | 5.87 |
| | | | | | | | | | |
| | 9 | 6.32 | 5.65 | 6.40 | | 9 | 6.56 | 5.62 | 6.60 |
| | | | | | | | | | |
| | 10 | 6.05 | 4.97 | 6.08 | | 10 | 6.72 | 5.79 | 6.76 |
| | | | Avg. | 6.41 | | | | Avg. | 6.46 |
| | | | St.Dev. | 0.23 | | | | St.Dev. | 0.25 |

789 N. Dixboro Rd. Ann Arbor, MI 48015, USA
1-800.NSF.MARK | +1-734.769.8010 | www.nsf.org

# TEST REPORT

**Table 3: Organism numbers on heated lots (Raw Data).**
The following table displays the number of organisms present for coupons that received heat treatment.

| Total Log CFU/mL (all organisms) | | | | | Total Log CFU/mL (all organisms) | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Sample | Replicate | *E. coli* | *Salmonella* | Sum | Sample | Replicate | *E. coli* | *Salmonella* | Sum |
| Lot 1 | 1 | <1 | <1 | <1 | Lot 2 | 1 | <1 | <1 | <1 |
| | | | | | | | | | |
| | 2 | <1 | <1 | <1 | | 2 | <1 | <1 | <1 |
| | | | | | | | | | |
| | 3 | <1 | <1 | <1 | | 3 | <1 | <1 | <1 |
| | | | | | | | | | |
| | 4 | <1 | <1 | <1 | | 4 | <1 | <1 | <1 |
| | | | | | | | | | |
| | 5 | <1 | <1 | <1 | | 5 | <1 | <1 | <1 |
| | | | | | | | | | |
| | 6 | <1 | <1 | <1 | | 6 | <1 | <1 | <1 |
| | | | | | | | | | |
| | 7 | <1 | <1 | <1 | | 7 | <1 | <1 | <1 |
| | | | | | | | | | |
| | 8 | <1 | <1 | <1 | | 8 | <1 | <1 | <1 |
| | | | | | | | | | |
| | 9 | <1 | <1 | <1 | | 9 | <1 | <1 | <1 |
| | | | | | | | | | |
| | 10 | <1 | <1 | <1 | | 10 | <1 | <1 | <1 |
| | | | Avg. | <1 | | | | Avg. | <1 |
| | | | St.Dev. | 0.00 | | | | St.Dev. | 0.00 |

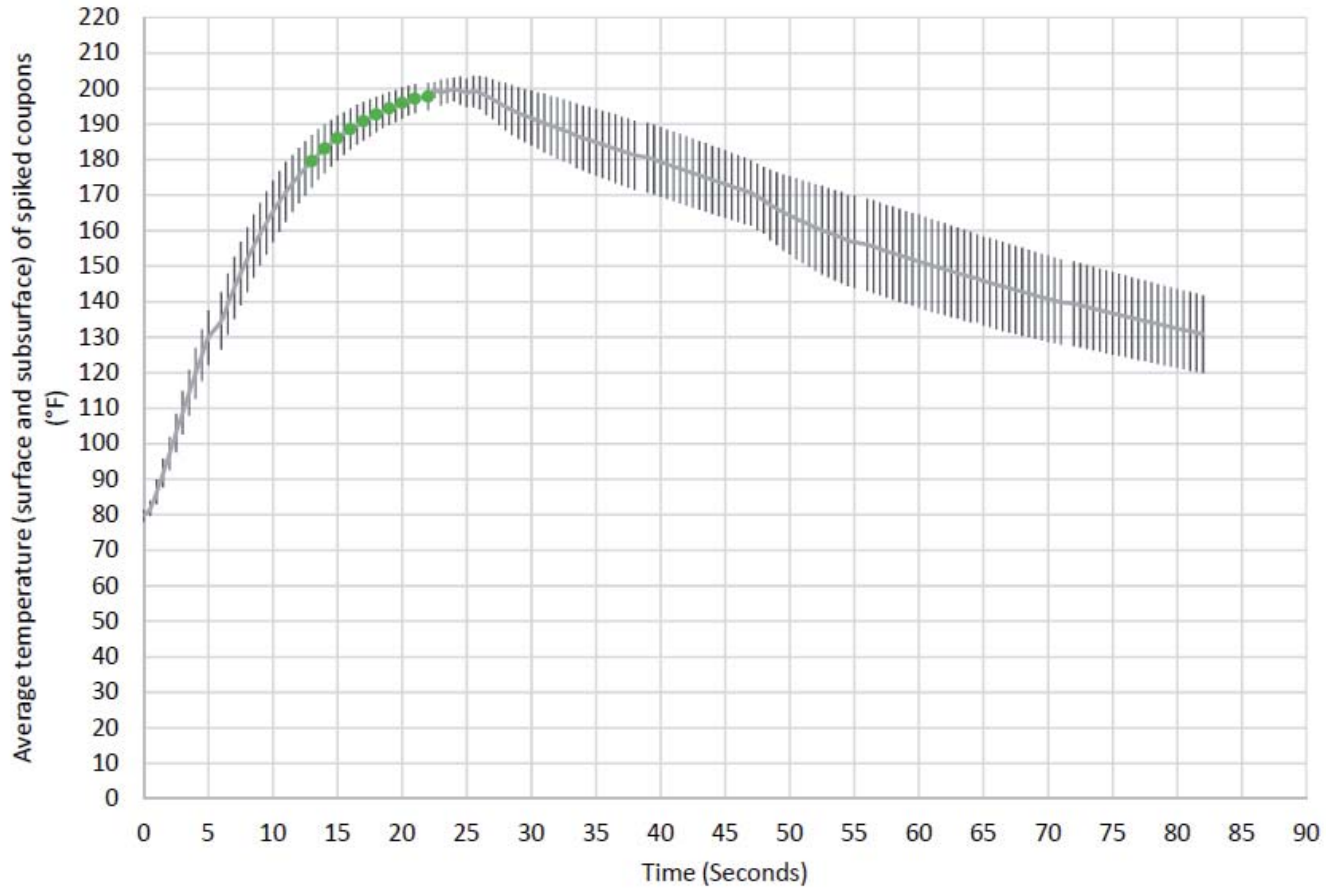



**Figure 1: Temperature profile of ideal simulated corrugation.**
The following chart shows the average ± standard deviation of 6 representative sample coupons, 3 monitored from the outer portion of the upper cardboard liner and 3 monitored from the inner portion of the upper cardboard liner). The coupons were wetted with 0.5mL of sterile BNaClPT with 50 mM Trehalose and dried for 10 minutes prior to testing. Green dots indicate the time points during the 22 second heated-plaque exposure, from 13 to 22 seconds, during which the upper cardboard liner reached the desired temperature required to simulate the corrugation process (180 – 200 °F).

**ATTACHMENT 2**

**Corrugator Effect on Microbial Contamination**
**NSF International**
**January 15, 2016**

**NSF International – Applied Research Center**

789 N. Dixboro Rd. Ann Arbor, MI 48015, USA
1-800.NSF.MARK | +1-734.769.8010 | www.nsf.org

# TEST REPORT

**Send to:** **Fibre Box Association**, a sponsor of the Corrugated Packaging Alliance
25 Northwest Point Boulevard, Suite 510
Elk Grove Village, Illinois 60007

**Result: COMPLETE** **Report Date: 15-January-2016**

| | |
|---|---|
| Customer Name: | Fibre Box Association |
| Location of Testing: | NSF Ann Arbor |
| Description: | Corrugator Effect on Microbial Contamination |
| Test Type: | Test Only |
| Job Number: | J-00205352 |
| Project Number: | 10028967 |
| NSF Corporate: | C0262787 |
| Project Manager: | J. Vantine |

**Executive Summary**:
Fibre Box Association contracted the Applied Research Center at NSF International to determine if the corrugation process is sufficient to mitigate microbial contamination on the container board that occurs prior to corrugation.
The surface of containerboard material was inoculated with a microbial challenge population of thermotolerant bacteria. This inoculated containerboard was then exposed to heat at a timed interval to simulate the corrugation process. The exposure of 150, 160 and 170 ± 10°F for 8-9 seconds was sufficient to eliminate microbial contamination.

**Thank you for working with the Applied Research Center! We hope to collaborate again with you soon!**

Please contact your Project Manager if you have any questions or concerns pertaining to this report.

**Report Authorization:**_____

Robert Donofrio – Director, Applied Research Center

**Scope of Test Report**

The surface of the containerboard material was inoculated prior to a simulation of the corrugation process with a known microbial load of a cocktail of thermotolerant organisms representative of food borne pathogens that are of concern in the produce industry.

Organism cocktail:
*Escherichia coli* ATCC 25922*
*Escherichia coli* ATCC 43890 (O157:H7)
*Escherichia coli* ATCC 51657 (O157:H7)
*Salmonella enterica* subsp. *enterica* serovar Enteritidis ATCC 13076
* According to Eblen et al. (J. Food Prot., 2005). *E. coli* ATCC 25922 is, relative to tested pathogenic *E. coli* strains, heat-labile but may be used as a surrogate to model the heat resistance of *Salmonella* strains like *Salmonella* Montevideo G4639 and *Salmonella* Poona RM 2350

The organism cocktail was applied to a 4 × 4 inch area of containerboard material (coupons) and allowed to dry for 10 minutes. 50 mM Trehalose was applied with the inoculum to protect against bacterial dessication (die-off) during the drying process (S.B. Leslie *et al.* 1995 *Appl. Environ. Microbiol.*). The corrugation process was simulated in the NSF Engineering laboratory utilizing 1 inch thick aluminum plaques and an oven. The temperature of the plaques was monitored and documented to record the required time of exposure to attain the target temperatures of 150, 160, 170 ± 10 °F for 8-9 seconds on the top-liner of the containerboard.

After simulated processing the containerboard coupons and surrounding foil press material (included to protect inoculated containerboard from contamination during the simulation) were stomached to determine the remaining population of challenge organisms.

The proposed processing regimen is as follows:

- 1 lot of container board was evaluated; (4 × 4 inch cut-outs; “coupons”).

Order of operation (designed to minimize required number of inoculated/unheated coupons)

| Coupon | Spike | Coupon target temperature (± 10°F) | Unheated controls used for decimal reduction calculation | | |
|---|---|---|---|---|---|
| 1 | Uninoculated | unheated | | | |
| 2 | Uninoculated | unheated | | | |
| 3 | Inoculated | unheated | X | | |
| 4 | Inoculated | 150 | | | |
| 5 | Inoculated | 150 | | | |
| 6 | Inoculated | 150 | | | |
| 7 | Inoculated | unheated | X | X | X |
| 8 | Inoculated | 160 | | | |
| 9 | Inoculated | 160 | | | |
| 10 | Inoculated | 160 | | | |
| 11 | Inoculated | unheated | X | X | X |
| 12 | Inoculated | 170 | | | |
| 13 | Inoculated | 170 | | | |
| 14 | Inoculated | 170 | | | |
| 15 | Inoculated | unheated | | X | X |

This workflow was initially designed to control for possible loss of challenge organism viability throughout the day of testing, since delays will be encountered as the oven is adjusted to reach the various set points. Accordingly, the decimal reduction observed for heated coupons would have been calculated based on the mean cellular density values collected from three unheated coupons that were processed in chronological order (see color coding in chart above). However, since there was no evidence of loss of challenge organism numbers over the course of the testing day, the decimal reduction observed for heated coupons was calculated based on the mean cellular density values collected from all four unheated coupons (#3, 7, 11, and 15 in the table above).

**Methodology**

Thermocouple Temperature Profile Method Development Study

1. 4” × 4”. cardboard coupons were spiked on the outer upper liner surface (rough material side) with 500 µL of sterile BNaClPT (per liter: FLUKA Peptone Hy-Soy® T, 1 g; Tween 80, 1 mL; KH2PO4, 3.6 g; Na2HPO4, 7.2 g; NaCl, 4.3 g; pH 7.0 +/- 0.2) containing 50 mM Trehalose. 50 mM Trehalose was included to protect the inoculum against dessication (die-off) during the drying process (S.B. Leslie et al.

1995 Appl. Environ. Microbiol.). The solution was immediately spread across the liner using a T-spreader and then allowed to dry for 10 minutes.

2. A thermocouple was affixed to the outer and inner portion of the upper liner of the coupons to monitor and document the temperature profile of the cardboard coupons during the heating process (≤23 seconds) and one minute of cooling.

   Note: After affixing the thermocouples, the coupon liner surface was topped with a 4" x 4" segment of heavy duty aluminum foil and then wrapped in heavy duty aluminum foil.

3. Mean temperature profiles (of three coupons monitored from the outer and inner portions of the upper liner; six profiles total) were calculated and plotted against time for each of the selected plaque temperatures.

4. This phase was deemed complete when the three plaque temperatures (monitored using a thermocouple attached to one of the two aluminum plaques) which quickly heated three consecutive replicate coupons to each of the desired target temperatures (150, 160, and 170 ± 10°F) within 14 seconds and maintains each temperature for 8-9 seconds, were identified. The selected plaque temperatures and exposure times were used in the full study.

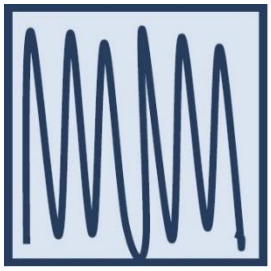
Full Study

1. For each test containerboard coupon, the following materials were UV-sterilized for ten minutes:
   a. One side of a 10" X 6" piece of heavy duty aluminum foil
   b. Both sides of a 4" x 4" piece of heavy duty aluminum foil
   c. Both sides of a 4" x 4" cardboard coupon

2. A master spike suspension mixture of the following organisms in BNaClPT + 50 mM Trehalose was created:
   a. *Escherichia coli* ATCC 25922
   b. *Escherichia coli* ATCC 43890 (O157:H7)
   c. *Escherichia coli* ATCC 51657 (O157:H7)
   d. *Salmonella enterica* ATCC 13076

3. A unique solution for each organism, with a target density of 1 x $10^9$ CFU/mL was combined in equal parts to result in the final inoculum suspension. This master suspension was used to inoculate spiked coupons. Two unspiked coupons were inoculated with the sterile diluent.

4. Using a calibrated pipette, 0.5 mL of the organism suspension mixture was inoculated onto the surface of the appropriate coupons following the pattern shown below; the inoculum was immediately spread using a T-spreader.



5. No more than two coupons were inoculated at a time and inoculations were staggered by at least 3 minutes to minimize variability in drying and processing time.

6. Coupons were allowed to dry for ten minutes and then covered with the 4" X 4" piece of corresponding sterile aluminum foil.

7. The large piece of aluminum foil was folded to cover the samples that are to be heated:

    a. Covered coupons were placed between two pre–heated aluminum blocks held at one of the three oven set points validated in method development.
    b. After the pre-determined hold time, the coupons were removed, allowed to rest for 1 minute and then processed using the procedure in step 9 (below)

8. The unheated coupons along with the 4" × 4" piece of corresponding aluminum foil were placed in a pre-labeled stomacher bag that contained 100 mL of room temperature Letheen broth and stomached for 30 seconds.

9. The letheen broth used to elute residual viable organisms from the unheated samples was diluted to $10^{-3}$, $10^{-4}$, and $10^{-5}$ and plated on 3M™ Petrifilm *E.coli*/Coliform Count Plates for detection and quantification of *E. coli* and to XLD agar for detection and quantification of *Salmonella.*

10. The letheen broth used to elute residual viable organisms from the heated samples was diluted to $10^{-1}$, and $10^{-2}$. A neat (undiluted sample of the broth along with the $10^{-1}$, and $10^{-2}$ dilutions were then plated on 3M™ Petrifilm *E.coli*/Coliform Count Plates for detection and quantification of *E. coli* and to XLD agar for detection and quantification of *Salmonella.*

11. The 3M™ Petrifilm *E.coli*/Coliform Count Plates and XLD agar plates were incubated aerobically for 48 ± 4 hours at 35 ± 1°C and 36 ± 1°C, respectively. XLD agar plates were pulled as early as 24 h if deemed appropriate to achieve the most accurate colony count.

### Results and Discussion

The challenge organism density on the inoculated, unheated coupons was 7.68 ± 0.30 and 7.16 ± 0.38 Log CFU per coupon for *E. coli Salmonella*, respectively. Neither challenge organism were recovered from control coupons spiked only with sterile diluent.

As can be seen from the data shown in Table 1 in Appendix A, the reduction of total cells from the cardboard coupons after exposure to the lowest target temperature of $150^0$ F had a greater than 99.995% reduction of viable cells detected. Table 3 provides that plating summary showing that only *E.coli* organisms were recovered after inoculated samples were heated at the $150^0$ and $160^0$ F targeted exposure. Exposure of *E.coli* at $170^0$ F and *Salmonella* at all target temperatures were less than the sampling detection limit.

Raw data are provided in Appendix A, including the temperature profile for the test as well as raw data averages for each sample tested.

## Appendix A

## Result Tables and Figures

**Table 1: Reduction in viable organisms from the heating process.**
The following table displays the number of organisms present after heat treatment and for coupons that received no heat treatment. Reduction percentages are shown as a calculation of heated organisms remaining to unheated.

| | Viable cells detected on coupons (Mean ± SD) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Total (*E. coli* and *Salmonella*) | | | *E. coli* | | | *Salmonella* | | |
| Target Coupon Temperature (°F) | Unheated (Log CFU) | Heated (Log CFU) (a,b) | Reduction (%) | Unheated (Log CFU) | Heated (Log CFU) (a) | Reduction (%) | Unheated (Log CFU) | Heated (Log CFU) (b) | Reduction (%) |
| 150 ± 10 | 7.80 ± 0.31 | 3.46 ± 0.70 | 99.995 | 7.68 ± 0.30 | 3.46 ± 0.70 | 99.994 | 7.16 ± 0.38 | <2.00 ± 0.00 | > 99.9993 |
| 160 ± 10 | | 2.21 ± 0.88 | 99.9997 | | 2.21 ± 0.88 | 99.9997 | | <2.00 ± 0.00 | > 99.9993 |
| 170 ± 10 | | <2.00 ± 0.00 | >99.9998 | | <1.70 ± 0.00 | >99.9999 | | <2.00 ± 0.00 | > 99.9993 |

[a]The limit of detection for *E. coli* was 50 CFU/coupon (1.70 Log CFU/coupon)(i.e., 50 CFU of viable challenge organism could have survived the simulated corrugation procedure and would not have been detected by the enumeration protocol).
[b]The limit of detection for *Salmonella* was 100 CFU/coupon (2.00 Log CFU/coupon) (i.e., 100 CFU of viable challenge organism could have survived the simulated corrugation procedure and would not have been detected by the enumeration protocol).

**Table 2: Organism numbers on unheated lots (Raw Data).**
The following table displays the number of organisms present for coupons that received no heat treatment.

| Inoculated Replicate | CFU / Coupon | Log CFU / coupon (Mean) | Log CFU / coupon (SD) |
|---|---|---|---|
| ***E.coli*** | | | |
| 1 | 8.35E+07 | 7.68 | 0.30 |
| 2 | 3.80E+07 | | |
| 3 | 2.03E+07 | | |
| 4 | 8.25E+07 | | |
| ***Salmonella*** | | | |
| 1 | 2.15E+07 | 7.16 | 0.38 |
| 2 | 6.95E+06 | | |
| 3 | 7.00E+06 | | |
| 4 | 4.00E+07 | | |

**Table 3: Organism numbers on heated lots (Raw Data).**
The following table displays the number of organisms present for coupons that received heat treatment.

| Target Coupon Temperature (°F) | Replicate | CFU / coupon | Log CFU / coupon (Mean) | Log CFU / coupon (SD) | Summary |
|---|---|---|---|---|---|
| ***E.coli*** | | | | | |
| 150 ± 10 | 1 | 6.50E+03 | 3.79 | 0.13 | **Log Reduction** |
| | 2 | 4.50E+03 | | | 3.89 |
| | 3 | 8.25E+03 | | | **% Kill** |
| | | | | | 99.987% |
| 160 ± 10 | 1 | <50 | 2.21 | 0.88 | **Log Reduction** |
| | 2 | <50 | | | 5.48 |
| | 3 | 1.65E+03 | | | **% Kill** |
| | | | | | 99.9997% |
| 170 ± 10 | 1 | <50 | 1.70 | >0 | **Log Reduction** |
| | 2 | <50 | | | 5.98 |
| | 3 | <50 | | | **% Kill** |
| | | | | | >99.9999% |
| ***Salmonella*** | | | | | |
| 150 ± 10 | 1 | <100 | 2 | >0 | **Log Reduction** |
| | 2 | <100 | | | 5.16 |
| | 3 | <100 | | | **% Kill** |
| | | | | | >99.9993% |
| 160 ± 10 | 1 | <100 | 2 | >0 | **Log Reduction** |
| | 2 | <100 | | | 5.16 |
| | 3 | <100 | | | **% Kill** |
| | | | | | >99.9993% |
| 170 ± 10 | 1 | <100 | 2 | >0 | **Log Reduction** |
| | 2 | <100 | | | 5.16 |
| | 3 | <100 | | | **% Kill** |
| | | | | | >99.9993% |

[a] The detection limit per coupon for *E.coli* is 50 CFU/Coupon. For samples with no detectable growth ("<50"), a value of 50 was used for calculating an average. Thus, these values are considered "less than or equal to" the reported mean. For samples with no detectable growth ("<50"), a value of 50 was used for calculating standard deviations. Thus, these values are considered "greater than or equal to" the reported standard deviation.
[b] The detection limit for *Salmonella* is 100 CFU/Coupon. For samples with no detectable growth ("<100"), a value of 100 was used for calculating an average. Thus, these values are considered "less than or equal to" the reported mean.
For samples with no detectable growth ("<100"), a value of 100 was used for calculating standard deviations. Thus, these values are considered "greater than or equal to" the reported standard deviation.

**Table 4: Coupon exposure duration.**
The total time that the coupons were exposed to the heated blocks in order to heat the coupons to (but not beyond) each target temperature range for a total of 8-9 seconds. These durations were validated in the temperature profile testing phase of this study.

| Target Coupon Temperature (°F) | Block Temperature (°F) | Coupon Exposure Duration (seconds) |
|---|---|---|
| 150 ± 10 | 160° | 17 |
| 160 ± 10 | 170° | 19 |
| 170 ± 10 | 190° | 15 |

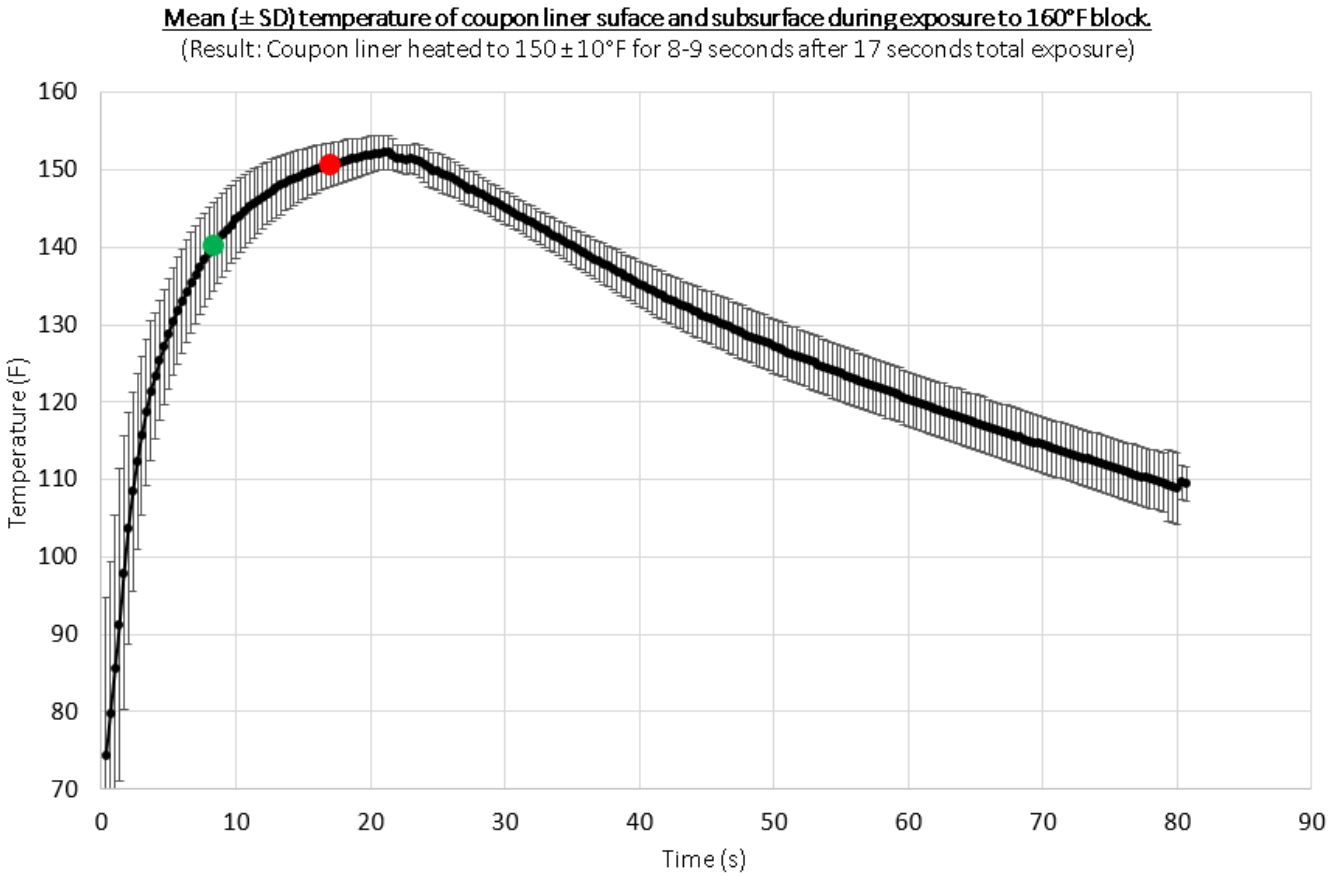



**Figure 1: Temperature profile of simulated corrugation for target temperature of 150° ± 10.**
This chart shows the average ± standard deviation of 3 representative sample coupons, monitored from the outer portion of the upper cardboard liner and from the inner portion of the upper cardboard liner). The coupons were wetted with 0.5mL of sterile BNaClPT with 50 mM Trehalose and dried for 10 minutes prior to testing. Green dot indicates the beginning of coupon liner exposure in the target temperature range (8.3 seconds; 140.1°F). Red dot indicates the end of coupon liner exposure in the target temperature range (17 seconds 150.7°F).

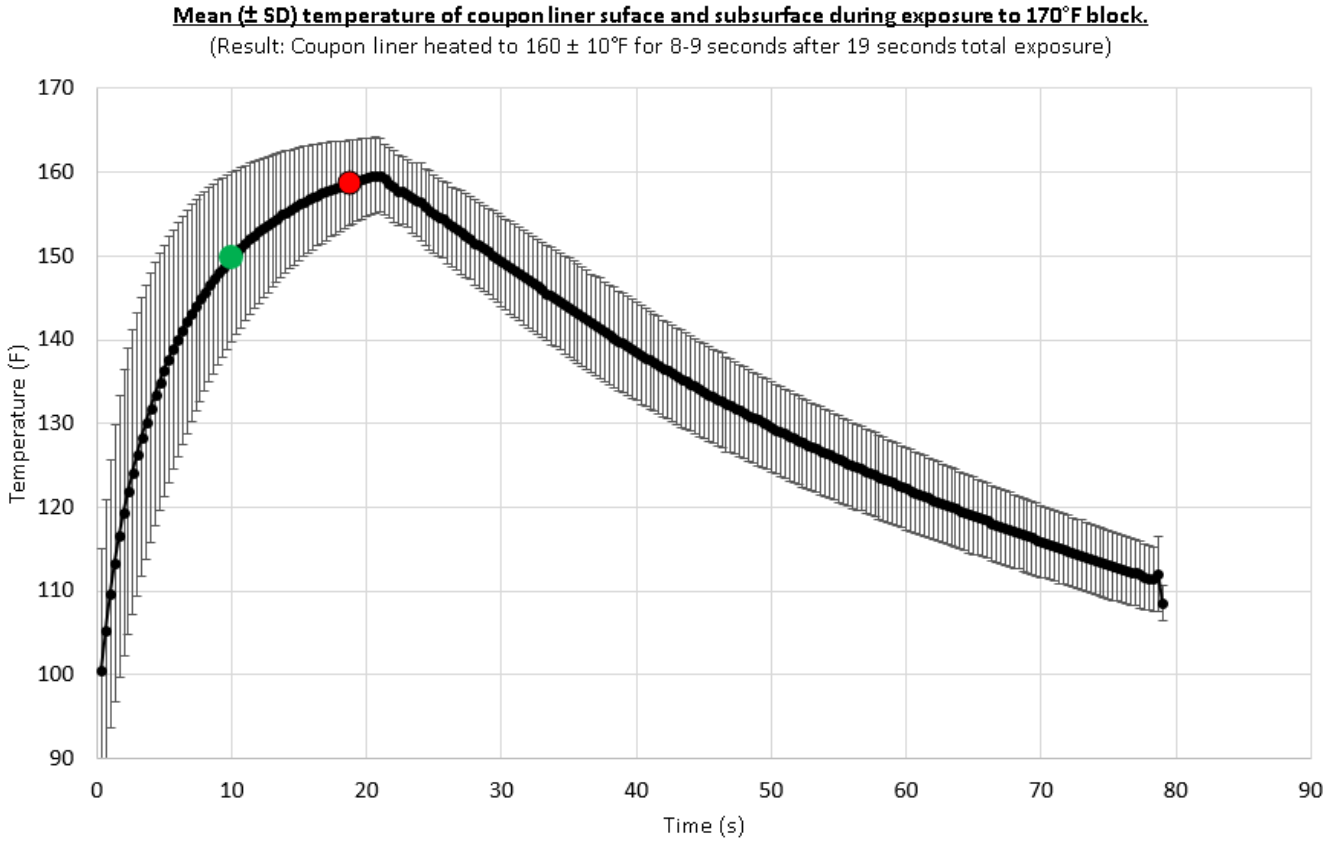



**Figure 2: Temperature profile of simulated corrugation for target temperature of 160° ± 10.**
This chart shows the average ± standard deviation of 3 representative sample coupons, monitored from the outer portion of the upper cardboard liner and from the inner portion of the upper cardboard liner). The coupons were wetted with 0.5mL of sterile BNaClPT with 50 mM Trehalose and dried for 10 minutes prior to testing. Green dot indicates the beginning of coupon liner exposure in the target temperature range (10.0 seconds; 149.88°F). Red dot indicates the end of coupon liner exposure in the target temperature range (19 seconds 158.86°F).

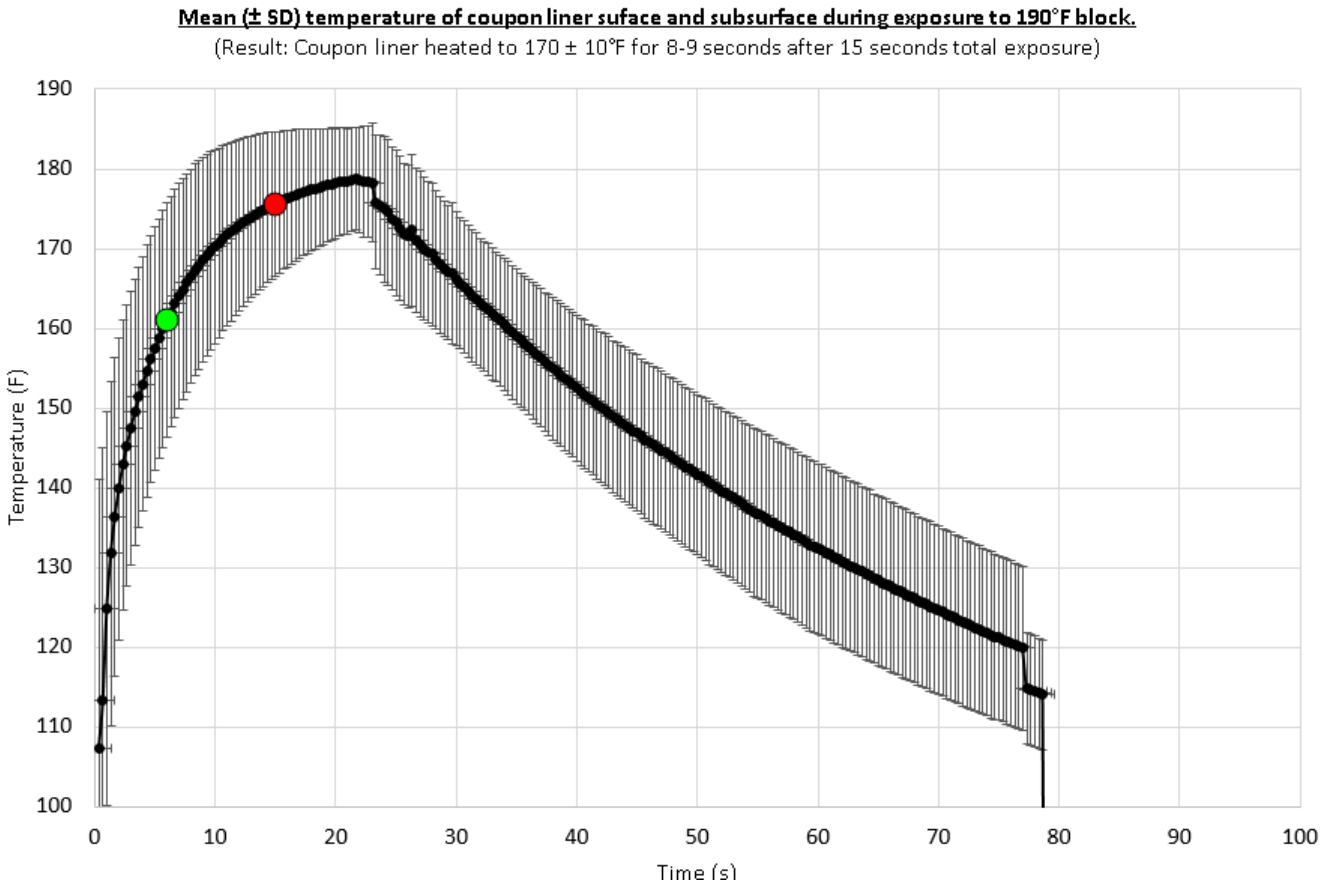



**Figure 3: Temperature profile of simulated corrugation for target temperature of 170° ± 10.**
This chart shows the average ± standard deviation of 3 representative sample coupons, monitored from the outer portion of the upper cardboard liner and from the inner portion of the upper cardboard liner). The coupons were wetted with 0.5mL of sterile BNaClPT with 50 mM Trehalose and dried for 10 minutes prior to testing. Green dot indicates the beginning of coupon liner exposure in the target temperature range (6.0 seconds; 161.00°F). Red dot indicates the end of coupon liner exposure in the target temperature range (15 seconds 175.59°F).