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Validation of the Bio-Response Solutions Human-28 Low-Temperature Alkaline Hydrolysis System

Gerald A. Denys[1]

Abstract

Introduction: High temperature alkaline hydrolysis (AH) is recognized as an alternative method for sterilization and disposition of animal carcasses and human remains. The aim of this study is to validate the low temperature (LT) AH process specific to its use in the Bio-Response Solutions, Inc. Human-28 LT System.

Methods: A 313-lb pig was process using the manufacturers recommended cycle parameters. Stainless steel sample vials containing spore suspensions of *Geobacillus stearothermophilus* were implanted into the pig's deep tissue to validate the efficacy of the process conditions. Spore suspensions of *Bacillus thuringiensis* was suspended in the vessel headspace to validate sterilization. The spore challenge was greater than the recommended 10^6 log used to determine sterilization. MALDI-TOF mass spectrometry analysis was used to validate the destruction of prion-sized particles in processed effluent.

Results: Complete inactivation of spores and digestion of animal tissue were achieved after processing in the Bio-Response Solutions Human-28 LT Alkaline Hydrolysis System. Complete inactivation of spores was achieved when exposed to heat in the animal carcass and headspace. No peptide fragments larger than 2500 Da were observed in the treatment effluent.

Discussion: The Bio-Response Solutions, Inc. Human-28 LT Alkaline Hydrolysis System was as effective as high-temperature alkaline hydrolysis for use on animal and human tissue.

Conclusion: LT AH for tissue and bodies exceeded the sterility assurance level III of the US State and Territorial Association on Alternative Treatment Technologies and sterility requirements for animal biosafety level–3 and –4 facilities. LT AH process validated destruction of prion-sized particles.

Keywords

alkaline hydrolysis, low temperature, sterilization, *Geobacillus stearothermophilus*, *Bacillus thuringiensis*, prions

Alkaline hydrolysis is recognized as an acceptable method for sterilization and disposition of animal carcasses and human remains.[1] Recently, alkaline hydrolysis has been considered an alternative method to cremation by incineration of humans and pets.[2] Alkaline hydrolysis is a thermochemical process used to breakdown proteins, fats, and carbohydrates. The remains of this process are liquid effluent and solid inorganic material of the bone and calcium phosphate.[3] Studies have shown that alkaline hydrolysis is effective in eliminating infectious agents, including infectious prion-size particles[1,4-6] and dissolving animal carcasses.[7]

The alkaline hydrolysis method for tissue disposal meets the sterility assurance level III of the US State and Territorial Association on Alternative Treatment Technologies (STAATT) that requires inactivation of bacteria, fungi, viruses, parasites and mycobacteria at 10^6 log reduction and inactivation of sporeforming bacteria at 10^4 log reduction.[8] The alkaline hydrolysis method also meets the sterility requirements for animal biosafety level 3 and 4 facilities which requires 10^6 log reduction of *G stearothermophilus*.[9]

Matrix assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) has emerged as a highly reliable technology for the evaluation of peptide fragment sizes. MALDI-TOF MS is advantageous for qualitative mass spectrometry due to its high dynamic range and resolving power to evaluate protein structure based on mass to charge ratios.[10] Infectious prion proteins are detectable by MALDI-TOF MS above 19 kDa, but have not been observed below10 kDa.[11]

Bio-Response Solutions, Inc (Danville, IN) is a commercial manufacturer of both high-temperature (HT) and low-temperature (LT) alkaline hydrolysis systems. The Bio-Response Solutions HT process is carried out in a stainless-steel vessel with measured parameters including a specified alkali

[1] Indiana University, Department of Pathology and Laboratory Medicine, University Hospital, School of Medicine, Indianapolis, IN, USA

Corresponding Author:
Gerald A. Denys, Indiana University, Department of Pathology and Laboratory Medicine, University Hospital, School of Medicine, 550 University Blvd., AOC Room 6129, Indianapolis, IN 46202 USA.
Email: gdenys@iupui.edu

Figure 1. Bio-Responses Solutions, Inc. Human-28 Low-Temperature Alkaline Hydrolysis System. The multistage process is carried out in a stainless-steel vessel with measured parameters including a specified alkali molarity, water-to-tissue ratio, rate of solution circulation, temperature, and operating time. A mesh basket is inserted into the vessel to hold the animal carcass. (Left) The mechanical control box is located on the far right. (Right) The vessel containing a mixing paddle is tilted into the operating position.

molarity, water-to-tissue ratio, rate of solution circulation, temperature, and operating time. The HT system operates at greater than 100°C (212°F) and must operate under pressure to achieve higher than boiling temperatures. In comparison, LT alkaline hydrolysis operates below 100°C (212°F), operates at atmospheric pressure, and does not require specialized pressure vessel equipment (Figure 1). LT alkaline hydrolysis is less expensive, safer to operate, and requires lower energy consumption than HT alkaline hydrolysis. However, LT alkaline hydrolysis does require longer processing time.[1,12] The purpose of this efficacy study is to validate the actual process parameters of the Bio-Response Solutions, Inc. Human-28 Low-Temperature Alkaline Hydrolysis System for animal and human use by inactivation of biological indicators, proteomic analysis of liquid effluent, and complete digestion of a large-animal surrogate carcass.

Materials and Methods

Study Design

This study addressed three separate experimental areas of concern in one processing run and was based on published literature for the validation of alkaline hydrolysis tissue treatment,[1,13,14] spore inactivation,[15] and prion destruction.[16] The objectives of these experiments are to demonstrate (1) sterilization of *Geobacillus stearothermophilus* under LT process conditions, (2) sterilization of *Bacillus thuringiensis* in the vessel headspace and LT process conditions in the absence of any chemical, and (3) absence of prion-size particles in the LT processed effluent.

Biological Indicators

A spore suspension of *G stearothermophilus* (7953) was obtained from Mesa Labs (Bozeman, MT) containing 1.8×10^7 spores/0.1 mL. A spore suspension of *B thuringiensis* (Thuricide) was obtained from Bonide (Oriskany, NY) containing at least 6 log spores/mg. *B thuringiensis* was used as a surrogate for *Bacillus anthracis*. The Bio-Response Solutions, Inc. Human-28 LT System process parameters does not allow for use of available standard biological indicator ampules.

Sample Test Vessels

A total of 9 sealed stainless-steel sample vials were used in this study (Table 1). Sample vials A to D contained tissue, water, alkali, and *G stearothermophilus* spore suspensions to simulate a range of tissue weights and molarities. The manufacturer's standard recommended chemical chart is presented in Table 2. Sample vials G and H were used as recovery controls of *G stearothermophilus* with and without tissue and held at room temperature. Sample vials E, F, and I contained *B thuringiensis* spore suspensions only. Sample vial E was exposed to heat in the pig carcass without alkali added. Sample vial F was suspended above the vessel headspace inside the Bio-Response Solution, Inc Human-28 LT System. Sample vial F was used to test the ability of the system to sterilize the air space inside the system, which is outside of the liquid process. Sample vial I was used for recovery control and held at room temperature.

Animal Tissue

A 313-lb pig carcass was used as surrogate for human tissue. The source of the pig was from a local farm in Indiana that had died of natural causes. The pig carcass was placed on ice prior to testing to simulate a refrigerated body condition. Stainless-steel sample vials containing biological indicators (BIs) were implanted into the pig's deep tissue. Refrigerated pork was also used in sample vials A-D and G to mimic experiment conditions of digestion of animal tissue and spore inactivation (Table 1).

Processing Cycle

The Bio-Response Solutions, Inc. Human-28 LT System consists of a stainless steel vessel with measured parameters including a specified alkali molarity, water-to-tissue ratio, rate of solution circulation, temperature, and operating time. The alkaline hydrolysis treatment is a multistage process that uses a combination of heat, time, atmospheric pressure, and high pH, rendering the animal carcass into a liquid effluent that can be drained into the sanitary sewer (Table 3). The operational parameters for the inactivation of a 313-lb pig were those recommended by the manufacturer.[12] The alkali concentration added was 40.69 lb of 90% anhydrous KOH (Tianjin Red Triangle, China) calculated at 13% tissue weight. The operator must calculate the amount of KOH based on the weight of the carcass and manually add this to the system. Manufacturer recommend PPE was used to handle the chemical as per practices in accordance with federal OSHA rules.

Table 1. Sample Vial Content.

Vial	Tissue, g	Water, mL/g	KOH, g	Spores, mL	Water-to-Tissue Ratio	Molarity of KOH	Location	Description
Geobacillus stearothermophilus								
A	0.74	8.08	0.18	1.0	10.89	0.50	Body	Simulates smallest body and lowest molarity[a]
B	1.78	6.99	0.23	1.0	3.94	0.78	Body	Simulates cycle actually run in the trial with 313-lb pig[a]
C	2.04	6.70	0.26	1.0	3.29	0.94	Body	Simulates largest body and highest molarity[a]
D	0.75	8.15	0.10	1.0	10.89	0.28	Body	Simulates smallest body and lowest molarity[b]
G	1.78	7.22	0.00	1.0	3.94	0.78	Room temperature	Control: Simulates cycle conditions actually run with 313-lb pig including tissue
H	0.00	9.00	0.00	1.0	N/A	0.00	Room temperature	Control: spores without tissue
Bacillus thuringiensis								
E	0	0	0	25	N/A	N/A	Body	Heat only
F	0	0	0	25	N/A	N/A	Headspace	Heat only
I	0	0	0	25	N/A	N/A	Room temperature	Control spores

[a] Based on current Chemical Chart supplied by manufacturer for recommended use.
[b] Based on previous version Chemical Chart B supplied by manufacturer for recommended use which may still be in use by some operators.

Table 2. Manufacturer's Recommended Chemical Specifications.

Tissue Weight, lb	90% KOH (g)	KOH% of Wt	Inches of Water in Vessel	Gallons of Water	Water-to-Tissue Ratio	Molarity of KOH
80	18.90	24%	30.00	105.00	10.89	0.5
313	40.69	13%	37.00	148.03	3.94	0.78
500	65.00	13%	45.04	198.25	3.29	0.94
80	10.40	13%	30.00	105.00	10.89	0.28

Table 3. Summary of Processing Cycle.

Stage	Time	Temperature
Add 90% anhydrous KOH		
Fill water to system vessel	00:30 min	59°F
Heat to processing temperature	00:40 min	59°F–204°F
Process	14:00 h	204°F
Paused to collect liquid effluent		
Drain fluid	00:35 min	204°F
Fill water to system vessel	00:30 min	59°F
Cold rinse	00:30 min	59°F–81°F
Drain fluid	00:35 min	—
Total test	17:20 h	

Table 4. Inactivation of *Geobacillus stearothermophilus* and *Bacillus thuringiensis* Spores after Processing in the Bio-Response Solutions Human-28 Low-Temperature Alkaline Hydrolysis System.

		Growth Observations (48–72 h)	
Vial	Location	Trypticase Soy Broth	Brain Heart Infusion Agar Colony Counts, CFU/mL
Geobacillus stearothermophilus			
A	Body: Left hind quarter	Negative	0
B	Body: Left shoulder tissue	Negative	0
C	Body: Right hindquarter	Negative	0
D	Body: Left hind quarter	Negative	0
G	Control: Room temperature	Positive	2.6×10^7
H	Control: Room temperature	Positive	1.4×10^8
Bacillus thuringiensis			
E	Body: Neck	Negative	0
F	Headspace	Negative	0
I	Control: Room temperature	Positive	4.5×10^{10}

A total of 148.43 gallons (561.86 L) of water was programmed and added to give a final molarity of 0.78 M KOH. The heat to processing temperature was set at 95.5°C (204°F), and the duration of this treatment time was set to 14 hours. The entire processing cycle duration was 17:20 hours from start to finish. The animal processing was performed at the manufacturer's site.

Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry

At the end of the 14-hour treatment time (15:10 hours including water fill and heat-up) the cycle was paused, the door opened to collect a composite sample of liquid effluent from the processing vessel, and the cycle resumed. Sample collection was necessary at this point because the equipment drains and performs clean water rinsing after this point. Liquid effluent were collected as grab samples, using several grabs at various liquid levels to create a composite sample. The composite sample was then

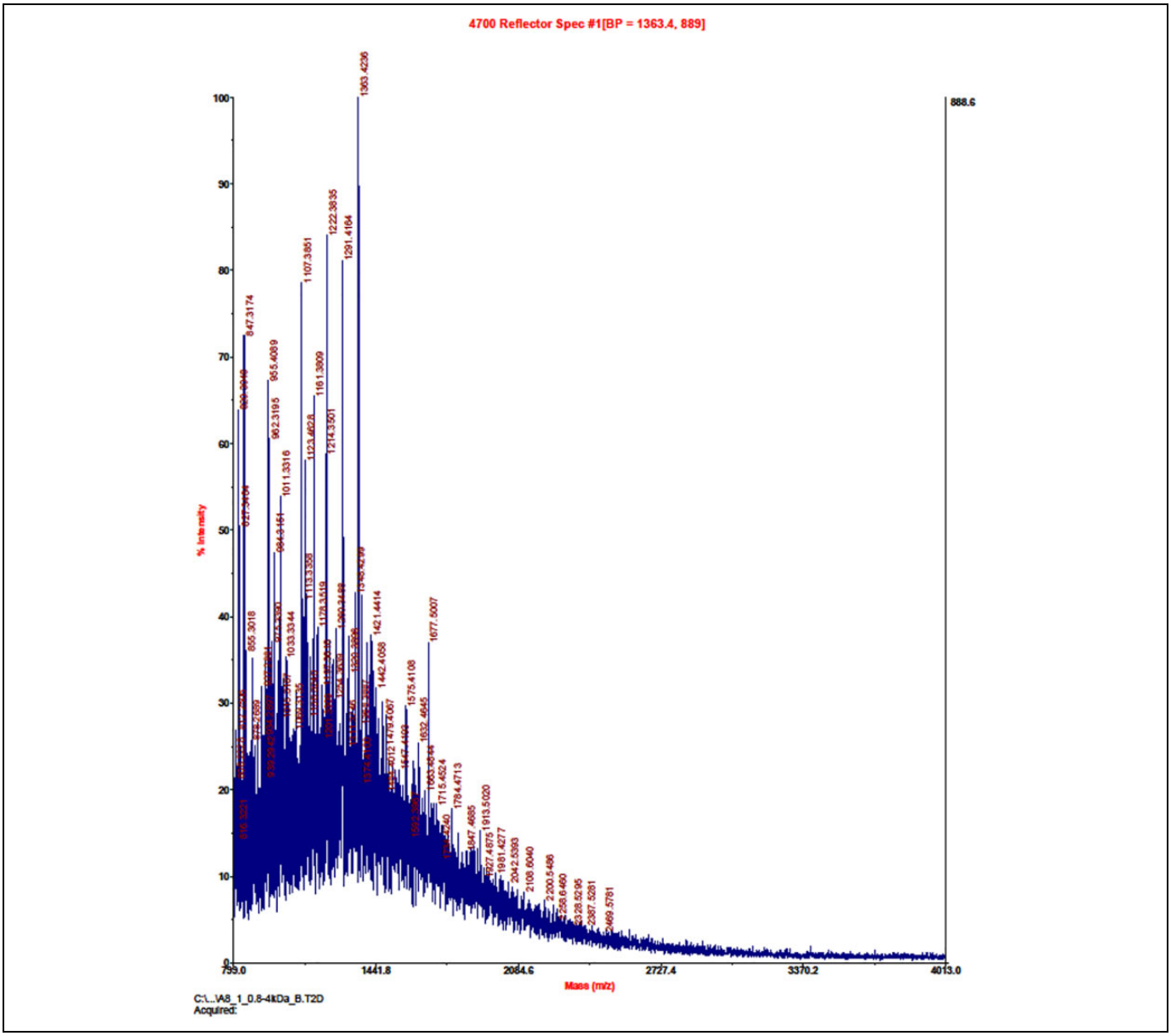

Figure 2. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MS) analysis of the effluent sample at 14 hours. MS spectra were analyzed for 0.8 to 4.0 kDa. The highest peptide fragment present in the sample was identified to be 2469.5781 Da in size.

placed on ice and sent immediately via courier for analysis to the Purdue University Proteomics Facility (Bindley Bioscience Center, Purdue University, West Lafayette, IN). The laboratory performed matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) analysis to determine the largest peptide sizing in the liquid fraction during the process. MS spectra were run in positive reflector and linear modes using alpha-cyano-4-hydroxycinnamic acid as a matrix. The sample was cleaned with C18 Zip Tip before being spotted on a MALDI plate. The sample was mixed in a 1:1 ratio (v/v) with the matrix solution. The MALDI 4800 TOF/TOF Analyzer from Sciex (formerly Applied Biosystems) was used to analyze these samples. MS spectra were collected in positive reflector and linear modes to cover the mass ranges of 0.8 to 4 kDa, 2 to 20 kDa, and 20 to 200 kDa. The MALDI analyzer was calibrated with standards provided by the manufacturer.

Bacterial Culture

Upon completion of the processing cycle, sealed stainless steel sample vials containing BIs were retrieved from the system. Vials were then mixed and immediately transported to Indiana University Health Pathology Laboratory (Indianapolis, IN) on ice. Cultures were performed in a biosafety cabinet. A 100-µL aliquot from each sample vial was placed in recovery broth (trypticase soy broth). An additional 100-µL aliquot of sample was serially diluted in sterile water and plated in duplicate onto brain heart infusion agar plates. The plates and tubes were

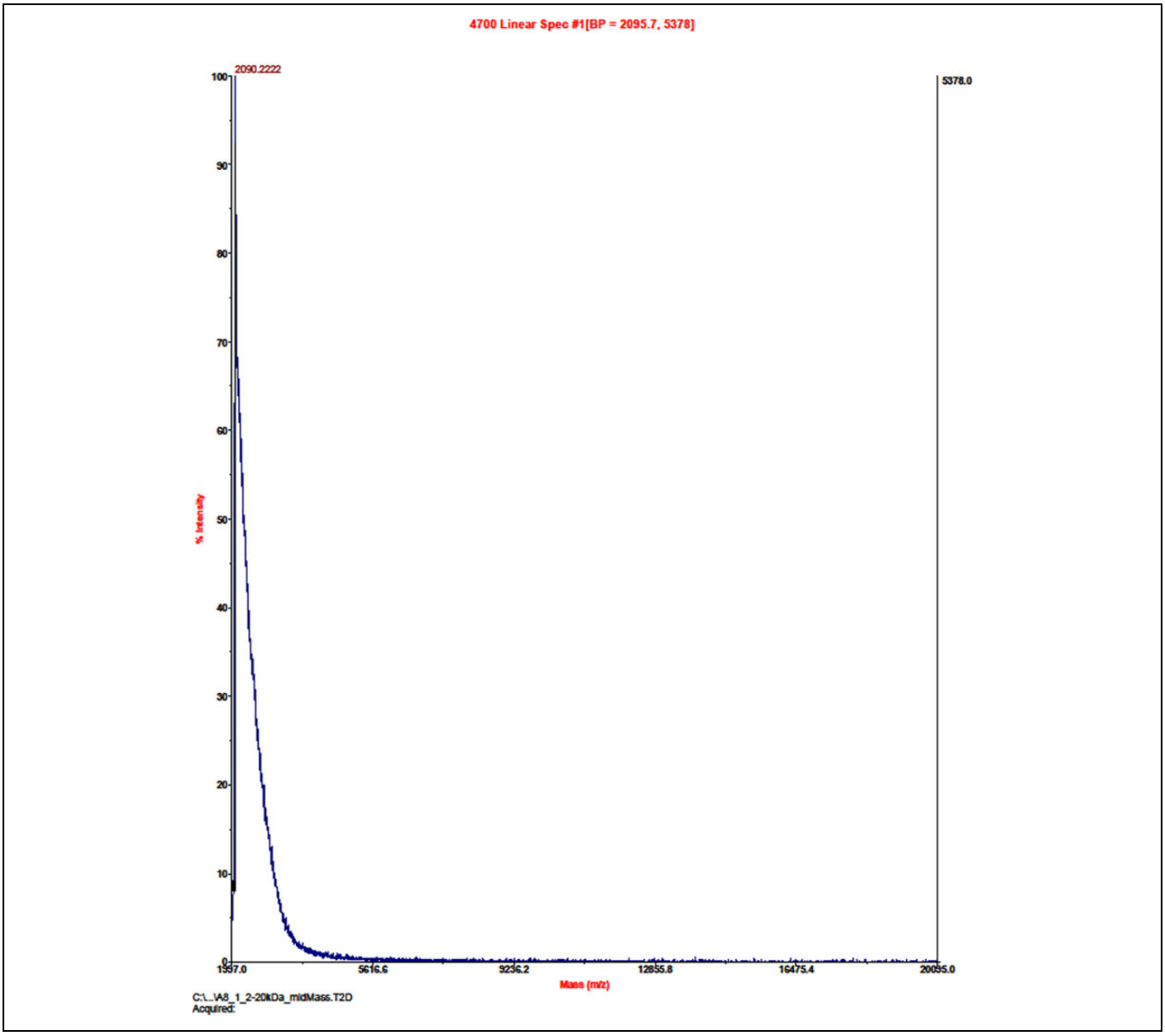

Figure 3. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MS) analysis of the effluent sample at 14 hours. MS spectra were analyzed for 2.0 to 20.0 kDa. No fragments greater than ~2500 Da in size were present.

incubated at 58°C (136.4°F) and 35°C (95°F) for *G stearothermophilus* and *B thuringiensis*, respectively, for 48 to 72 hours. After incubation, the plates were evaluated for spore colonies (CFU/mL) and compared with recovery control spore counts tested under similar conditions.

Results

BIs and Animal Tissue

Table 4 summarizes the results of spore testing after a 14-hour processing cycle in the Bio-Responses Solutions, Inc. Human-28 LT Alkaline Hydrolysis System. The standard LT processing cycle inactivated *G stearothermophilus* spore suspensions combined with tissue (vial B). When spore suspensions of *G stearothermophilus* were diluted in KOH and tissue representing low body weight and low molarity (vials A and D) and high body weight and high molarity (vial C), spores were completely inactivated. No colonies were observed on isolation plates or in recovery broth from the test vials. In contrast, simulated controls in the presence or absence of tissue had spore counts of 2.6×10^7 CFU/mL (vial G) and 1.4×10^8 CFU/mL (vial H), respectively. Spore suspensions of *B thuringiensis* were implanted in the animal carcass (vial E) and vessel headspace (vial F) without the addition of KOH and tissue. Heat alone from the standard LT processing cycle inactivated *B thuringiensis* spores. No colonies were observed on isolation plates or in recovery broth from the

test vials, whereas the simulated control spore count was 4.5×10^{10} CFU/mL (vial I). Complete digestion of pig carcass was achieved after the 14-hour processing cycle.

MALDI-TOF MS Analysis

The results of MALDI-TOF MS testing of the composite samples of liquid effluent from the processing vessel after 14 hours validates peptide destruction. Most peptide fragments in the effluent ranged from 1.1 to 1.8 kDa in size. Samples analyzed at 0.8 to 4 kDa showed the largest peptide size to be 2469.5781 Da (Figure 2). No peptide fragments greater than 2500 Da in size were observed. Samples analyzed at 2 to 20 kDa (Figure 3) and 20 to 200 kDa (not shown) did not identify the presence of higher fragments present in the samples. Complete digestion of peptides below 2500 Da is well below the standard range for prions (19-26 kDa).

Discussion

The finding of this study demonstrated complete inactivation *G stearothermophilus* and *B thuringiensis* spores when tested in the Bio-Response Solutions, Inc. Human-28 LT Alkaline Hydrolysis System. *G stearothermophilus* spores were chosen as the challenge BI because they are more highly resistant to moist heat than *B subtilus*.[17] LT alkaline hydrolysis achieved sterilization using the manufacturer's recommended process cycle and selected test conditions. Test conditions represented by simulated smallest body weight, simulated largest body weight, and simulated smallest body weight with lowest molarity were chosen to represent the worst-case scenarios for alkaline hydrolysis that could be encountered in the funeral industry. Implanting spores at various internal locations within the pig carcass broadened the range of temperatures tested. The use of a chilled pig carcass ensured complete digestion at the end of the processing cycle. These validation studies indicate that LT alkaline hydrolysis not only can fully digest a large animal carcass but also can inactivate high microbial loads within contaminated carcasses under various test conditions.

Heat alone produced in the process cycle achieved complete inactivation of *B thuringiensis* in the headspace and body cavity. *B thuringiensis* spores are considered the overall best surrogate for *B anthracis* spores, or Anthrax.[18,19] Bacterial spores are more resistant than any other type of human microorganism. *B thuringiensis* is also considered an acceptable BI for validation testing of heat inactivation for the range of temperatures used in LT alkaline hydrolysis as well as demonstrate the ability to sterilize human infectious agents.[15]

The process cycle in the Bio-Response Solutions, Inc. Human-28 LT Alkaline Hydrolysis System was able to break down tissue and protein material into peptides smaller than the smallest infectious prion particle.[16,20] The demonstration that extremely small fractions of proteins and no polypeptide chains are present in a sample of digestate has been accepted as sufficient evidence of the absence of infectious prions.[20,21] Previous studies have provided further evidence for alkali inactivation of prions at subboiling temperatures.[22-26] Liquid effluents from the Bio-Response Solutions, Inc. LT Alkaline Hydrolysis process are unlikely to contain viable infectious agents.

In conclusion, results of this study using LT alkaline hydrolysis for tissue and bodies exceeded the sterility assurance level III of the US State and Territorial Association on Alternative Treatment Technologies (STAATT). Second, the results of this study exceeded the sterility requirements for animal biosafety level–3 and –4 facilities. Finally, the data showed that prion-sized proteins could not survive the LT alkaline hydrolysis process under real-world conditions using the Bio-Response Solutions, Inc. Human-28 LT Alkaline Hydrolysis System.

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Ethical Approval Statement

Not applicable

Statement of Human and Animal Rights

Not applicable

Statement of Informed Consent

Not applicable

Declaration of Conflicting Interests

The author declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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ORCID iD

Gerald A. Denys https://orcid.org/0000-0003-1627-4818

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