I&EC research
Industrial & Engineering Chemistry Research

Article

Cite This: Ind. Eng. Chem. Res. 2018, 57, 4680−4687

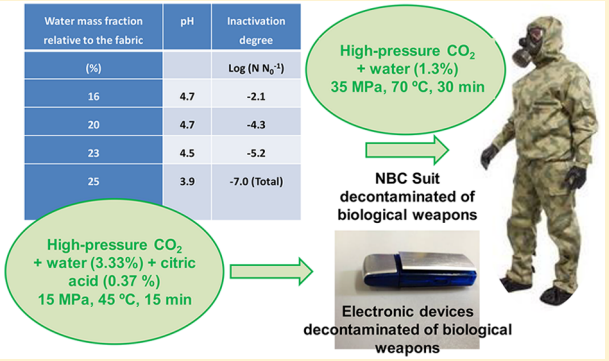
pubs.acs.org/IECR

Sterilization of Biological Weapons in Technical Clothing and Sensitive Material by High-Pressure CO_2 and Water

Lourdes Calvo* and Javier Casas

Departamento de Ingeniería Química, Universidad Complutense de Madrid, Avenida Complutense s/n, 28040 Madrid, Spain

ABSTRACT: This work explores the effectiveness of high-pressure CO_2 on inactivation of *Bacillus thuringiensis* spores as a surrogate of *B. anthracis* spores (anthrax). The investigation was done in combatant equipment and several electronic devices. It was possible to sterilize this sensitive material by the environmentally friendly mixture formed by high-pressure CO_2 and water. The presence of water in mass proportions ranging from 20 to 30% relative to the material was essential. However, it was continuously introduced in small amounts (<4% in relation to CO_2), entrained by the CO_2, so as not to have an adverse impact on materials. A low external pH enhanced the inactivation. Therefore, by using CO_2 mixed with water acidified with citric acid to 3.3, the spores were killed at only 45 °C. The much less aggressive conditions of the treatment allowed preservation of the quality of the technical fabrics and the functionality of the electronic devices.



1. INTRODUCTION

A biological weapon, also known as a bioweapon or bacteriological weapon, is any pathogen (bacteria, virus, fungus, or other organisms) that causes disease and can therefore be used as a weapon of war. It may be destined to kill, disable, or seriously impair an individual or entire areas such as cities.

There are different types of biological agents, but among them those agents most likely to be used as a biological weapon are anthrax (*Bacillus anthracis*), *Francisella tularensis* (tularemia), *Clostridium botulinum* (botulism), *Vibrio cholera* (cholera), *Brucella* spp. (brucellosis), *Yersinia pestis* (plague), and *Shigella* spp. (shigellosis). There are also several viruses that can be used as biological weapons, for example, the variola virus (smallpox).[1]

Anthrax is considered to be one of the most powerful bioweapons for several reasons. First, it forms strong spores, perfect for dispersion in aerosols. Second, it begins with the symptoms of an ordinary cold, but it quickly becomes lethal, with a mortality rate of 90% or higher. Also, while other biological agents break down rapidly when exposed to sunlight and other environmental factors, anthrax spores persist for extended periods of time, even for several years.[1]

Gaseous chemical sterilization is primarily used for biological warfare agent decontamination of equipment and materials within enclosed spaces.[2] The most extensively used sterilants are the alkylating agents, ethylene oxide (EO) and formaldehyde. EO can inactivate most bacteria, molds, yeasts, and viruses, and it has a high penetrating capability in porous products. For EO treatment, this inactivation involves heating the load to the desired sterilization temperature (38−55 °C) and humidifying the load, usually to 50−80% relative humidity. Exposure time is from 4.5 h at 38 °C to 1 h at 55 °C.[3] Nowadays, a steam−formaldehyde gas mixture is used at concentrations higher than 2% supplied at temperatures of 60−80 °C for 30−10 min, respectively.[4] A precise humidity control rendering humidity between 75 and 100% is required. It is also possible to use oxidizing agents such as hydrogen peroxide and peracetic acid. The concentrations at which hydrogen peroxide is sporicidal range from 25 to 60%. Its application at low temperature is done in a plasma state, for which purpose it is necessary to apply radio frequency energy. In modern equipment, 45−75 min cycles at temperatures of 45−55 °C are needed.[5] Similar to hydrogen peroxide gas systems, sterilization processes have been developed based on gaseous peracetic acid (PAA). A proposed sterilization cycle is 10 mg L^{-1} at 45 °C for 60 min. The water content of the PAA solution can be sufficient to provide the necessary humidity (80%) for optimal antimicrobial activity.[3] All these methods involve the use of highly toxic and aggressive additives, the application of heat, the presence of a quite humid atmosphere, and quite extended exposure times. If applied to sensitive or hydrolytic materials, these treatments may damage them, which means that it is impossible to reuse them, with the financial burden this situation entails.

This paper explores the viability of the destruction of biological weapons in military suits and electronic devices using high-pressure CO_2. The study was conducted on *Bacillus thuringiensis* since it shares the same genome as anthrax[6] and has a similar thermal resistance based on *D* and *z* values.[7]

Received: November 20, 2017
Revised: February 12, 2018
Accepted: March 15, 2018
Published: March 15, 2018

DOI: 10.1021/acs.iecr.7b04794
Ind. Eng. Chem. Res. 2018, 57, 4680−4687

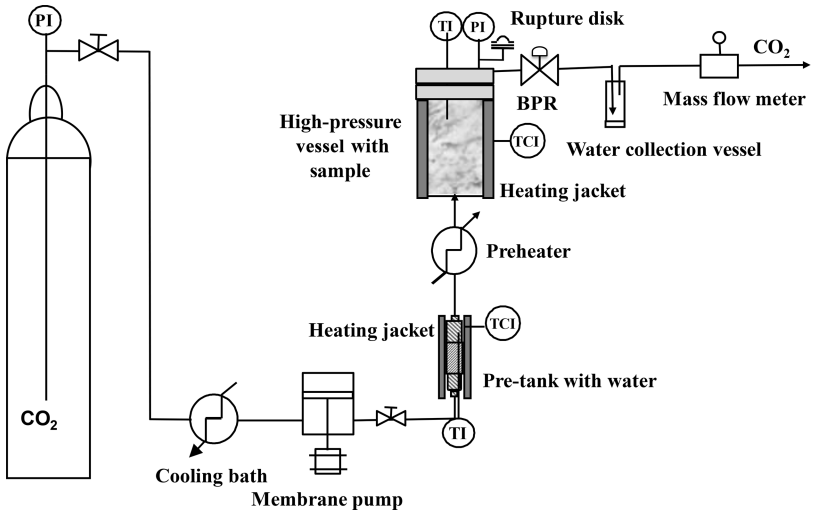


Figure 1. Diagram of HPCD treatment equipment.

Furthermore, *B. thuringiensis* is a risk group 1 microorganism (not associated with disease in healthy adult humans or animals) that can be manipulated in class I biosafety laboratories with standard microbial practices.

Nuclear, biological, and chemical (NBC) suits for military use are conceived for protecting the body from direct exposure to chemical and biological agents and for preventing contact with radioactive particles. They are usually made of a green or camouflage outer waterproof layer, a layer of synthetic fibers treated with fire retardants (carbonized fabric), and an inner cotton layer impregnated with activated carbon (activated carbon foam). The suit is complemented with other elements for protecting the limbs, such as butyl gloves and boot covers. For the face, it is vital to use filtering systems such as gas masks or self-contained breathing apparatus, which have cylinders containing breathable air. The physical and chemical characteristics of the elements of the NCB suit and the appearance and functional capacity of the electronic devices were analyzed after the high-pressure CO_2 tests to demonstrate the viability of the procedure as a sterilization technique.

2. EXPERIMENTAL SECTION

2.1. Materials. The strain used was *B. thuringiensis* (CECT 197T; ATCC 10792; CIP 53.137; DSM 2046) from the Spanish Type Culture Collection. Pressurized liquid carbon dioxide at its vapor pressure, 99.998%, was provided by Air Products, Spain. Citric acid (>99.5%, Sigma-Aldrich), peroxyacetic acid solution (39% in acetic acid, Sigma-Aldrich), hydrogen peroxide (6% solution, Scharlau Chimie S.A.), and isopropyl alcohol (Multisolvent, Scharlau Chimie S.A.) were used as additives.

2.2. Nutrient Broth. The specific medium recommended by the supplier was used for culturing *B. thuringiensis*: "nutrient broth/agar I," comprising 5 g of meat extract, 10 g of peptone, 5 g of NaCl, 15 g of agar (only for solid medium), and 1 L of distilled water, with the pH being adjusted to 7.2. To generate spores, 0.1 g of $MnSO_4 \cdot H_2O$ was added. All the reagents were supplied by Sigma-Aldrich, Spain.

2.3. Generation and Concentration of *B. thuringiensis* Spores. The microorganism was recovered from an Eppendorf tube in a flask with about 100 mL of specific medium and cultured in a bath with stirring (150 rpm) for 8 h at 45 °C. Next, 0.1 mL of this culture was added by means of surface seeding on Petri dishes with the specific medium furthermore containing 0.1 g L^{-1} Mn ($MnSO_4 \cdot H_2O$, Panreac). They were then incubated in the incubation chamber for 2 days at 45 °C.

After this time, the dishes were washed once by adding two batches of 7.5 mL of distilled H_2O on the surface of each dish. During each washing, a sterile slide was dragged over the entire surface of the dish to collect the formed spores. The washing water was centrifuged at 10 000 rpm for 10 min. The supernatant was removed, and the pellet was resuspended in sterile distilled H_2O. The resulting product was then subjected to a thermal treatment (90 °C for 15 min) to inactivate the vegetative forms that may remain in the suspension, obtaining a concentration of 10^8 colony forming units (CFU) per milliliter of viable spores. For testing the spore suspensions in water, a diluted solution of 10^6 CFU mL^{-1} was used.

2.4. Contamination of Equipment Materials. A 5 g sample of each tested fabric was introduced in the concentrated suspension of the spores for 10 min, and the fabric was left to dry in a laminar flow chamber. The contamination achieved was of 10^7 CFU g^{-1}. NATO standards require the total elimination of this biocontamination.

In the electronic materials, contamination was performed by attempting to simulate, as much as possible, a terrorist attack. Specifically, it was performed as follows: in a laminar flow chamber, a diluted suspension containing 10^7 CFU mL^{-1} of *B. thuringiensis* spores was aerosolized with a hand-operated spray bottle on the electronic material, covering the entire surface thereof, and the material was left to dry at room temperature, also inside the laminar flow chamber. The contamination achieved was of 10^6 CFU g^{-1}.

2.5. Determination of the Degree of Inactivation in the Fabrics of the NBC suit and the Electronic Devices. After high-pressure CO_2 treatment, the fabric was introduced in a bag with 10 mL of the nutrient medium and left to soak in said medium for 1/2 h. Taking 1 mL of that medium, a serial dilution table was prepared for obtaining the spore concentration by counting the colony forming units in Petri dishes with the specific medium and Mn salt.

Contamination in the electronic material was determined by passing a sterile swab over the surface of said material. The swab was then introduced in 10 mL of specific medium, and the serial dilution table was prepared in the same manner. These analyses were done in triplicate.

DOI: 10.1021/acs.iecr.7b04794
Ind. Eng. Chem. Res. 2018, 57, 4680−4687

In both cases, the results were expressed as the logarithm of the count after treatment (N) divided by the initial count (N_0) that was read before each test. The maximum standard deviation in the readings was 0.3 log cycle. Total inactivation means that no growth was detected.

2.6. High-Pressure Carbon Dioxide (HPCD) Treatment Equipment. The schematics of the installation used is shown in Figure 1. The CO_2 supplied in the liquid state was passed through a thermally controlled bath (Selecta, Frigiterm-30, Spain) to cool it to −10 °C before reaching the membrane pump (Milroyal MD23G6M250, Dosapro Milton Roy, France). CO_2 cooling prevented cavitation during pressurization. The pressurized CO_2 was preheated in a tube wound into a heating jacket before entering a high-pressure vessel (50 or 250 mL) where the material to be sterilized was introduced. The temperature was read by a K-type thermocouple positioned within the vessel to an accuracy of ±1 °C, while the pressure was read at the outlet of the vessel with a Bourdon gauge with an accuracy of ±0.2 MPa. In tests where modified water was mixed with CO_2, there was a pretank located before the high-pressure vessel. It also had a temperature control to ensure that the entrainment of the water by the CO_2 was done at the same temperature conditions as the subsequent sterilization operation. Pressure and the CO_2 flow rate were controlled by the combined action of a final pressure regulator (BPR, Tescom, Series 26-1761-24-161, Germany) and the pump flow regulator, respectively. The equipment had a rupture disk tared to 38 MPa to avoid problems of overpressure. Total CO_2 mass and flow rate were measured using a mass flow meter (Alicat Scientific, M-10 SLPM-D, USA) connected to the outlet.

2.7. Treatment of Samples with HPCD for the Inactivation of *B. thuringiensis*. The vessel was loaded with the material to be treated in a laminar flow chamber. For tests on the fabrics of the NBC suit, fragments were introduced into a 50 mL vessel. In the experiments that aimed to examine the effect of variations in moisture on the fabrics, water was previously atomized into the piece of the fabric to obtain increasing moisture levels of up to 59%, which was determined by weight. For electronic material, the tests were performed in a 250 mL container with an inner diameter of 6×10^{-2} m. The corresponding vessel was fitted in place and closed. For tests on spore suspensions in water, stirring was achieved by an impeller with a speed of 500 rpm. The CO_2 supply was then opened, and the pump was put into operation to reach the working pressure. The CO_2 was pumped at a high rate of 4 g min^{-1} for the purpose of generating turbulence. It was subsequently preheated by means of passage through a heated coil. The CO_2 entered the lower part of the container and went through the suspension, the contaminated fabric, or the electronic material. After the established operating time, the pump was shut off, the CO_2 supply was closed, and depressurization commenced very slowly to prevent freezing. Once the container was depressurized and separated from the equipment, it was brought to a laminar flow chamber, where the treated sample was collected to perform subsequent microbiological analyses.

A similar procedure was followed in the tests where the CO_2 was mixed with water (pure or modified) except that in those cases, after being pumped to the operating pressure, the CO_2 passed through the water pretank before entering the vessel where the materials were loaded. The amount of water introduced by the CO_2 was on average 1.3% by mass in the fabric tests and 3.3% in the electronic material tests, which was much higher than the theoretical solubility of the water in CO_2, at most 0.5%,[8] within the interval of explored conditions. This was due to the high CO_2 flow rate.

2.8. Thermal Treatments. To study the separate effect of temperature on spore inactivation, several non-CO_2 tests were carried out. In these experiments, the operating procedure was the same, except that there was no CO_2 flow in the vessel. Instead, the suspension of the spores was kept inside the vessel at the desired temperature for the same time as in the comparative tests with CO_2.

2.9. Measuring pH. The pH was measured during treatment using reactive strips (Merck, Spain) introduced in the sterilization vessel together with the materials, where said pH was of two ranges: 4.0−7.0 and 2.5−4.5 (MColorpHast, sensitivity 0.3 pH unit). The pH of the spore suspension in water was measured with a Crison pH-Meter Basic 20 Plus and was 4.7 ± 0.1.

2.10. Determination of Properties of HPCD-Treated Fabrics. The treated fabrics were sent to the Technological Institute of Marañosa of the Ministry of Defense of Spain to evaluate the mechanical and permeability properties according to the standardized methods of this center: traction and tear test, thickness, repellency to oils, resistance to surface wetting, weight per square meter, resistance to penetration of aggressive chemicals, air permeability, permeability to water vapor, resistance to water penetration under pressure, and hydrostatic water absorption.

2.11. Statistical Analysis. The experiments were replicated at least three times. Microbial analyses were performed in duplicate for each replicate (n = 3·2). Means and standard deviations were computed for all data.

3. RESULTS AND DISCUSSION

First, inactivation of *B. thuringiensis* in an aqueous suspension was investigated. The goal was to evaluate the effect of the main operating variables: pressure, temperature, and contact time. Subsequently, the efficacy of HPCD in the inactivation of *B. thuringiensis* spores was investigated, studying different fabrics and components of the NBC suit, as well as electronic material that the soldier may carry in the field, once they were contaminated. Since NBC suits are expensive, being able to reuse them after CO_2 treatment would be extremely favorable for the viability of the technology. For this reason, mechanical and physical tests established by standards were performed on the treated materials. On the other hand, the electronic material could lose capabilities due to the treatment at high pressure and temperature. Hence the effect of CO_2 treatment on the functional capacity and aspect of three electronic devices was analyzed.

3.1. Inactivation of *B. thuringiensis* in Aqueous Suspension. *3.1.1. Effect of Operating Conditions.* Figure 2 shows the effects of temperature and pressure on the inactivation of *B. thuringiensis* spores suspended in water. The degree of inactivation achieved when only heat was applied to assess the effect of heat per se is also included for comparison.

First, it was observed that at 70 °C total inactivation (>6 log cycles) was achieved if operated at 35 MPa, although at 60 °C 5 log cycles were already achieved and at 50 °C 4 log cycles were achieved.

Second, if the results obtained with CO_2 are compared with those obtained with the heat treatment, we found that CO_2 has a lethal effect on its own. This fact was previously published by other authors[9−11] and by us in earlier papers on the inactivation of *Alicyclobacillus acidoterrestris*[12] and *Bacillus cereus*.[13] At 80 °C,

DOI: 10.1021/acs.iecr.7b04794
Ind. Eng. Chem. Res. 2018, 57, 4680−4687

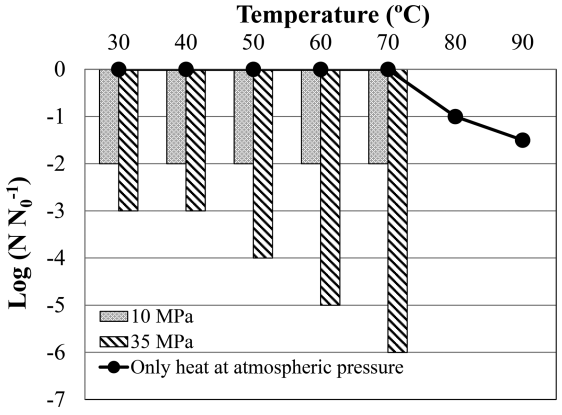


Figure 2. Inactivation of *B. thuringiensis* with HPCD applied at different pressures and temperatures and comparison with heat treatment. Other operating conditions: treatment time, 60 min; CO_2 flow rate, 4 g·min^{-1}; stirring speed, 500 rpm.

a temperature that coincides with those typically established for thermal "awakening" of the spores, when only heat was applied, some inactivation of *B. thuringiensis* began.[14,15]

The explanation for why HPCD has a lethal effect per se could be that, in media with high water activity, it could promote the activation and germination of spores,[11,16] reducing their tolerance to heat[17] and CO_2. This phenomenon would also be strongly associated with the reduction of the pH of the medium by dissolution of CO_2, as is shown in section 3.1.2.

There were significant differences in the inactivation of *B. thuringiensis* upon increasing pressure. While total spore inactivation was achieved at 35 MPa, only 2 log cycles were achieved at 10 MPa operating at 70 °C (see Figure 2). However, other authors found in other sporulated forms that hydrostatic pressure in the intervals used in the HPCD method does not affect this aspect.[18]

Therefore, the impact of operating pressure must be associated with facilitating the solubilization of CO_2 in the water, lowering the pH, and generating turbulence in the medium, facilitating good contact between the gaseous CO_2 and the spores. This proved to reduce the necessary time and the aggressiveness of the conditions for the inactivation of other bacillus type spores.[12,13]

3.1.2. pH Effect. The solubilization of CO_2 in the aqueous suspension of the spores caused a reduction in pH close to a value of 4. To assess the effect of the medium acidity on the lethal power against *B. thuringiensis* spores, comparative tests were performed applying HPCD under the same conditions but using a buffered medium at pH 7. As shown in Table 1 in the buffered samples, only 1 log reduction was achieved versus the 6 log reductions of the nonbuffered samples. Tests were also carried out using N_2O applied under the same conditions. This gas has critical, physical and chemical properties very close to those of CO_2; however, its dissolution in water does not reduce pH. When the suspension was treated with this gas, the pH remained at 4.6, which was the original acidity of the suspension. Consequently, only 1 logarithmic reduction was achieved. Therefore, there is a significant effect of the external pH on *B. thuringiensis* spore inactivation.

Table 1. Effect of Extracellular pH on the Inactivation of *B. thuringiensis*[a]

treatment	press. (MPa)	pH	degree of inactivation log(NN_0^{-1})
CO_2 on buffered samples	35	6.9	−1.1
CO_2	35	3.9	−6.0[b]
N_2O	35	4.6	−1.2
citric acid solution	0.1	3.7	0.0

[a]Operating conditions: temperature, 70 °C; treatment time, 60 min. [b]Total.

However, when tests were conducted in which the spores were suspended in a citric acid solution at a pH similar to that obtained in the CO_2 treatment, operating with the same equipment and under the same conditions of temperature and agitation (except pressure), the combination of lowered pH and temperature did not achieve any reduction in the contamination of *B. thuringiensis*. This is indicative that CO_2 has a sporicidal effect per se and that the mechanism by which it deactivates *B. thuringiensis* spores involves several phenomena improved by a low pH medium. Similar results were obtained in the inactivation of *B. cereus*.[13]

3.1.3. Effect of Operating Time. The above tests where sterilization was achieved were performed with 1 h of operation. The next investigation intended to reduce that time. This variable is crucial for the technical and economic viability of the process.

The results are shown in Figure 3 when the time was reduced to 15 min. The treatment time to achieve sterility at 35 MPa and 70 °C could be reduced to 30 min. Below this time, it was no longer possible to totally inactivate the *B. thuringiensis* spores in aqueous suspension.

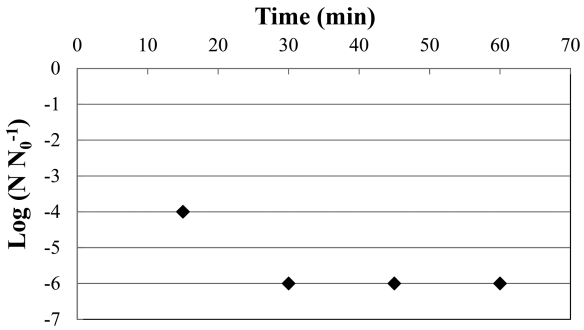


Figure 3. Inactivation of *B. thuringiensis* with HPCD applied at different treatment times. Other operating conditions: pressure, 35 MPa; temperature, 70 °C.

3.2. Inactivation of *B. thuringiensis* Spores in NBC Fabrics. The following tests were conducted relating to the inactivation of *B. thuringiensis* spores that contaminated the different elements of the NBC suit and electronic devices.

3.2.1. Effect of Moisture. Earlier work on inactivation of the natural microbial load of solid foods such as herbs and spices,[19] cocoa,[20] and paprika[21] demonstrated that high pressure is only sporicidal in the presence of a given amount of water. Likewise, Kamihira et al.[22] proved that pure HPCD was not effective in the elimination of dry spores, and Furukawa et al.[23] proved that a reduction in water activity in a model food system protected *Geobacillus stearothermophilus* spores from CO_2 inactivation because it reduced the rate of germination.

Table 2 shows the amount of water in mass fraction relative to the material which was necessary to achieve increasing degrees of inactivation of *B. thuringiensis* in NBC suit fabrics. As noted, a minimum moisture percentage of 25% was required to

DOI: 10.1021/acs.iecr.7b04794
Ind. Eng. Chem. Res. 2018, 57, 4680−4687

Table 2. Inactivation of *B. thuringiensis* in the Fabrics of the NBC Suit at Different Percentages of Moisture[a]

water mass fraction relative to fabric (%)	pH	degree of inactivation, $\log(NN_0^{-1})$
16	4.7	−2.1
20	4.7	−4.3
23	4.5	−5.2
25	3.9	−7.0[b]
40	3.9	−7.0[b]
59	3.9	−7.0[b]

[a]Other operating conditions: pressure, 35 MPa; temperature, 70 °C; operating time, 60 min. [b]Total.

achieve total inactivation. The results were the same in the three types of fabrics. Water was most likely necessary to promote germination of spores and allow the pH to drop to values less than 4, which is a synergistic factor in this process, as demonstrated in section 3.1.2. An acidic medium hinders growth[3,24] and even inhibits the synthesis of proteins that must necessarily be produced for germination.[25]

However, the strong need for water could be detrimental to maintaining the properties of the fabrics and the electronic material. Therefore, the introduction of water together with CO_2 instead of prewetting the material was further investigated. The operation was carried out as follows. The CO_2 entrained the water from the pretank, and then the mixture at 35 MPa and 70 °C entered the high-pressure vessel where the material was located. Table 3 shows the results obtained for one of the

Table 3. Inactivation of *B. thuringiensis* in the Outer Fabric of the NBC Suit with Wet HPCD at Different Treatment Times[a]

time (min)	water mass fraction relative to fabric (%)	pH	degree of inactivation, $\log(NN_0^{-1})$
60	59	3.9	−7.0[b]
30	35	3.9	−7.0[b]
15	19	4.5	−3.1

[a]Other operating conditions: pressure, 35 MPa; temperature, 70° C. [b]Total.

NBC fabrics treated at decreasing operating times. It includes the water proportion relative to the fabric. Even though the total proportion is quite high (ranging from 19 to 59%), the fabric after the treatment was almost dry when it was collected due to the small quantity of water in contact with the fabric at all times.

At 60 and 30 min operating times, the amount of water that was introduced was sufficient to lower the pH to values close to 4, resulting in total inactivation. However, with 15 min of operation, the pH only dropped to 4.5 and inactivation was 3 log reductions.

Therefore, the importance of a low pH to achieve the inactivation of *B. thuringiensis* and, on the other hand, the need to provide a quantity of water between 19 and 35% were confirmed (in previous tests when the material was initially wetted, it was 25%). Under these conditions, the required treatment time was 30 min, which is a significantly reduced period.

3.2.2. Impact of the Use of Additives. In the continuous attempt to reduce the amount of water and given the importance of pH, in this group of tests, the NBC outer fabric was treated with CO_2 mixed with water acidified with citric acid, which is a somewhat aggressive and nontoxic organic acid that is used as a food preservative. The citric acid was added to the water to lower its pH to 3.9. The results on *B. thuringiensis* spore inactivation at different operating conditions are shown in Table 4.

Table 4. Impact of the Use of Water Acidified with Citric Acid Added to the CO_2 on the Inactivation of *B. thuringiensis* Spores in the NBC Outer Fabric under Different Operating Conditions[a]

press. (MPa)	temp (°C)	degree of inactivation, $\log(NN_0^{-1})$
35	70	−7.0[b]
15	70	−7.0[b]
15	60	−7.0[b]
15	50	−4.2
15	40	−3.3

[a]The total amount of the water meant a mass fraction of 30% of the fabric, and it was introduced in a 15 min treatment time. The pH of the citric acid water solution was 3.9. [b]Total.

The addition of acidified water allowed reducing the aggressiveness of the operating conditions to obtain a sterilized fabric. This fact is fundamental to reducing the impact of the treatment on the quality of the materials and the installation and operating costs. Thus, at the rather mild conditions of 15 MPa, 60 °C, and 15 min, it was possible to sterilize NBC fabrics contaminated with biological weapons.

Table 5 shows the results obtained by applying this same treatment to a pen drive, previously sprayed with an aqueous

Table 5. Impact of the Use of Water with Several Additives Added to CO_2 on the Inactivation of *B. thuringiensis* Spores Contaminating a Pen Drive at Decreasing Temperatures[a]

additive	temp (°C)	pH	additive proportion in water (%)	additive proportion in CO_2 (%)	degree of inactivation, $\log(NN_0^{-1})$
citric acid	60	3.9	10.00	0.333	−6.0[b]
	55	3.9	10.00	0.333	−6.0[b]
	45	3.3	11.00	0.367	−6.0[b]
	40	3.3	11.00	0.367	−5.9
peracetic acid	50	3.9	0.59	0.020	−6.0[b]
	45	3.9	0.59	0.020	−6.0[b]
	40	3.9	0.59	0.020	−5.2
	40	3.3	0.78	0.026	−6.0[b]
H_2O_2	45	4.0	0.29	0.010	−5.8
isopropyl alcohol	45	4.7	5.00	0.167	−6.0[b]
	40	4.7	5.00	0.167	−5.9

[a]The total amount of the modified water meant a mass fraction of 20% of the pen drive and was introduced in 15 min treatment time. The operating pressure was 15 MPa. [b]Total.

suspension of spores until reaching a contamination of $>10^6$ CFU g^{-1}. Similarly, it was possible to sterilize this device at 15 MPa in 15 min at 60 °C. When citric acid was added to lower the pH to 3.3, the spores were killed at 45 °C.

Peracetic acid is a strong oxidizing agent which decomposes in water, giving hydrogen peroxide and acetic acid. In previous works, this acid yielded good results as an additive of high-pressure CO_2. In the amount of 55 ppm, it brought about the sterilization of *Bacillus atrophaeus* spores in 27 min, of porcine

DOI: 10.1021/acs.iecr.7b04794
Ind. Eng. Chem. Res. 2018, 57, 4680−4687

viruses in 120 min, and of several fungi (*Penicillium*, *Aspergillus*, and *Verticillium*) inoculated in a biomaterial in only 15 min, supplying the CO_2 at 10 MPa and 41 °C.[26] It was also effective in inactivating *G. stearothermophilus* spores.[27]

We added peracetic acid to the water to achieve pH values ranging from 3.9 to 3.3 for the same reasons provided in relation to the citric acid tests. The results are shown in Table 5. PAA was even more efficient than citric acid because at a lower proportion (260 vs 3670 ppm) it allowed total inactivation of the spores on the pen drive at 40 °C in 15 min of treatment. This is probably because the PAA exerts an oxidizing action, in addition to lowering the pH (note that PAA is available stabilized in acetic acid).

Recently, Setlow et al. published evidence on the mechanism of inactivation of CO_2 plus PAA in *Bacillus subtilis* spores by comparing dry and water tests.[28] They concluded that this mixture inactivates spores by damaging their inner membrane. DNA protective proteins provided resistance to treatment only in dry spores. Perhaps this is why the presence of water enhanced inactivation.

Another oxidant, hydrogen peroxide (H_2O_2), was then tested. As a CO_2 additive, it was previously tested on anthrax spores, achieving sterilization with 200 ppm operating at 27.5 MPa and 40 °C in 4 h.[29] The authors studied the mechanism of action of the mixture and observed that there were no signs of germination and that the spores were practically intact, with almost no release of dipicolinic acid into the medium. Nevertheless, they did observe that about half of the inactivated spores had lost membrane integrity.

The CO_2 plus H_2O_2 mixture was also successful in the complete inactivation of *B. atrophaeus* spores inoculated into three-dimensional structures of biomaterials used for implants at concentrations ranging from 200 to 1250 ppm, depending on whether the treatment lasted more (4 h) or less (2 h) time, operating at very mild temperatures of 35−37 °C.[30,31] In the amount of 600 ppm at 40 °C, it achieved the inactivation of *G. stearothermophilus* spores in thermoset materials in 2 h.[32] Mixed with water and acetic anhydride (0.25% water, 0.15% H_2O_2, 0.5% acetic anhydride), it allowed the inactivation of three different types of spores (*G. stearothermophilus, B. atrophaeus*, and *B. cereus*) that contaminated bioresorbable agarose/alginate structures and mineralized collagen at 38 °C in 45 min.[33]

The results of Table 5 show that the water−H_2O_2 mixture was very efficient as an HPCD modifier at very low H_2O_2 concentration of 100 ppm. The presence of water (3.33% water) could justify the smaller amount and the less time needed to achieve sterility compared to the results obtained by other authors with CO_2 + pure H_2O_2 mixtures. In any case, the concentrations were much less than those used in a plasma state.

3.2.3. Study of a Possible Combined Process of Sterilization and Dry Cleaning of the NBC Suits. In the past two decades, CO_2 has been introduced as a solvent for the dry cleaning of fabrics to replace perchloroethylene (PERC) and solvents of petroleum origin, which present both health and safety risks and are harmful to the environment. The process is carried out under a pressure of 5−7 MPa and at a temperature between 10 and 20 °C, i.e., in conditions where CO_2 is still liquid.[34] The operation lasts for 30 to 45 min.[35] CO_2 is used with small concentrations of wash enhancers, such as surfactants and/or cosolvents. Surfactants are valid for the elimination of soil particles from the textile garment.[36] As for cosolvents, their mission is to increase the solubility of polar spots. In addition to a small amount of water (0.4%), the most suitable cosolvent is isopropyl alcohol (IPA) at concentrations of approximately 4%.[37] The presence of IPA has another effect: the inactivation of pathogenic bacteria. For example, in a study on cotton garments contaminated with *Escherichia coli*, it was shown that introducing a 5% volume fraction of IPA into CO_2 was sufficient for complete mortality of this bacterium at only 7 MPa and 20 °C.[38]

Although IPA has no sporicidal activity per se, we found that introducing this alcohol (3.33%) dissolved in the HPCD allowed a reduction of 5 log cycles in the contamination of *B. thuringiensis* spores in the outer fabric of the NBC suit at 70 °C in 15 min (results not shown). The operating pressure was 15 MPa. Results improved when this alcohol was introduced mixed with water (3.17% water + 0.17% IPA) in the HPCD stream, as shown in Table 5. Total inactivation of the *B. thuringiensis* spores was achieved at 45 °C, probably because water favored spore germination, reducing their resistance to CO_2 and IPA. The pH remained at 4.7 since IPA is a high p*K* compound.

3.3. Quality Analysis of the Treated Materials. Samples of the various NBC fabrics and elastomers treated under the conditions shown in Tables 2−4 were subjected to chemical and mechanical quality tests. The results are summarized in Table 6.

Table 6. Summary of the Results of the Quality Tests Done on the Fabrics, Gloves, and Boot Covers by Standard Tests of the Technological Institute of Marañosa

material	quality observations
carbonized fabric active carbon foam outer fabric	no significant changes in fundamental tests such as air or water vapor permeability, chemical repellency, and traction−tear tests
gloves	loss of tensile strength; bubbles disappeared after 24 h
boot covers	no significant changes

There were no major changes in fundamental tests for the NBC fabrics, such as air permeability, water vapor permeability, chemical and oil repellency, and mechanical tests, when comparing untreated fabrics to fabrics treated with HPCD in both the presence and the absence of water. No modifications were observed either on the samples of the outer fabric treated with acidified water. On the other hand, the few deformations viewed in the elastomeric materials (gloves), such as an increase in size and bubble formation, virtually disappeared after 24 h. This is because they were primarily caused by trapped CO_2 that was gradually released. However, this caused a loss of tensile strength.

We generally observed that the electronic materials left with malfunctions in test treatments run at 70 °C, but they suitably resisted the impact of pressure even at 35 MPa. Deformations were observed mainly in the plastic covers. However, if the temperature was below 60 °C, no modifications were observed. Figure 4 shows images of a mobile phone treated with HPCD mixed with water acidified with citric acid to pH 3.9 at 15 MPa, 60 °C, and 15 min.

Due to the short treatment time required, deformations in the mobile phone were minimal. Only minor swelling of the closing tabs of the flip top was observed. The mobile phone was

DOI: 10.1021/acs.iecr.7b04794
Ind. Eng. Chem. Res. 2018, 57, 4680−4687

Figure 4. Appearance of a mobile phone after treatment with HPCD mixed with acidified water at 15 MPa, 60 °C, for 15 min.

Figure 5. Appearance of a pen drive subjected to many treatments with CO_2 modified with water and several additives at 15 MPa and temperatures ranging between 40 and 60 °C.

then switched on normally. The low pH of the environment seemed not to cause any negative impact.

Photographs of the pen drive treated with HPCD and its modifiers under the conditions summarized in Table 5 are shown in Figure 5. After all these tests there were no significant alterations except a slight detachment of the plastic case. Moreover, the pen drive maintained its functional capacity. The files that it contained were accessible and could be opened and manipulated. The same results were obtained by other authors when treating a pen drive with HPCD modified with water and hydrogen peroxide at 10 MPa, 50 °C, for 45 min.[39]

4. CONCLUSIONS

It is possible to sterilize military material contaminated with biological weapons by the environmentally friendly and simple mixture formed by high-pressure CO_2 and water according to the most restrictive NATO standards. The presence of water in mass proportions ranging from 20 to 30% with respect to the material was essential to achieve this level. The water was best introduced progressively, in small amounts (<4% in relation to CO_2) entrained by the CO_2, to avoid the initial wetting of the material and to promote better contact. Water probably favored the germination of the spores, making them more sensitive to inactivation by CO_2. A reduction of the external pH, which was a key parameter, is also attributed to the dissolution of CO_2. Therefore, CO_2 mixed with small amounts of acidified water could inactivate more than 6 orders of magnitude of bacteria spores that could be used as biological weapons at 45 °C in a short period of 15 min. If pH reduction was not desirable, water solutions with hydrogen peroxide or isopropyl alcohol (0.2%) could be alternatively used. These mild conditions allowed preserving the quality of the treated materials, a fact that is pivotal to reusing them with important savings. The method was suitable for electronic components.

Although tests were done on spores of a bacterium, the treatment would surely be effective for the inactivation of other microorganisms that could be used as potential biological weapons, such as some viruses, since their resistance to HPCD treatment is generally much smaller, as is concluded in the specialized bibliography. This process could also be applied to individual protection equipment of other groups that are in contact with biological agents, such as sanitary personnel, or in microbiology laboratories, biowaste disposal plants, etc.

AUTHOR INFORMATION

Corresponding Author

*E-mail: lcalvo@ucm.es.

ORCID

Lourdes Calvo: 0000-0003-3184-4954

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

This work was supported by the Dirección General de Armamento y Material, Ministry of Defense of Spain, Contract No. 1003211003200.

REFERENCES

(1) Sistema de Observación y Prospectiva Tecnológica (SOPT). *Detección e Identificación de Agentes de Guerra Biológica. Estado del Arte y Tendencia Futura*; Ministerio de Defensa: Madrid, Spain, 2010.

(2) Hawley, R. J.; Eitzen, E. M. Protection against Biological Warfare Agents. In *Disinfection, Sterilization, and Preservation*; Block, S. S., Ed.; Lippincott Williams & Wilkins: Philadelphia, PA, USA, 2001; p 148.

(3) McDonnell, G. E. *Antisepsis, Disinfection, and Sterilization: Types, Action, and Resistance*; ASM Press: Washington, DC, 2007.

(4) Matachana. Low temperature sterilizer. https://www.matachana.com/downloads/CA_IC_130LF_14_00_01_en_arx.pdf (accessed June 30, 2017).

(5) Matachana. Low temperature hydrogen peroxide & plasma sterilizer. https://www.matachana.com/downloads/CA_IC_130HPO_01_05_en_arx.pdf (accessed March 30, 2017).

(6) Helgason, E.; Okstad, O. A.; Caugant, D. A.; Johansen, H. A.; Fouet, A.; Mock, M.; Hegna, I.; Kolsto, A.-B. *Bacillus anthracis, Bacillus cereus*, and *Bacillus thuringiensis*—One Species on the Basis of Genetic Evidence. *Appl. Environ. Microbiol.* **2000**, *66* (6), 2627.

DOI: 10.1021/acs.iecr.7b04794
Ind. Eng. Chem. Res. 2018, 57, 4680−4687

(7) Montville, T. J.; Dengrove, R.; De Siano, T.; Bonnet, M.; Schaffner, D. W. Thermal Resistance of Spores from Virulent Strains of *Bacillus anthracis* and Potential Surrogates. *J. Food Prot.* **2005**, *68* (11), 2362.
(8) Wiebe, R.; Gaddy, V. L. The Solubility in Water of Carbon Dioxide at 50, 75 and 100°, at Pressures to 700 atm. *J. Am. Chem. Soc.* **1939**, *61* (2), 315.
(9) Watanabe, T.; Furukawa, S.; Hirata, J.; Koyama, T.; Ogihara, H.; Yamasaki, M. Inactivation of *Geobacillus stearothermophilus* Spores by High-Pressure Carbon Dioxide Treatment. *Appl. Environ. Microbiol.* **2003**, *69* (12), 7124.
(10) Ballestra, P.; Cuq, J.-L. Influence of Pressurized Carbon Dioxide on the Thermal Inactivation of Bacterial and Fungal Spores. *LWT - Food Sci. Technol.* **1998**, *31* (1), 84.
(11) Spilimbergo, S.; Bertucco, A.; Lauro, F. M.; Bertoloni, G. Inactivation of *Bacillus subtilis* Spores by Supercritical CO_2 Treatment. *Innovative Food Sci. Emerging Technol.* **2003**, *4* (2), 161.
(12) Casas, J.; Valverde, M. T.; Marín-Iniesta, F.; Calvo, L. Inactivation of *Alicyclobacillus acidoterrestris* Spores by High Pressure CO_2 in Apple Cream. *Int. J. Food Microbiol.* **2012**, *156* (1), 18.
(13) Casas, J.; Calvo, L. Inactivation of Spores by High Pressure CO_2. Presented at the 6th International Symposium on High Pressure Process Technology, 2013.
(14) Finley, N.; Fields, M. L. Heat Activation and Heat-Induced Dormancy of *Bacillus stearothermophilus* Spores. *Appl. Microbiol.* **1962**, *10* (3), 231.
(15) Levinson, H. S.; Hyatt, M. T. Effects of Temperature on Activation, Germination, and Outgrowth of *Bacillus megaterium* Spores. *J. Bacteriol.* **1970**, *101* (1), 58.
(16) Furukawa, S.; Watanabe, T.; Tai, T.; Hirata, J.; Narisawa, N.; Kawarai, T.; Ogihara, H.; Yamasaki, M. Effect of High Pressure Gaseous Carbon Dioxide on the Germination of Bacterial Spores. *Int. J. Food Microbiol.* **2004**, *91* (2), 209.
(17) Watanabe, T.; Furukawa, S.; Tai, T.; Hirata, J.; Narisawa, N.; Ogihara, H.; Yamasaki, M. High Pressure Carbon Dioxide Decreases the Heat Tolerance of the Bacterial Spores. *Food Sci. Technol. Res.* **2003**, *9* (4), 342.
(18) Norton, T.; Sun, D.-W. Recent Advances in the Use of High Pressure as an Effective Processing Technique in the Food Industry. *Food Bioprocess Technol.* **2008**, *1* (1), 2.
(19) Haas, G. J.; Prescott, H. E.; Dudley, E.; Dik, R.; Hintlian, C.; Keane, L. Inativation of Microorganisms by Carbon Dioxide under Pressure. *J. Food Saf.* **1989**, *9* (4), 253.
(20) Calvo, L.; Muguerza, B.; Cienfuegos-Jovellanos, E. Microbial Inactivation and Butter Extraction in a Cocoa Derivative Using High Pressure CO_2. *J. Supercrit. Fluids* **2007**, *42* (1), 80.
(21) Calvo, L.; Torres, E. Microbial Inactivation of Paprika Using High-Pressure CO_2. *J. Supercrit. Fluids* **2010**, *52* (1), 134.
(22) Kamihira, M.; Taniguchi, M.; Kobayashi, T. Sterilization of Microorganisms with Supercritical Carbon Dioxide. *Agric. Biol. Chem.* **1987**, *51* (2), 407.
(23) Furukawa, S.; Watanabe, T.; Koyama, T.; Hirata, J.; Narisawa, N.; Ogihara, H.; Yamasaki, M. Inactivation of Food Poisoning Bacteria and *Geobacillus stearothermophilus* Spores by High Pressure Carbon Dioxide Treatment. *Food Control* **2009**, *20* (1), 53.
(24) *Russell, Hugo & Ayliffe's: Principles and Practice of Disinfection, Preservation and Sterilization*, 5th ed.; Fraise, A. P., Maillard, J.-Y., Sattar, S. A., Eds.; Wiley-Blackwell: Oxford, U.K., 2013.
(25) András, C. D.; Csajági, C.; Orbán, C. K.; Albert, C.; Ábrahám, B.; Miklóssy, I. A Possible Explanation of the Germicide Effect of Carbon Dioxide in Supercritical State Based on Molecular-Biological Evidence. *Med. Hypotheses* **2010**, *74* (2), 325.
(26) Qiu, Q.-Q.; Leamy, P.; Brittingham, J.; Pomerleau, J.; Kabaria, N.; Connor, J. Inactivation of Bacterial Spores and Viruses in Biological Material Using Supercritical Carbon Dioxide with Sterilant. *J. Biomed. Mater. Res., Part B* **2009**, *91B* (2), 572.
(27) White, A.; Burns, D.; Christensen, T. W. Effective Terminal Sterilization Using Supercritical Carbon Dioxide. *J. Biotechnol.* **2006**, *123* (4), 504.
(28) Setlow, B.; Korza, G.; Blatt, K. M. S.; Fey, J. P.; Setlow, P. Mechanism of Bacillus Subtilis Spore Inactivation by and Resistance to Supercritical CO_2 plus Peracetic Acid. *J. Appl. Microbiol.* **2016**, *120* (1), 57.
(29) Zhang, J.; Dalal, N.; Matthews, M. A.; Waller, L. N.; Saunders, C.; Fox, K. F.; Fox, A. Supercritical Carbon Dioxide and Hydrogen Peroxide Cause Mild Changes in Spore Structures Associated with High Killing Rate of B*acillus anthracis*. *J. Microbiol. Methods* **2007**, *70* (3), 442.
(30) Ellis, J. L.; Titone, J. C.; Tomasko, D. L.; Annabi, N.; Dehghani, F. Supercritical CO_2 Sterilization of Ultra-High Molecular Weight Polyethylene. *J. Supercrit. Fluids* **2010**, *52* (2), 235.
(31) Herdegen, V.; Felix, A.; Haseneder, R.; Repke, J. U.; Leppchen-Fröhlich, K.; Prade, I.; Meyer, M. Sterilization of Medical Products from Collagen by Means of Supercritical CO_2. *Chem. Eng. Technol.* **2014**, *37* (11), 1891.
(32) Donati, I.; Benincasa, M.; Foulc, M.-P.; Turco, G.; Toppazzini, M.; Solinas, D.; Spilimbergo, S.; Kikic, I.; Paoletti, S. Terminal Sterilization of BisGMA-TEGDMA Thermoset Materials and Their Bioactive Surfaces by Supercritical CO_2. *Biomacromolecules* **2012**, *13* (4), 1152.
(33) Bernhardt, A.; Wehrl, M.; Paul, B.; Hochmuth, T.; Schumacher, M.; Schütz, K.; Gelinsky, M. Improved Sterilization of Sensitive Biomaterials with Supercritical Carbon Dioxide at Low Temperature. *PLoS One* **2015**, *10* (6), e0129205.
(34) Pallado, P. Dry Cleaning with Liquid Carbon Dioxide. In *High Pressure Process Technology*; Bertucco, A., Vetter, G., Eds.; Elsevier: Amsterdam, Netherlands, 2001; pp 641−649.
(35) De Walle, F.; Van den Otter, W. *DETECTIVE Laymen's Report: LCO_2 Textile Cleaning. Demonstration Pilot Scale Testing of Textile Dry-Cleaning with Sub/supercritical Carbon Dioxide*; LIFE: 2005.
(36) van Roosmalen, M. J. E.; Woerlee, G. F.; Witkamp, G. J. Surfactants for Particulate Soil Removal in Dry-Cleaning with High-Pressure Carbon Dioxide. *J. Supercrit. Fluids* **2004**, *30* (1), 97.
(37) van Roosmalen, M. J. E.; Woerlee, G. F.; Witkamp, G. J. Dry-Cleaning with High-Pressure Carbon Dioxide—the Influence of Process Conditions and Various Co-Solvents (Alcohols) on Cleaning-Results. *J. Supercrit. Fluids* **2003**, *27* (3), 337.
(38) Schmidt, A.; Beermann, K.; Bach, E.; Schollmeyer, E. Disinfection of Textile Materials Contaminated with *E. coli* in Liquid Carbon Dioxide. *J. Cleaner Prod.* **2005**, *13* (9), 881.
(39) Shieh, E.; Paszczynski, A.; Wai, C. M.; Lang, Q.; Crawford, R. L. Sterilization of *Bacillus pumilus* Spores Using Supercritical Fluid Carbon Dioxide Containing Various Modifier Solutions. *J. Microbiol. Methods* **2009**, *76* (3), 247.

DOI: 10.1021/acs.iecr.7b04794
Ind. Eng. Chem. Res. 2018, 57, 4680−4687