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High voltage atmospheric cold plasma decontamination of *Salmonella enteritidis* on chicken eggs

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ABSTRACT

Salmonella enteritidis (SE) accounts for more than 70% of *Salmonella* spp. infections in humans with a primary source being chicken eggs, that can result from post-lay SE cross-contamination of the shell from contaminated equipment or the environment. The objective of this study was to apply a HVACP treatment that can achieve a minimum 5-log reduction in SE on the surface of artificially inoculated shell eggs with an initial bacterial load of 10^8 CFU/egg, after a previous disinfection. Optimized HVACP treatment conditions were an indirect treatment with air at 60% humidity at 100 kV for one minute treatment and six hours post-treatment or alternatively, five minutes of treatment and four hours post-treatment. Egg quality parameters of Haugh unit (HU), pH, color, and vitelline membrane and shell strength were tested under the optimized conditions and showed no significant difference ($p > 0.05$) between treated and untreated eggs.

Industrial relevance: Missing information for a possible scale up of a cold plasma system for egg surface decontamination has been addressed by an optimization of HVACP treatment focused on treatment and post-treatment time, essential parameters to have into account in the food industry. These results demonstrate that HVACP is an effective decontamination method for SE on chicken shell eggs and provides a baseline for a future scale up of the process, showing that different combinations of treatment variables can achieve the desired decontamination without affecting to key quality parameters of the egg such as Haugh Unit or vitelline membrane strength.

1. Introduction

Salmonellosis is caused by the bacteria *Salmonella* spp. and constitutes a major source of the foodborne illnesses with more incidence in the world, having 26,500 cases annually reported in the United States (CDC, 2021) and 6500 cases in Canada (Public Health, G. of C., 2019). Salmonellosis is claimed to be the second most commonly gastrointestinal infection in humans, and among all the different *Salmonella* serovars, *Salmonella enteritidis* (SE) is the one with the highest incidence, accounting with more than the 70% of salmonellosis infections (EFSA, 2021). Eggs and eggs products are pointed as the main food vehicles for the transmission of SE to humans. The interior contamination of SE in eggs can occur through two different pathways, direct or indirect contamination. The first one occurs when the reproductive tract of the hen is colonised by SE, which will infect the egg during its formation. The indirect contamination is the most frequent among those two, and takes place once the egg has been laid, showing a higher frequency in unwashed eggs. In this case, *Salmonella* spp. present in the surface of the egg penetrates through the shell pores, infecting the interior (Whiley & Ross, 2015).

Current practices of egg washing and sanitizing provide some reduction in SE shell contamination; however, SE outbreaks from eggs still occur. According to the Center for Disease Control, they estimate that SE is approximately found in one in 20,000 eggs (0.005%) (CDC, 2021). Thus, alternative technologies are being investigated to further reduce SE shell contamination including pulsed light technology (Lasagabaster, Arboleya, & De Marañón, 2011), ultraviolet light (Turtoi & Borda, 2014), far-infrared radiation (Alkaya, Erdogdu, Halkman, & Ekiz, 2016) or ozone (Braun, Fernandez, & Fuhrmann, 2011) among others. The primary limitation of the first three technologies are their line-of-sight requirement and limited penetration into the egg shell pores. Ozone gas can penetrate into the pores, but in solution, oxidation and denaturation of the protein can occur after prolonged exposure (Cataldo, 2003), what can impact egg protein functional performance.

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Cold plasma is presented as a preferred alternative for egg surface decontamination because it can generate a range of reactive gas species including peroxides, nitrates, and ozone that can be formulated to maximize the SE reduction while minimizing egg quality impact. Those species have been proved effective for microbial decontamination over a broad range of microorganisms, including spores and viruses (Misra, Tiwari, Raghavarao, & Cullen, 2011). Cold plasma consists of a partially ionized gas composed of electrons, ions and elements in their fundamental and excited states generated after the application of a high voltage electric field into any gas at room temperature and pressure (Sarangapani, Patange, Bourke, Keener, & Cullen, 2018).

Recent studies where different plasma devices and configurations are used for egg shell decontamination have shown to be effective on SE reduction. Although effective, usually the reported experiments in those studies require complex or expensive gas mixtures, such as helium (Apostol, Georgescu, Vatuiu, & Gaceu, 2015), argon with oxygen (Moritz, Wiacek, Koethe, & Braun, 2017), or helium with oxygen (Georgescu, 2015) among others. Frequently, extended treatment times and post-treatment times are used to achieve the desired microbial reduction, as for example 90 min treatment (Ragni et al., 2010) or 24 h of post-treatment (Wan, Chen, Pankaj, & Keener, 2017). Some of the used plasma systems also have a high power consumption, such as 350 W (Hertwig et al., 2017), which can represent a high cost for their future scale up. Looking into an industrial application of egg shell decontamination using a plasma treatment, this study aims to perform an optimization of the process conditions, where the importance of different treatment parameters are evaluated and optimized according to the industry requirements and preferences, focusing on a high effectiveness with a minimum cost. In this regard, the objective of this study is to optimize a high voltage atmospheric cold plasma (HVACP) process for a minimum five log reduction of SE on the eggshell, while maintaining the quality of the treated eggs.

2. Methods

2.1. HVACP set up

HVACP treatments were performed using a dielectric barried discharge (DBD) set-up. Plasma was generated using a high voltage transformer (Phenix Technologies, Inc., USA) (30–130 kV RMS, 60 Hz) in the gap between two circular aluminum electrodes of 15 cm diameter, with two polypropylene layers of 1 mm of thickness acting as dielectric barriers. For each treatment, one large egg was placed inside a polypropylene ArtBin® box of 5 cm height sealed in high barrier nylon pouches, and treated both under direct or indirect treatment. In the direct mode, the egg is directly placed between the two electrodes, and in the indirect mode the egg is placed out of the plasma field (Fig. 1).

For the HVACP process optimization, two different gases were used, air (21% O_2, 78% N_2, 1% other gases) and modified air MA65 (65% O_2, 30% CO_2 and 5% N_2) (Praxair Canada Inc., Canada), both at 0 and 80% relative humidity. Both gases were supplied dry, and relative humidity was increased by passing the dry gas through a water bubbler. The treatment time ranged from 0.5 min to 15 min, and post-treatment time from 0 h to 24 h at refrigeration temperature of 4 °C. Two different applied voltages were tested of 80 kV and 100 kV RMS.

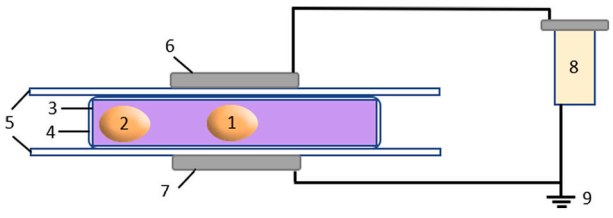

Fig. 1. HVACP set up used in the study, where: 1. Position of the egg for the direct treatment, 2. Position of the egg for the indirect treatment, 3. ArtBin® box, 4. Packaging film, 5. Polypropylene dielectric barriers, 6. High voltage electrode, 7. Ground electrode,8. High voltage transformer, 9. Ground.

Power consumption, voltage and current were collected for each of the plasma treatments. Voltage (kV) and current (mA) values were provided in real time by the monitor on the high voltage transformer panel control, being the average values during the experimental design and model validation of 99.6 ± 3.6 kV RMS (281.7 ± 10.2 kV peak-to peak) and 4.6 ± 0.1 mA, respectively. For the collection of power values (W), the transformer was connected to a power meter in its main electric source, with an average value of 205.3 ± 9.1 W when running experiments at 100 kV, what translates in 1.162 ± 0.051 W/cm^2.

2.2. Experimental design

The optimized process was defined as the combination of HVACP factors where a five log reduction of *Salmonella enteritidis* is achieved on the eggshell. For the optimization of the treatment conditions, a combination of Plackett-Bürman (P—B) method and Central Composite Rotatable Design (CCRD) with surface response methodology was applied. P—B method uses a fractional factorial design which allows the researcher to discard non-contributing factors (Plackett & Burman, 1946). The chosen experimental design was performed with six factors and each factor fixed at two levels: Treatment time (2 and 15 min), post-treatment time (0 and 24 h), mode of exposure (direct and indirect), relative humidity (0% and 80%), gas for plasma generation (air and MA65) and voltage (80 and 100 kV). Total combination of factors gave 12 different assays, and each of them was performed in triplicate, having the design a total of 36 experiments. Based on the screening design results, a final experimental design was performed using CCRD combined with surface response methodology. CCRD is commonly used for optimization of processes, since it studies the effects of the different parameters involved in the process and together with response surface modelling, it allows the researcher to predict an effect under conditions that have not been experimentally tested. The independent variables studied were the treatment and post-treatment time. Treatment time levels were 0.5 min (−1) and 5 min (+1), and post-treatment time levels were 0 h (−1) and 6 h (+1). Considering the axial points (−1.41 and + 1.41) and 3 central points, the design totaled 11 treatments (Table 2). For the selection of axial points, α =1.41, following the calculations for rotatability when two factors are studied ((NIST, 2022a). The equations describing SE log reduction in relation to the combination of treatment and post-treatment time were obtained using a multiple linear regression analysis based on which response surfaces were generated. Following NIST recommendations for validation of design of experiments results (NIST, 2022b), three treatment conditions were randomly selected within the optimized region and performed in triplicate to validate the model. All sets of experiments and their results can be found in the Results section.

2.3. Inoculum preparation, inoculation and recovery method

Salmonella enterica serovar Enteritidis was obtained from the Department of Food Science, University of Guelph. The working culture was prepared by inoculating frozen culture (culture with 50% glycerol at −80 °C) in 40 ml Tryptic Soy Broth (TSB)(OXOID Ltd.) and incubated at 37 °C for 24 h. The culture was streak platted in Tryptic Soy Agar (TSA) (MiliporeSigma) and incubated for 24 h at 37 °C. Fresh inoculum was prepared each 7 days by inoculating one isolated colony from the streaked plate in 40 ml of TSB, and after gentle stirring, incubated at 37 °C for at least 20 h. The bacteria concentration was determined by its optical density at 600 nm, and prior to the inoculation, the culture was centrifuged (Thermo Scientific Sorval ST40R) at 3500 rpm for 20 min at 4 °C. The resulting pellet was suspended in TSB and adjusted to a final cell concentration of 10^9 CFU/ml.

Eggs were purchased in a local store (Guelph, Canada), and

disinfected by wiping them with a 70% ethanol solution to avoid the presence of background microflora. An area of 3.00 cm × 3.00 cm of the egg surface was spot inoculated with 100 μl of the prepared SE suspension and allowed to dry for approximately 30 min in a laminar flow cabinet. Final bacterial concentration was approximately 10^8 CFU/egg.

For the recovery, eggs were individually placed in sterile stomacher bags with 20 ml of Phosphate Buffered Saline (PBS) pH 7.4 at 37 °C. Eggs were then hand rubbed for 3 min and the recovered solution was serially diluted and spread plated in a non-selective media (TSA) and *Salmonella* spp. selective media, Xylose Lysine Deoxycholate (XLD) Agar. The resulting colony forming units (CFU) were counted after incubation overnight at 37 °C. The difference in counts between TSA and XLD were considered as injured cells (Kang & Fung, 2000). The detection limit of the applied recovery and enumeration method was 3-log (CFU/egg), therefore, when no colonies were found in the recovered solution, log reduction was expressed as >5-log.

2.4. Characterization of the reactive gas species (RGS)

2.4.1. Ozone measurement in the gas

Ozone concentration was measured using an ozone monitor (106-MH, 2B Technologies, USA) after each treatment and post-treatment time. This equipment allows to measure up to 10,000 ppm ozone. A small perforation was made in the packaging film and ozone concentration was immediately measured through an opening in the box containing the sample, which was sealed until the measurement.

2.4.2. Nitrate, nitrite and peroxides measurement in the egg

The presence and quantification of nitrates, nitrites and peroxides was performed in the treated eggs after their treatment under the optimized conditions in triplicate. Indicator strips (Bartovation, United States) were used for all species measurements, providing different concentration ranges; nitrates (0–500 ppm), nitrites (0–25 ppm) and peroxides (0–100 ppm).

2.5. Egg quality determination

The most characteristic quality parameters of eggs were evaluated both in untreated and treated eggs. Eggs were treated under the optimized HVACP conditions and analyzed after the stablished post-treatment time. In total, 12 eggs were used for the quality study after each treatment. All eggs were maintained at room temperature for at least two hours before the quality measurements, with an average temperature of 14.0 ± 0.8 °C to prevent any variation caused by differences in egg temperature.

An Egg Analyzer (ORKA Food Technologies LLC, USA) was used for the determination of egg weight, albumen height, Haugh Unit and yolk color based on the YolkFan™ color (DSM Co., Netherlands).

The color of the yolk was additionally evaluated using a hand colorimeter (CSM 4, PCE Inst., USA) in the CIELAB scale using a glass petri dish where the yolk had been previously homogenized. L*, a* and b* values were collected to compare the color of control (untreated) and treated eggs.

The pH measurements of both albumen and yolk were performed using a hand pH meter (Oakton pH Testr® 5).

Shell strength and Vitelline Membrane Strength (VMS) were measured using an Instron texture analyzer (Model 5969). For eggshell strength test, eggs were placed in a disposable polystyrene weighting dish with square shape (4.4 cm * 4.4 cm) with the blunt and round tips of the eggs being horizontal. A 10-kg load cell was used, along with a 75 mm aluminum compression plate. The egg was compressed until the shell cracked, and the strength applied was recorded. For the VMS test, the same load cell was applied along with a 1-mm round-tipped probe. The setting was based on Return to Start mode with some modifications from Wan et al. (2017), with test speed of 0.5 mm/s with a trigger force of 0.1 g. Additionally, pre-test and post-test speeds were 1 mm/s and 10 mm/s, respectively, and the distance was 16 mm. Prior to VMS test, egg albumen was removed using an egg separator. Considering that chalazae identified as the strongest part of the vitelline membrane (Lyon, Newell, & Morrison, 1972), caution was taken to ensure measurements were not made near this area. Punction of the yolk was performed in its middle, and the required strength to break the vitelline membrane was reported.

2.6. Statistical analysis

Regarding the experimental design for microbial reduction analysis, for the P—B screening design, based on the results of each treatment, it was possible to calculate the effect of each variable and a Pareto's chart was plotted to facilitate the visualization. In order to optimize the HVACP conditions for the inactivation of the SE in chicken eggshell, the log reduction data were fitted against the treatment time and post-treatment time to a response surface model using multiple linear regression. The model was selected based on its $R^2 > 0.90$ and p values indicated significant regressions ($p \leq 0.05$). A few points in the optimized region were selected to validate the predictive capacity of the model. Calculations and plots of both designs were carried out using the software Chemoface (Nunes, Freitas, Pinheiro, & Bastos, 2012).

Regarding the egg quality measurements, data was presented as mean ± standard deviation. The results were evaluated by analysis of variance (ANOVA) and Tukey's post-hoc test with a 95% significance level using SPSS software (IBM® SPSS® Statistics 20).

3. Results and discussion

3.1. Screening design results

To calculate the microbial reduction after each treatment, CFU counts from recovered SE of treated eggs was subtracted from the SE counts present in the inoculated egg not subjected to HVACP treatment (positive control). For each experiment, two positive controls were recovered along with the treated eggs and taken as reference for initial load for that experiment. Additionally, two disinfected and non inoculated eggs were used in each experiment as negative controls, to assure the absence of microorganisms. Although not treated by HVACP, positive controls were also packed under the same gas and relative humidity conditions for the same time as the treated samples, including the treatment and post-treatment time. Longer post-treatment times showed lower SE recovery due to the effect of storage time. Positive controls recovered after 24 h at 4 °C showed 6.40 ± 0.12 $\log_{10}$ and 5.64 ± 0.39 $\log_{10}$CFU/egg in TSA and XLD media, respectively, regardless of the gas or humidity used. When SE was recovered right after inoculation (0 h of storage), the recovery was 7.98 ± 0.13 $\log_{10}$ and 7.43 ± 0.18 $\log_{10}$ CFU/egg in TSA and XLD, respectively. More than 1.5-$\log_{10}$ CFU/egg were lost due to the effect of storage at 4 °C.

In order to be able to compare the microbial reduction between experiments with different post-treatment times, 6.40 $\log_{10}$CFU/egg and 5.64 $\log_{10}$CFU/egg were taken as the reference for positive control in TSA and XLD media, respectively, in order to normalize all results and don't overestimate the ones with 24 h post-treatment. The set of experiments tested in the Plackett-Bürman design can be seen in Table 1.

For the statistical analysis of the microbial reduction in the screening design, only TSA media SE counts were used, since they allow the growth of injured but still viable colonies. XLD media only allows the growth of healthy SE cells, and were used for an estimation of injured cells, calculated as the difference between log reduction in TSA and XLD media. If optimization of the process was performed with XLD media, it would overestimate the effect of plasma treatments, as it would not represent the reality of SE cells death. SE log reduction in TSA under the tested conditions ranged from 0.62 ± 0.08 to higer than 5.00.

After the statistical analysis of the results, it was determined that all the tested variables showed a significant effect ($P < 0.05$) on SE reduction. In order to understand the significance of the effect of each

Table 1
Screening design set of experiments (P—B).

#	Treatment time (min)	Post-treatment time (h)	Mode of exposure*	Relative humidity (%)	Gas	Voltage (kV)
1	15	0	I	0	Air	80
2	15	24	D	80	Air	80
3	2	24	I	0	MA65	80
4	15	0	I	80	Air	100
5	15	24	D	80	MA65	80
6	15	24	I	0	MA65	100
7	2	24	I	80	Air	100
8	2	0	I	80	MA65	80
9	2	0	D	80	MA65	100
10	15	0	D	0	MA65	100
11	2	24	D	0	Air	100
12	2	0	D	0	Air	80

* (I) indirect and (D) direct mode of exposure.

factor and what conditions favoured the bacterial reduction, the Pareto's Chart, also generated using the same software, can be seen in Fig. 2.

Post-treatment time and voltage are the two HVACP treatment variables with the highest significant effect on SE reduction. According to the values reflected in Fig. 2, if the post-treatment time is increased from 0 to 24 h in an experiment, regardless of the other parameters, SE reduction would increase by 2.45-log$_{10}$. Something similar would happen with the voltage, where an increase from 80 to 100 kV would mean a 2.27 log$_{10}$ further reduction of SE.

Looking into the effect values, it can be determined that longer treatment times, longer post-treatment time, indirect more of exposure (−1 coded direct, +1 coded indirect), higher relative humidity, air as gas (−1 coded MA65, +1 coded air), and higher voltage have a higher effect on microbial reduction under the tested conditions.

3.2. Optimization results

Fill gas and mode of exposure are variables that can not be changed gradually, therefore, to continue with the CCRD, air was fixed as the gas, and the mode of exposure was chosen to be indirect mode, due to their higher impact on SE reduction. Furthermore, these selected conditions resulted to be favourable to perform in the case of a real treatment in the industry. Regarding the relative humidity, an intermediate value was chosen at approximately 60%, since maintaining the relative humidity at 80% results in condensation on the eggs after treatment. As higher voltage showed a greater impact on SE reduction, its value was fixed at 100 kV, since choosing this value could significantly reduce the treatment and post-treatment time of the process. Therefore, for the CCRD, only treatment and post-treatment time were evaluated as variable factors. The fixed factors were 100 kV, air at 60% RH and indirect mode of exposure. With the aim of choosing industry viable times, and based on preliminary testing, the treatment time range was set between 0.5 and 5 min, and the post-treatment time between 0 and 6 h. Treatment times higher than 5 min and post-treatment times longer than 6 h showed greater SE reduction, but were considered unacceptable for integration into existing processing facilities that process over 12,000 eggs per hour. Therefore, these conditions were selected as the upper limits for the optimization process. The full set of experiments along with the coded and experimental conditions for the CCD, and the SE log reduction in TSA and XLD can be found in Table 2.

As it can be seen in Table 2, variable SE reduction values were obtained when combining the proposed conditions for treatment and post-treatment time. Slightly higher reduction was observed in XLD plates, although only significant difference was observed in treatment #2, suggesting that although there exists a number of colonies that were injured but still viable, plasma treatments had a lethal effect on SE cells. When comparing with similar studies, Wan et al. (2017) used a similar plasma generation system and set up for the treatment of the eggs. In their study, they applied 85 kV during various treatment times and post-treatment time of 24 h at 5 °C, using MA65 and air both in direct and indirect mode of exposure. Differently to our study, they obtained the highest reduction using MA65 as gas, in the direct mode. When using air in the indirect mode, after 15 min of treatment, and 24 h of post-treatment, less than 1-log$_{10}$ SE reduction was observed in TSA media and less than 2-log$_{10}$ in XLD, respectively. In the current study, these conditions would produce a > 5-log$_{10}$ SE reduction. The difference between these two studies is the relative humidity of the gas, Wan et al. (2017) maintained it lower than 5% RH, while the value in our experiments was 60% RH. This suggests that high relative humidity has significant effect on SE reduction in a plasma treatment. In a different

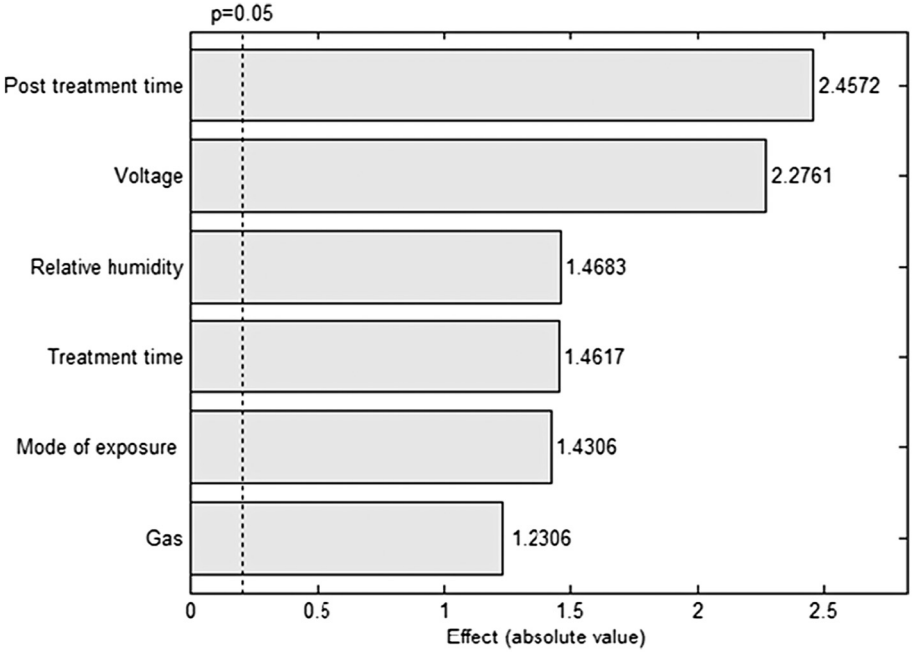

Fig. 2. Pareto's Chart showing the effect in absolute value for each treatment variable ($p = 0.05$).

Table 2
Central composite rotatable design (CCRD) set of experiments and SE log reduction using an indirect treatment at 100 kV in air at 60% humidity.

#	Codified variables		Experimental variables		SE log reduction	
	Treatment Time (min)	Post-treatment Time (hours)	Treatment Time (min)	Post-treatment Time (hours)	TSA	XLD
1	−1.00	−1.00	1.17	0.9	1.01 ± 0.37[a]	1.15 ± 0.25[a]
2	−1.00	1.00	1.17	5.15	3.02 ± 0.21[a]	> 5.00[b]
3	1.00	−1.00	4.4	0.9	2.14 ± 0.29[a]	2.01 ± 0.28[a]
4	1.00	1.00	4.4	5.15	> 5.00[a]	> 5.00[a]
5	−1.41	0.00	0.5	3	1.83 ± 0.31[a]	1.81 ± 0.33[a]
6	1.41	0.00	5	3	2.79 ± 0.67[a]	3.58 ± 1.1[a]
7	0.00	−1.41	2.78	0	0.58 ± 0.23[a]	0.58 ± 0.31[a]
8	0.00	1.41	2.78	6	> 5.00[a]	> 5.00[a]
9	0.00	0.00	2.78	3	2.39 ± 0.68[a]	2.30 ± 0.56[a]
10	0.00	0.00	2.78	3	2.02 ± 0.22[a]	2.41 ± 0.56[a]
11	0.00	0.00	2.78	3	2.53 ± 0.71[a]	3.07 ± 0.59[a]

Means followed by the same letter do not differ statistically among each other in the same row ($p > 0.05$; Tukey test).

study, the effect of initial relative humidity in air was tested against SE reduction on egg shells. The formation of OH radicals were measured by its irradiance in the emission spectrum during plasma treatment, and a higher concentration was observed in a treatment using air with 65% RH when compared to one with 35% RH, proving its direct relation to the higher initial concentration of water in the gas mixture (Ragni et al., 2010), also showing a slightly higher microbial reduction, being 2.5-log at 35% RH and 4.5-log at 65% RH. It is important to mention that Ragni et al. (2010) operated at 15 kV up to 90 min, so it is possible that a higher OH radical production occurs at 100 kV, due to a higher ionization of the gas at higher voltage (Bismo, Irawan, Karamah, & Saksono, 2013). OH radicals are known to easily interact with microbial cells and produce oxidizing reactions, being one of the main oxidant species responsible for microbial reduction (Cho, Chung, Choi, & Yoon, 2004). Therefore, a OH radical concentration measurement in the plasma gas should be performed to completely characterize the process.

SE reduction results in TSA media was subjected to response surface methodology regression and fit into a quadratic model. The model showed a R^2 value of 0.912, and significant regression ($p \leq 0.05$), showing its adequacy for log reduction predictions (Henika, 1982). The 3D plot for the modelled log reduction over treatment and post-treatment time is shown in Fig. 3.

The predicted model equation is as follows:

$$Y = 1.5641 - 0.1768X_1 - 0.7808X_2 + 0.2042X_1X_2 + 0.0111{X_1}^2 + 0.1985\,{X_2}^2$$

Where:
X_1 = Treatment time.

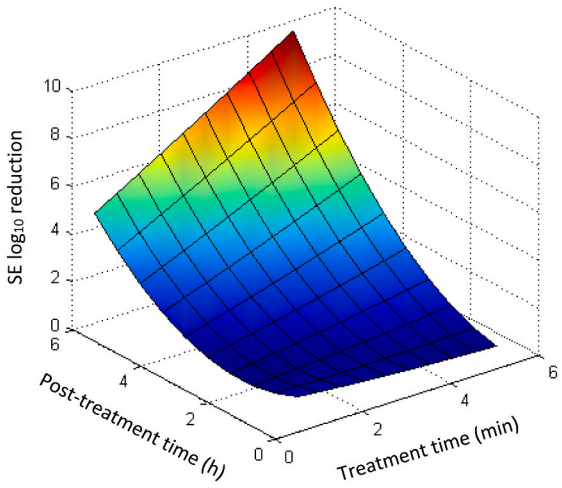

Fig. 3. 3D Quadratic model plot of SE log reduction (Y), over treatment time in minutes (X_1) and post-treatment time in hours (X_2).

X_2 = Post-treatment time.
Y = Response (log_{10} reduction).

For the validation of the model to make predictions, three random experimental conditions where tested and compared to the predicted ones. Selected experiments were chosen in regions were a reduction between approximately 2.5 and 5-logs were expected, since higher reductions can not be determined due to the limit of detection of the methodology employed, and lower reductions were considered to be of less interest. Selected experiments were: 1) 0.5 min treatment +4.5 h post-treatment, 2) 4 min treatment +5 h post-treatment, 3) 5 min treatment +2.5 h post-treatment. All of them where performed in triplicate and results are shown in Table 3.

Experimental data obtained showed higher log_{10} reduction in all tested points when compared to the one predicted by the model. Therefore the model was considered as valid for an estimation of SE log reduction on egg shell surface when treated with HVACP under the specified conditions.

Using the model, it was possible to select two different optimized conditions for further analysis related to the quality of the eggs. As mentioned before, rather a short treatment time or a short post-treatment time could be beneficial for a scale up of this application to the food industry. Therefore, as optimized conditions, one combination using the shortest treatment time and one using the shortest post-treatment time, while achieving a SE reduction >5-log_{10}, were selected.

As per the model equation and posterior experimental confirmation in triplicate, the optimized conditions proposed were: 1) 1 min treatment +6-hourours post-treatment and, 2) 5 min treatment +4 h post-treatment at 100 kV using air at 60% RH.

3.3. Gas chemistry characterization of the optimized HVACP treatment

3.3.1. Ozone concentration results

As previously mentioned, reactive gas species generated during the plasma treatment have been proven to have bactericidal effect, being the reactive oxygen species (ROS) the ones with a higher impact on most

Table 3
Model validation data collection for SE log reduction.

Treatment	Treatment time (min)	Post-treatment time (hours)	Predicted log reduction	Experimental log reduction
1	0.5	4.5	2.45	3.17 ± 0.70
2	4.0	5.0	> 5.00	> 5.00
3	5.0	2.5	2.80	3.44 ± 0.36

bacteria, due to their oxidizing effect (Stoffels, Sakiyama, & Graves, 2008). When one or more of these species crosses the bacteria cell wall, the oxidation of the cytoplasmic membrane, proteins and DNA strands may occur, causing the death of the cell (Gallagher et al., 2007).

Due to its high generation during air plasma treatments, ozone was quantified after each of the performed treatments and post-treatment periods included in the screening and final experimental design.

The ozone generation followed a logarithmic trend ($R^2 = 0.9990$) over treatment times, and as it can be appreciated in Fig. 4(a), the generation rate is much higher in the initial part of the treatments, where the ozone generated in the first 30 s was 797.5 ± 29.0 ppm, and reached a value of 1491.5 ± 85.6 ppm after 70 s of treatment. These values represent a 87% increase in the ozone concentration in just 40 s of treatment. After this, as long as treatment time increases, the generation rate of ozone decreases, showing just a 7.7% of ozone concentration increase between 4 and 5 min of treatment. After a certain treatment time, an ozone concentration plateau is reached, and although treatment time increases, ozone concentration is maintained constant, which according to other studies, could be related to an increase of the nitrogen oxidized species with the present oxygen, rather than ozone generation (Shimizu, Sakiyama, Graves, Zimmermann, & Morfill, 2012).

The ozone concentration decrease followed a similar trend, fitting to a logarithmic model ($R^2 = 0.9998$). In Fig. 4(b), the ozone decrease rate has been plotted versus the post-treatment time after it was measured. As it can be seen, in less than an hour (54 min) of storage at 4 °C, a 41.02 ± 7.18% of the ozone in the packed sample had decreased, and reached a reduction of 65.61 ± 3.59% of the ozone concentration after just 3 h. Ozone decrease after this point was constant but slower, showing a reduction of 76.71 ± 6.61% of ozone after 5 h of post-treatment, and 80.46 ± 6.67 after 6 h. Although a detailed study of the ozone decrease after 6 h of post-treatment has not been performed, it has been proved that after 24 h of post-treatment, no residual ozone is present in any sample. This agrees with a previous study where spinach was also treated in-package with a similar HVACP set up, and ozone concentration was measured during the post-treatment time, finding that more than 50% of the ozone concentration had decreased in 2 h, and no ozone was present in the package after 24 h (Klockow & Keener, 2009). Once the process is scaled up and implemented, the producers will need to ensure that the time between the process and the product available to the consumers respects the necessary time to completely degrade the ozone, which will not go beyond 24 h. If a faster ozone reduction would be needed, HVACP treatments could be performed with an increased relative humidity, which has been shown to significantly increase the ozone decay rate (McClurkin, Maier, & Ileleji, 2013).

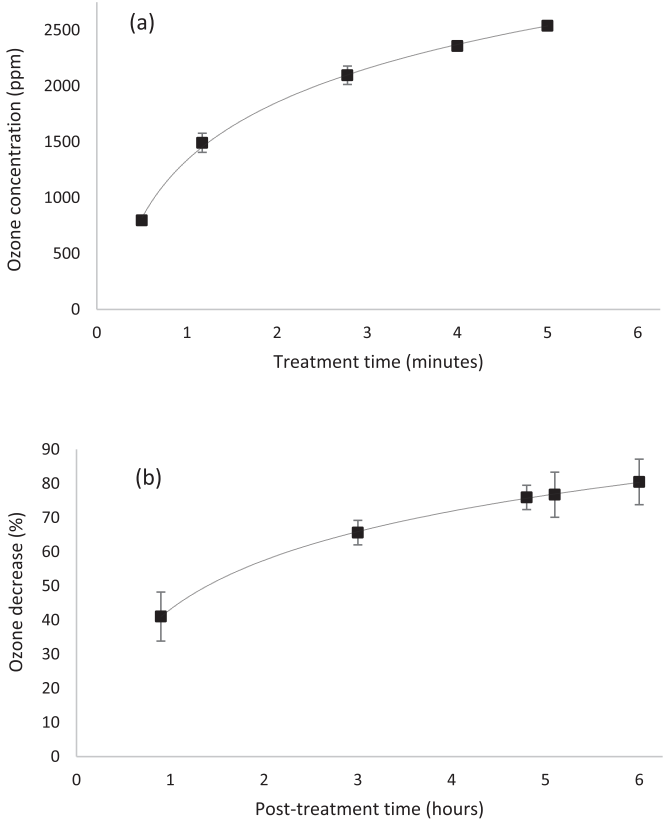

Fig. 4. (a) Ozone generation (ppm) after different treatment times and (b) Ozone decrease rate (%) after plasma treatments and post-treatment time completion. Plasma treatment conditions were 100 kV, air with 60% relative humidity as working gas, and eggs placed in indirect treatment position. Filled symbols represent experimental data average and standard deviation, while dashed line represents a logarithmic model.

As previously observed in the study of the significance of the treatment variables over SE reduction, the parameter showing the higgest effect was the post-treatment time, increasing the bacterial reduction when it was extended. While during the plasma treatment the main agents against bacteria are the short lived species, the UV light and the electric field, these components are not present during the post-treatment time, where the long lived gas species, such as ozone, will be the responsibles for further SE reduction on the egg shell. In this way, we can directly relate the ozone with SE inactivation, since it has been seen that the longer the post-treatment time, the higher the inactivation, and as it has been seen, high concentrations of ozone are present in contact with the sample during the post-treatment time. Even a lower amount of ozone is enough for total reduction of SE, if an appropriate post-treatment time is applied. In preliminary testing not included in this publication, it was observed that with a treatment of just 30 s (797.5 ± 29.0 ppm O_3) under the stablished treatment conditions, more than 5-$\log_{10}$ reduction of SE were achieved when combining it with 24 h post-treatment at 4 °C. Therefore, we can hypothesize that although ozone concentration is an important factor, it is the time of contact with it which has the crucial effect on bacteria reduction.

Nevertheless, we need to mention that in a HVACP treatment, ozone is not the only species related to bacterial inactivation, and although not measured in this study, the complete characterization of the plasma gas should be done in order to completely understand SE inactivation process on eggs surface.

3.3.2. *Nitrate, nitrite and peroxides concentration in the eggs*

To ensure that no residues of nitrates, nitrites or peroxides formed in the plasma gas would penetrate to the inner part of the egg, those were measured in both the yolk and albumen of treated eggs under the optimized conditions. The measurements showed no presence of any of these species in the inside of the egg. Measurements were performed in triplicate in each egg.

3.4. *Quality of the egg after HVACP treatment under optimized conditions*

Some internal quality parameters for HVACP treated and untreated eggs are shown in this section. Eggs were treated under the two sets of optimized conditions, being 1 min treatment +6-h post-treatment (Treatment 1) and, 5 min treatment +4-h post-treatment (Treatment 2) at 100 kV using air at 55–65% RH.

3.4.1. *Albumen and yolk pH*

Lower pH values in egg yolk and albumen is linked to higher freshness, since, as time passes, CO_2 scapes through the eggshell pores and increases the pH of the inner egg (Lin et al., 2021). Eggs submitted to HVACP treatments didn't show a significant difference neither in albumen nor yolk pH (Table 4) after none of the treatments, with average values of 9.35 ± 0.08 and 6.07 ± 0.04, respectively. No changes in egg components pH after plasma treatments have been also found in other studies (Moritz et al., 2021; Wan et al., 2017). Lin et al. (2021)

Table 4
Some internal quality parameters of HVACP treated and untreated eggs.

Egg sample	Albumen pH	Yolk pH	Haugh Unit (HU)
Control	9.34 ± 0.07[a]	6.09 ± 0.05[a]	75.88 ± 2.26[a]
Treatment 1	9.32 ± 0.09[a]	6.07 ± 0.02[a]	72.73 ± 8.50[a]
Treatment 2	9.40 ± 0.08[a]	6.05 ± 0.03[a]	76.78 ± 5.02[a]

Means followed by the same letter do not differ statistically among each other in the same column ($p > 0.05$; Tukey test). Control: untreated eggs; Treatment 1: HVACP treated eggs for 1 min with 6-hour post-treatment; Treatment 2: HVACP treated eggs for 5 min with 4-hour post-treatment.

compared untreated commercial washed eggs and eggs treated under non thermal argon plasma in a conveyor belt. They did not observe a significant difference in albumen pH, 8.94 ± 0.00 and 9.10 ± 0.01 for untreated and treated eggs, respectively. Their yolk albumen pH values were 6.05 ± 0.04 and 6.10 ± 0.07 for control and treated eggs, respectively, showing very similar values to the obtained ones in the present work.

3.4.2. Haugh unit

Haugh unit is another indicator of freshness in eggs, as well as egg albumen protein quality measure. Haugh unit values are used for the grading of eggs, and according to the USDA - US Standards, Grades, and Weight Classes for Shell Eggs, a firm white has a Haugh unit value of 72 or higher when measured between 7 and 15.5 °C (United States Department of Agriculture, 2000). Therefore, as it can be seen in Table 4, HVACP treatments didn't significantly change the HU of the eggs, and grade of the eggs was not reduced from ΄AA΄, with an average among samples of 75.13 ± 5.70. Similar findings were reported in literature, where after a 5 min treatment in a semidirect plasma source using air at 5 kV, not significant changes were reported, and eggs showed an average of 75.57 ± 6.16 (Moritz et al., 2021). Haugh unit values have a great variability between studies, due to the origin and age of eggs used. Lin et al. (2021) untreated eggs showed a HU of 89.68 ± 2.27, and after non thermal plasma treatment, it didn't significantly change, being 91.54 ± 1.78.

3.4.3. Color

YolkFan™ color (DSM Co., Netherlands) values were directly provided by the Egg Analyzer. This scale gives values from 1 to 16 according to the color density of the yolk, being the lower values the ones related to a pale yellow yolk, and increasing in redness to a dark orange yolk color in level 16.

Analyzed yolks using the YolkFan™ color scale showed an average among samples of 3.8 ± 1.3, looking like a light yellow yolk. No significant difference was found between the untreated sample and the treated ones (Table 5). Standard deviation of measurements in each sample was high, due to the natural color variability in eggs. Wan et al. (2017) also reported a non significant difference after treatment of eggs at 85 kV, where their untreated eggs had a YolkFan™ color of 3.0 ± 2.8, and the treated ones 9.5 ± 7.8. This case also shows the high variability in yolk color among eggs.

Table 5
Color parameters of HVACP treated and untreated eggs.

Egg sample	YolkFan color	Yolk color		
		L*	a*	b*
Control	3.80 ± 0.84[a]	58.89 ± 0.46[a]	9.26 ± 1.08[a]	63.26 ± 3.67[a]
Treatment 1	3.20 ± 1.30[a]	57.79 ± 0.61[b]	11.51 ± 1.74[a]	68.99 ± 3.72[a]
Treatment 2	4.40 ± 1.67[a]	58.23 ± 0.54[ab]	11.53 ± 5.24[a]	66.56 ± 7.36[a]

Means followed by the same letter do not differ statistically among each other in the same column ($p > 0.05$; Tukey test). Control: untreated eggs; Treatment 1: HVACP treated eggs for 1 min with 6-hour post-treatment; Treatment 2: HVACP treated eggs for 5 min with 4-hour post-treatment.

The grading of yolk color was also identified using a colorimeter. Redness (a*), and yellowness (b*) didn't show a significant difference in eggs after HVACP treatments, although a slight increasing tendency can be appreciated in both (Table 5). A significant difference only emerged between the Treatment 1 yolk color, with a lower value of L* (darker color) and untreated eggs. Regardless of this difference, not noticeable by the human eye, the overall color did not significantly change, as it can be confirmed by the YolkFan™ color measurements.

3.4.4. Shell strength and vitelline membrane strength

The shell strength and VMS results for the untreated and HVCAP treated eggs are indicated in Table 6. No significant difference ($p > 0.05$) was observed in none of these parameters after HVCAP treatments. These results were comparable with the ones from Wan et al. (2017), where after a DBD plasma treatment at 85 kV, no significant differences where observed in the VMS, being 0.0026 ± 0.0001 for untreated eggs and 0.0024 ± 0.0001 for the HVACP treated ones.

With these results, it can be concluded that the natural strength of the eggshell and the yolk membrane were not degraded, maintaining the quality and firmness of the egg.

Overall, no significant differences were observed in the main quality parameters of eggs after being treated under two selected different HVACP treatments. However, further investigation is needed to analyze the effect of plasma treatments on key egg nutritional components such as protein, lipids, vitamins and antioxidant compounds. Effect of storage time over shelf life of eggs also needs to be performed for a future scale up of the proposed technology in this study.

4. Conclusions

Using a combination of a Plackett Burman screening design and a Central Composite Design along with surface response methodology analysis, it was possible to determine the effect of different HVACP variables over SE reduction on egg surfaces and propose optimized treatment conditions based on the generated and validated model for SE reduction. This model can be used for further research and taken as a reference for a future scale up of HVACP treatments to the egg industry. Furthermore, optimized HVACP treatments did not significantly change any of the key quality parameters in eggs, while assuring a $\log_{10}$ reduction ≥5.0, proving it as a promising alternative technology for egg surface disinfection. Further studies on the plasma gas characterization and on the egg quality stability during shelf life should be considered for future works, as well as a more detailed analysis of ozone decay during post-treatment time.

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CRediT authorship contribution statement

Alba E. Illera: Conceptualization, Methodology, Validation, Formal

Table 6
Shell strength and VMS parameters of HVACP treated and untreated eggs.

Egg sample	Shell Strength (N)	VMS (N)
Control	35.025 ± 4.889[a]	0.021 ± 0.005[a]
Treatment 1	38.305 ± 6.469[a]	0.030 ± 0.006[a]
Treatment 2	37.360 ± 7.099[a]	0.028 ± 0.004[a]

Means followed by the same letter do not differ statistically among each other in the same column ($p > 0.05$; Tukey test). Control: untreated eggs; Treatment 1: HVACP treated eggs for 1 min with 6-hour post-treatment; Treatment 2: HVACP treated eggs for 5 min with 4-hour post-treatment.

analysis, Investigation, Writing – original draft. **Vanessa R. Souza:** Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing – review & editing. **Nooshin Nikmaram:** Methodology, Validation, Investigation. **Linyi Tang:** Methodology, Validation, Investigation. **Kevin M. Keener:** Conceptualization, Methodology, Validation, Supervision, Writing – review & editing.

Declaration of Competing Interest

The authors have no conflicts of interest to disclose.

Data availability

Data will be made available on request.

References

(NIST), National Institute for Standards and Technology. *Central Composite Designs (CCD)*. Retrieved February 12, 2022, from https://www.itl.nist.gov/div898/handbook/pri/section3/pri3361.htm.

(NIST), National Institute for Standards and Technology. How to confirm DOE results (confirmatory runs). Retrieved February 12, 2022, from https://www.itl.nist.gov/div898/handbook/pri/section4/pri46.htm.

Alkaya, G. B., Erdogdu, F., Halkman, A. K., & Ekiz, H. I. (2016). Surface decontamination of whole-shell eggs using far-infrared radiation. *Food and Bioproducts Processing, 98*, 275–282. https://doi.org/10.1016/j.fbp.2016.02.006

Apostol, L., Georgescu, N., Vatuiu, I., & Gaceu, G. (2015). Egg surface decontamination by using high voltage pulsed, cold atmospheric plasma jets. *Journal of EcoAgriTourism, 11*(2), 45–47.

Bismo, S., Irawan, K., Karamah, E. F., & Saksono, N. (2013). On the production of OH radical through plasma electrolysis mechanism for the processing of ammonia waste water. *Journal of Chemistry and Chemical Engineering, 7*(January), 6–12.

Braun, P. G., Fernandez, N., & Fuhrmann, H. (2011). Investigations on the effect of ozone as a disinfectant of egg surfaces. *Ozone Science and Engineering, 33*(5), 374–378. https://doi.org/10.1080/01919512.2011.589359

Cataldo, F. (2003). On the action of ozone on proteins. *Polymer Degradation and Stability, 82*(1), 105–114.

Centers for Desease Control and Prevention. (2021). Salmonella. https://www.cdc.gov/salmonella/index.html. (Accessed 12 February 2022).

Cho, M., Chung, H., Choi, W., & Yoon, J. (2004). Linear correlation between inactivation of E. coli and OH radical concentration in TiO_2 photocatalytic disinfection. *Water Research, 38*(4), 1069–1077. https://doi.org/10.1016/j.watres.2003.10.029

European Food Safety Authority. (2021). The European Union one Health 2019 Zoonoses report. *EFSA Journal, 19*(2). https://doi.org/10.2903/j.efsa.2021.6406

Gallagher, M. J., Vaze, N., Gangoli, S., Vasilets, V. N., Gutsol, A. F., Milovanova, T. N., … Fridman, A. A. (2007). Rapid inactivation of airborne bacteria using atmospheric pressure dielectric barrier grating discharge. *IEEE Transactions on Plasma Science, 35*(5 II), 1501–1510. https://doi.org/10.1109/TPS.2007.905209

Georgescu, N. (2015). Dielectric barrier discharges for egg decontamination with cold atmospheric plasma: Physical and chemical characteristics. *Romanian Journal of Physics, 60*(9–10), 1561–1573.

Henika, R. G. (1982). Use of Response Surface Methodology in Sensory Evaluation. *Food Technology, 36*(11), 96–101.

Hertwig, C., Leslie, A., Meneses, N., Reineke, K., Rauh, C., & Schlüter, O. (2017). Inactivation of *Salmonella enteritidis* PT30 on the surface of unpeeled almonds by cold plasma. *Innovative Food Science and Emerging Technologies, 44*, 242–248. https://doi.org/10.1016/j.ifset.2017.02.007

Kang, D., & Fung, D. Y. C. (2000). *Application of thin agar layer method for recovery of injured Salmonella typhimurium. 54* pp. 127–132).

Klockow, P. A., & Keener, K. M. (2009). Safety and quality assessment of packaged spinach treated with a novel ozone-generation system. *Lwt, 42*(6), 1047–1053. https://doi.org/10.1016/j.lwt.2009.02.011

Lasagabaster, A., Arboleya, J. C., & De Marañón, I. M. (2011). Pulsed light technology for surface decontamination of eggs: Impact on salmonella inactivation and egg quality. *Innovative Food Science and Emerging Technologies, 12*(2), 124–128. https://doi.org/10.1016/j.ifset.2011.01.007

Lin, C. M., Herianto, S., Syu, S. M., Song, C. H., Chen, H. L., & Hou, C. Y. (2021). Applying a large-scale device using non-thermal plasma for microbial decontamination on shell eggs and its effects on the sensory characteristics. *LWT, 142*(November 2020), Article 111067. https://doi.org/10.1016/j.lwt.2021.111067

Lyon, C. E., Newell, G. W., & Morrison, R. D. (1972). Vitelline membrane strength and egg yolk mottling. *Poultry Science, 51*(2), 480–487. https://doi.org/10.3382/ps.0510480

McClurkin, J. D., Maier, D. E., & Ileleji, K. E. (2013). Half-life time of ozone as a function of air movement and conditions in a sealed container. *Journal of Stored Products Research, 55*, 41–47. https://doi.org/10.1016/j.jspr.2013.07.006

Misra, N. N., Tiwari, B. K., Raghavarao, K. S. M. S., & Cullen, P. J. (2011). Nonthermal plasma inactivation of food-borne pathogens. In *, Vol. 3, Issues 3–4. Food engineering reviews* (pp. 159–170). Springer. https://doi.org/10.1007/s12393-011-9041-9.

Moritz, M., Wiacek, C., Koethe, M., & Braun, P. G. (2017). Atmospheric pressure plasma jet treatment of *Salmonella enteritidis* inoculated eggshells. *International Journal of Food Microbiology, 245*, 22–28. https://doi.org/10.1016/j.ijfoodmicro.2017.01.004

Moritz, M., Wiacek, C., Weihe, T., Ehlbeck, J., Weltmann, K. D., & Braun, P. G. (2021). Effect of cold atmospheric pressure plasma treatment of eggshells on the total bacterial count inoculated *Salmonella enteritidis* and selected quality parameters. *Plasma Processes and Polymers, 18*(1), 1–9. https://doi.org/10.1002/ppap.202000061

Nunes, C. A., Freitas, M. P., Pinheiro, A. C. M., & Bastos, S. C. (2012). Chemoface: A novel free user-friendly interface for chemometrics. *Journal of the Brazilian Chemical Society, 23*(11), 2003–2010. https://doi.org/10.1590/S0103-50532012005000073

Plackett, R. L., & Burman, J. P.. The design of optimum multifactorial experiments author (s): R. L. Plackett and J. P. Burman Published by: Oxford University Press on behalf of Biometrika Trust Stable. URL: https://www.jstor.org/stable/2332195.

Public Health, G. of C. (2019). Salmonellosis surveillance. https://www.canada.ca/en/public-health/services/diseases/salmonellosis-salmonella/surveillance.html.

Ragni, L., Berardinelli, A., Vannini, L., Montanari, C., Sirri, F., Guerzoni, M. E., & Guarnieri, A. (2010). Non-thermal atmospheric gas plasma device for surface decontamination of shell eggs. *Journal of Food Engineering, 100*(1), 125–132. https://doi.org/10.1016/j.jfoodeng.2010.03.036

Sarangapani, C., Patange, A., Bourke, P., Keener, K., & Cullen, P. J. (2018). Recent advances in the application of cold plasma technology in foods. *Annual Review of Food Science and Technology, 9*(1), 609–629. https://doi.org/10.1146/annurev-food-030117-012517

Shimizu, T., Sakiyama, Y., Graves, D. B., Zimmermann, J. L., & Morfill, G. E. (2012). The dynamics of ozone generation and mode transition in air surface micro-discharge plasma at atmospheric pressure. *New Journal of Physics, 14*. https://doi.org/10.1088/1367-2630/14/10/103028

Stoffels, E., Sakiyama, Y., & Graves, D. B. (2008). Cold atmospheric plasma: Charged species and their interactions with cells and tissues. *IEEE Transactions on Plasma Science, 36*(4 PART 2), 1441–1457. https://doi.org/10.1109/TPS.2008.2001084

Turtoi, M., & Borda, D. (2014). Decontamination of egg shells using ultraviolet light treatment. *World's Poultry Science Journal, 70*(2), 265–278. https://doi.org/10.1017/S0043933914000282

United States Department of Agriculture. (2000). *United States standards, grades, and weight classes for shell eggs. 56* p. 12). United States Department of Agriculture.

Wan, Z., Chen, Y., Pankaj, S. K., & Keener, K. M. (2017). High voltage atmospheric cold plasma treatment of refrigerated chicken eggs for control of *Salmonella enteritidis* contamination on egg shell. *LWT - Food Science and Technology, 76*, 124–130. https://doi.org/10.1016/j.lwt.2016.10.051

Whiley, H., & Ross, K. (2015). Salmonella and eggs: From production to plate. *International Journal of Environmental Research and Public Health, 12*(3), 2543–2556. https://doi.org/10.3390/ijerph120302543