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Inactivation of *Staphylococcus aureus* in raw salmon with supercritical CO_2 using experimental design

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Abstract

Considering the microbial safety of consumption of raw foods (Asian food), this study aimed to explore the inactivation *S. aureus* in raw salmon by supercritical CO_2 treatment (SC-CO_2). For this purpose, experimental design methodology was employed as a tool to evaluate the effects of pressure (120-220 bar), the depressurization rate (10 to 100 bar.min^{-1}) and the salmon:CO_2 mass relation (1:0.2 to 1:1.0). It was observed that the pressure and the depressurization rate was statistically significant, i.e. the higher the system pressure and depressurization rate, the greater the microbial inactivation. The salmon: CO_2 mass relation did not influence the *S. aureus* inactivation in raw salmon. There was a total reduction in *S. aureus* with 225 bar, a depressurizing rate of 100 bar.min^{-1}, a salmon: CO_2 mass relation of 1:0.6, for 2 hours at 33 °C.

Keywords: *Staphylococcus aureus*; supercritical carbon dioxide; microbial inactivation.

Practical Application: The use of carbon dioxide is an alternative to thermal treatment of food, being able to inactivate microorganisms and increasing shelf life of foods.

1 Introduction

Bacteria of the *Staphylococcus* genus are Gram-positive cocci and have great importance in public health, in particular in the sanitary surveillance of food. Outbreaks are associated with the consumption of ready-made foods such as dairy products, meat products, fish and seafood and vegetables in general. In this sense, it is essential to seek new procedures to preserve food and increase shelf-life (Tironi et al., 2010).

S. aureus is a pathogenic bacterium which does not form a part of the normal microbial flora of fish. However, this microbial group is responsible for about 45% of the world's intoxication and is one of the most common pathogens responsible for foodborne outbreaks; it is usually transmitted by food handlers (Gonçalves, 2011; Germano & Germano, 2008). The safety of fish, particularly the microbiological standard, is very important, since foodborne illnesses occur as a result of lack of care and control from the selection of raw materials up to handling and processing (Marques et al., 2009).

Salmon (*Oncorhynchus* spp.) is a fish of the *Salmonidae* family, much appreciated for its pink flesh. It is peculiar to European seas and rivers, and is also used in aquaculture (Garcia, 2011). It is one of most commonly used fish as a raw material for establishments specializing in Japanese cuisine.

Fresh salmon is a high quality product, with considerable economic importance. It is usually sold vacuum packed and stored frozen without further treatment. The shelf life is difficult to estimate because of the lack of standardized criteria for determining freshness, but the commercial shelf life is often limited to just one week when stored at 2 to 8 °C. The loss of quality is reflected in changes in appearance, odor, color or texture.

Consumption of raw fish in the form of sushi and sashimi occurs in exclusive Japanese cuisine restaurants, and is increasingly found in commercial self-service restaurants. As these establishments are not specialized in Oriental cuisine, in some cases the sushi and sashimi are prepared with the same tools and the same employees who handle other foods, increasing the risk of contamination of raw fish. Additionally, establishments do not always provide adequate food storage, with regard to temperature, time and place of exposure, thus posing a risk to the health of those who consume it (Leisner et al., 2014).

New conservation strategies that prolong life while ensuring microbial safety would allow salmon distribution beyond current markets. Processing at high pressures has been used for the preservation of chicken, pork, surimi and salmon products, with considerable positive effects on the activity of proteases, textural properties, taste and flavor (Amanatidou et al., 2000; Yağız et al., 2009).

Innovative methods for microbiological stabilization in foods as alternatives to the traditional methods, which can provide nutritional and sensory loss to food, are becoming a common object study of researchers all over the world. The use of supercritical carbon dioxide is an alternative to traditional thermal techniques, because it has been found to be effective in reducing the number of microorganisms, causing little change in the original properties of the product. Supercritical

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technology is considered an innovation for food preservation. Processing with supercritical carbon dioxide (SC-CO_2) is used to inactivate pathogenic microorganisms and enzymes, resulting in minimal degradation of heat-labile nutrients, preserving the sensory and nutritional characteristics, and also increasing shelf-life (Choi et al., 2008; Norton & Sun, 2008). In SC-CO_2 treatment, food comes into contact with pressurized CO_2 for a period of time such that CO_2 diffuses through the sample and exerts bactericidal action. In this context, the aim of this study was to evaluate the effects of pressure, the depressurization rate and the proportion of the mass of cells in the inactivation of *S. aureus* previously impregnated in salmon.

2 Materials and methods

The Gram-positive bacterium *Staphylococcus aureus* (ATCC 6538) was first incubated on Luria Bertani broth (Merck) for 24 hours at 35-37 °C. To assess the inactivation of bacteria in pressurized CO_2, we used a factorial design 2^3 where the independent variables were pressure - X_1 (120-220 bar), the depressurization rate - X_2 (10 to 100 bar.min^{-1}) and the salmon:CO_2 mass relation - X_3 (1:0.2 to 1:1.0). The independent variables were fixed at 5 g of salmon contaminated with a bacterial solution of *S. aureus* yielding 1.0×10^5 UFC.g^{-1}, a constant pressurization time of 2 hours and temperature of 33 °C, close to the minimum temperature for the supercritical CO_2 (Soares et al., 2013). The dependent variable (response) was the reduction in the microorganism count in relation to the initial count (Log UFC.g^{-1}).

To evaluate the isolated effect of pressure on the inactivation of *S. aureus* using supercritical CO_2, experiments were conducted with pressures ranging from 90 to 250 bar. All experiments had a total duration of 2 hours (pressurization and depressurization stationary phases), a salmon to CO_2 mass relation of 1:0.6, at a temperature of 33 °C and a depressurization rate of 100 bar.min^{-1}.

After each treatment, the salmon samples were submitted to microbiological analysis, with successive dilutions and plating on Baird-Parker agar (Merck) to count the surviving microorganisms. All experiments were performed in triplicate and the results are expressed as log UFC g^{-1}. The results of microbial inactivation were statistically assessed using the planning experiments methodology in Statistica version 8.0, with a significance level of 90%.

3 Results and discussion

Table 1 shows the matrix of the factorial design (real and coded values) with the independent variables of the study and responses in terms of a reduction in the bacterial count (Log UFC.g^{-1}). Microbial counts ranged between 0.15 and 5.0 log UFC g^{-1}, which corresponds to log reductions of 5.3 and 0.14, respectively (Experiments 4 and 5, respectively). We evaluated the results we found that the best conditions for reducing the microorganism count (Experiment 4) was with a pressure of 220 bar and a depressurizing rate of 100 bar.min^{-1}; the worst performance (Experiment 5) was observed when the pressure was 120 bar and the depressurizing rate was 10 bar.min^{-1}, with a salmon:CO_2 mass relation of 1:0.2 and 1:1.0, respectively.

Microbial reduction data were reported by Erkmen (2000), who investigated the inactivation of *Brochothrix thermosphacta* by SC-CO_2 (610 bar.min^{-1}, 45 °C, 150 rpm) in minced meat and reported a reduction of 1 Log UFC.g^{-1}. According to this author, the nature of the matrix and the presence of compounds such as carbohydrates and fats may play an important role in microorganism protection. Similar results were also obtained by Sirisee et al. (1998), who tested the SC-CO_2 tolerance (35 to 50 °C and 100 to 310 bar) of *Escherichia coli* and *S. aureus* inoculated into ground beef and higher pressures and temperatures significantly decreased the D-values of both bacteria and no cells survived at 50 °C. Meujo et al. (2010) studied the effect of the supercritical CO_2 in microbial inactivation of entire oysters, obtaining 2 and 3 logarithmic reductions in the aerobic microorganisms count, under the conditions 100 bar and 37 °C for 30 minutes and 172 bar and 60 °C for 60 minutes, respectively. These results indicate effect of temperature (pasteurization), not only of pressure. In the present study the temperature effect was not evaluated (fixed close by the minimum temperature for the supercritical CO_2) seeking to prevent a possible pasteurization effect interfering with the evaluation of other evaluated variables.

Table 1. Matrix of the central composite design 2^3 (coded and real values) and the reduction in the count (Log UFC.g^{-1}) of *S. aureus* using SC-CO_2.

Experiment	Independent Variables*			Reduction (Log UFC.g^{-1})
	(X1) (bar)	(X2) (bar.min^{-1})	(X3) (salmon mass:CO_2 mass)	
1	(120) -1	(10) -1	(0.2) -1	0.70
2	(220) +1	(10) -1	(0.2) -1	0.47
3	(120) -1	(100) +1	(0.2) -1	1.04
4	(220) +1	(100) +1	(0.2) -1	5.30
5	(120) -1	(10) -1	(1.0) +1	0.14
6	(220) +1	(10) -1	(1.0) +1	2.96
7	(120) -1	(100) +1	(1.0) +1	0.98
8	(220) +1	(100) +1	(1.0) +1	4.10
9	(160) 0	(55) 0	(0.6) 0	1.14
10	(160) 0	(55) 0	(0.6) 0	1.10
11	(160) 0	(55) 0	(0.6) 0	1.30

*X_1= pressure (bar); X_2= despressurization ratio (bar.min^{-1}); X_3= relation of salmon mass: CO_2 mass. Fixed variables: inoculum: 5×10^5 UFC.g^{-1} of *S. aureus*, total pressurization time 2 hours, temperature 33 °C.

Figure 1 shows a Pareto chart of the estimated effects of the variables for the inactivation of *S. aureus* with SC-CO_2. It was noted that the mass ratio of salmon and CO_2 was not significant ($p>0.10$), indicating that this variable do not interfere with the inactivation of bacteria. The pressure and depressurization rate were positive and significant ($p<0.10$), i.e., the higher the system pressure and depressurization rate, the greater the microbial inactivation.

Divergent data were found by Soares et al. (2013) in which the proportion by mass of the cell suspension and CO_2 was statistically significant, indicating that an increase in the proportion of CO_2 in the system favored microbial inactivation. However, these authors used a liquid matrix in which inactivation was facilitated due to cell spreading; in comparison solid food has a more complex matrix, which impairs the bactericidal effect of CO_2 (Ferrentino & Spilimbergo, 2011). There were no studies demonstrating the effect of SC-CO_2 depressurizing rate in the inactivation of microorganisms in solid foods. Silva et al. (2013) observed effect of pressure and depressurization rate, beyond the pressure cycling, on the inactivation of *Escherichia coli* by supercritical carbon dioxide in the liquid culture media.

To evaluate the individual effect of pressure on the inactivation of *Staphylococcus aureus* using supercritical CO_2, the pressure was varied from 90 to 250 bar. As the depressurization rate had significant positive effect on the inactivation of *S. aureus* and its increase has no influence on process cost, this was fixed at 100 bar.min^{-1}. The relation mass salmon:CO_2 was fixed at the central point of the factorial design (Table 1). The results are shown in Figure 2, where a linear reduction can be seen in the number of surviving microorganisms as the pressure increased. A total reduction in *S. aureus* was observed up to 225 bar, with a depressurizing rate of 100 bar.min^{-1}, a salmon: CO_2 mass relation of 1:0.6, and a process time of 2 hours at 33 °C.

According to Patterson (2005), it is the cell membrane of the microorganism that suffers most from the action of high pressure. It causes changes in the molecular organization of the peptide-lipid complex, breaking the structure of membrane bilayer phosphatidic acids. When the reorganization of the membrane occurs, changes occur in the functions of the proteins that control ion permeability, which causes the membrane to lose its functionality.

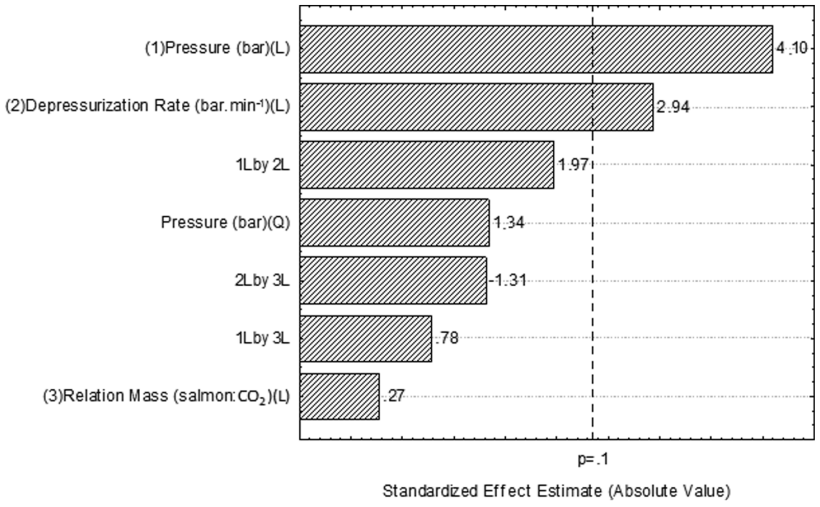

Figure 1. Pareto chart with estimated effect (absolute value) of the variables tested in the factorial design 2^3 for the inactivation of *S. aureus* in SC-CO_2.

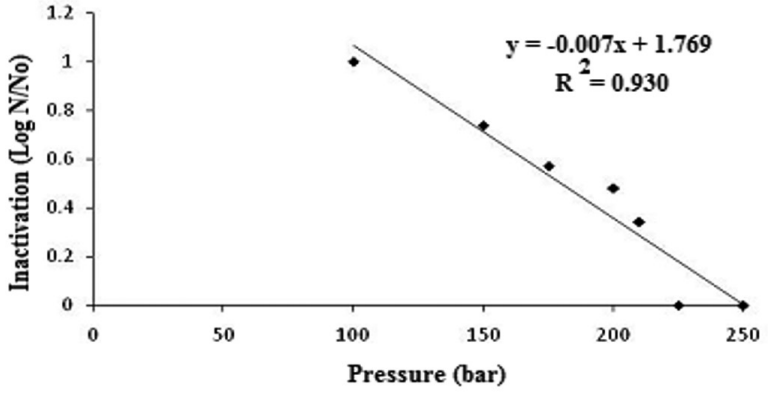

Figure 2. Effect of CO_2 pressure on the inactivation of *Staphylococcus aureus*.

In general, microbial inactivation is accelerated with increasing pressures of CO_2. As a result, higher pressures mean less exposure time is required to inactivate the same number of microbial cells (Lin et al., 1993; Hong & Pyun, 1999). The pressure controls the rate of CO_2 solubility and its total solubility in the suspension medium. Therefore, a higher pressure increases the solubility of CO_2 to facilitate both the acidification of the external environment as well as contact with cells. Moreover, higher CO_2 pressures generally exhibit a greater degree of dilution. Microbial inactivation is also sensitive to temperature. In general, the inactivation rate increases with increasing temperature (under equal conditions). Higher temperatures stimulate the diffusivity of CO_2, and may also increase the fluidity of the cell membrane to facilitate penetration (Lin et al., 1993; Hong & Pyun, 1999).

4 Conclusion

The use of SC-CO_2 technology was effective at inactivating *S. aureus*, where the pressure was the most significant variable in the process. The best experimental conditions to inactivate *S. aureus* were 225 bar of pressure, a depressurizing rate of 100 bar. min^{-1}, a salmon: CO_2 mass relation of 1:0.6, and a process time of 2 hours at 33 °C.

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