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Magali Ritz, Philippe Courcoux, Michel Semenou, Michel M. Federighi. High hydrostatic pressure inactivation of *Salmonella typhimurium*: effects of pressure, duration, pH and temperature studied by analysis of variance. *Veterinary Research*, 1998, 29 (6), pp.547-556. <hal-00902547>

HAL Id: hal-00902547

<https://hal.science/hal-00902547v1>

Submitted on 11 May 2020

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High hydrostatic pressure inactivation of *Salmonella typhimurium*: effects of pressure, duration, pH and temperature studied by analysis of variance

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(Received 7 April 1998; accepted 2 July 1998)

Abstract – High hydrostatic pressure treatments are regarded as possible alternative methods for food preservation. One of the primary considerations for industrial applications is the ability of these methods to eradicate pathogenic microorganisms. This study subjected *S. typhimurium* suspensions, first in a phosphate buffer (pH 7.0) and then in a citrate phosphate buffer (pH 5.6), to high hydrostatic pressure treatments relative to the following variables: pressure (200–400 MPa), duration (3, 10 and 20 min), temperature (4, 20 and 40 °C) and the pH of the suspension medium (5.6 and 7.0). An optimal design of 40 runs was obtained using the Fedorov algorithm, and responses were studied by analysis of variance in terms of cell survival on plate count agar. Efficiency was determined by Log₁₀ comparisons of the numbers of live cells before and after treatment. A statistically significant relationship was found between the four variables considered (pressure, pH, duration and temperature), their interactions (duration × pressure, pH × temperature, pH × pressure) and the inactivation of *S. typhimurium*. R-squared statistical analysis indicated that the linear model used accounted for more than 98 % of the variability in the inactivation of *S. typhimurium*. © Inra/Elsevier, Paris.

Salmonella typhimurium / high pressure / inactivation / model / interaction

Résumé – Inactivation par haute pression hydrostatique de *Salmonella typhimurium* : étude par analyse de la variance des effets de la pression, durée, pH et température. Le traitement hautes pressions des aliments apparaît comme une alternative aux méthodes de conservation traditionnelles. En vue d'applications industrielles, il est important de connaître sa réelle capacité à détruire les micro-organismes pathogènes. Lors de cette étude, des suspensions de *Salmonella typhimurium* placées dans deux tampons : phosphate (pH 7.0) et citrate phosphate (pH 5.6) sont soumises à des traitements hautes pressions. Ces traitements possèdent les caractéristiques suivantes : pression (de 200 à 400 MPa), durée (3, 10 et 20 min), température (4, 20 et 40 °C) et le pH du milieu de traitement (5,6

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ou 7,0). Un plan expérimental de 40 essais a été obtenu par l'algorithme de Fedorov et les réponses (dénombrements des cellules survivantes) ont été étudiées par analyse de la variance. L'efficacité du traitement a été estimée par différence entre les dénombrements (en Log_{10}) avant et après traitement. On a observé une relation statistiquement significative entre l'inactivation de *Salmonella typhimurium* et les quatre variables (pression, durée, température, et le pH du milieu de traitement), mais également avec leurs interactions du premier ordre (durée \times pression, pH \times température, pH \times pression). Le R^2 de l'analyse statistique indique que le modèle linéaire choisi expliquerait plus de 98 % de l'inactivation de *Salmonella typhimurium*. © Inra/Elsevier, Paris.

Salmonella typhimurium / hautes pressions / inactivation / modèle / interaction

1. INTRODUCTION

Salmonella is a leading foodborne pathogen. Traditional physical preservation treatments such as ionizing radiation or heat are currently used to ensure food safety and security. High hydrostatic pressure is now regarded as a promising alternative preservation process for inactivating microbes in food [4, 8]. One of the primary considerations in evaluating the effectiveness of any preservation treatment is its ability to eradicate pathogenic microorganisms and thus ensure product safety. This study tested the efficiency of high-pressure treatments to inactivate a *Salmonella* population. Most studies evaluating the effects of such treatments on bacterial cells have considered the individual effect of treatment time alone [15, 18], the pH of the treatment medium [9], or pressure [15, 17], without determining statistical significance or whether interactions exist between different variables. Some mathematical models have been used for different types of microorganisms to account for specific growth rates, acidification rates or production of bacteriocins [5, 16]. These models have indicated that interactions between variables may exist and show significance. In the present study, the treatment variables (temperature, duration, pressure and pH of the treatment medium) were considered as possible influences on the effectiveness of treatment in a linear model. An experimental design was used to organize the combinations of the different variables.

The purpose of the study was to evaluate the significance of each variable and

determine by analysis of variance how their interactions relate to the inactivation of *S. typhimurium*.

2. MATERIALS AND METHODS

2.1. Bacteria culture preparation

S. typhimurium (ATCC 13311) was obtained from Collection de l'Institut Pasteur (Institut Pasteur, Paris, France) and stored at -30°C in cryobeads (AES, Combourg, France). The first bacterial culture was obtained by inoculating a cryobead into brain–heart infusion (BHI; Biokar, Beauvais, France) incubated at 37°C for 24 h. This first culture was used to prepare a subculture by inoculating 1 p.1 000 (v/v) into fresh BHI for incubating 18 h at 37°C . In these conditions, the count for the initial cell population was 8.1 Log_{10} CFU/mL, with a standard error of 0.1.

2.2. Inoculation of the suspending medium

The pressurized samples were composed of 1 mL of subculture diluted in 9 mL of buffer. Two buffers were used: a phosphate buffer (pH 7.00) composed of Na_2HPO_4 (0.2 mol.L^{-1}) and NaH_2PO_4 (0.2 mol.L^{-1}), and a citrate buffer (pH 5.6) composed of Na_2HPO_4 (0.2 mol.L^{-1}) and $\text{C}_6\text{H}_8\text{O}_7$ (0.1 mol.L^{-1}) (Merck, Darmstadt, Germany). The samples were placed in a sterile ministomacher bag (AES, Combourg, France).

2.3. Enumeration of surviving cells

The number of cells was determined before and after treatment, and the efficiency of treat-

ment was calculated by comparing these two counts in Log_{10} CFU/mL. Cells were enumerated by plating 0.1 mL volumes twice on plate count agar (PCA) (Biokar, Beauvais, France), which were then incubated for 48 h at 37 °C. For low numbers of viable cells, 10 mL of cell suspension were divided between two plates and poured with PCA. Thus, the pitch limit was 0.1 c/mL, corresponding to the result 0.

2.4. High-pressure treatment

The stomacher bags were sealed after eliminating the air inside and placed in a hydrostatic pressure vessel (3 L) in a high-pressure apparatus (Gec Alstom, Nantes, France). High-pressure levels (200–400 MPa) were generated using water pressurized by a hydrostatic pump. Each treatment was a combination of a pressure level, duration, temperature and pH as determined by an experimental design.

2.5. Experimental design

A fractional design was performed to determine the effect of individual variables and their interactions on the inactivation of *S. typhimurium*. Four variables were studied: pressure, pH, duration and temperature. In these experiments, the response, i.e. the extent of inactivation of *S. typhimurium* as measured by Log_{10} CFU/mL (number of viable cells), was assumed to be influenced by the four variables. The levels for each variable were selected on the basis of previous studies. The optimal design of 40 runs was obtained using the Fedorov algorithm with SAS Software [6]. Temperature, pH, duration and pressure were varied as indicated in *table 1*.

2.6. Statistical analysis

For each treatment combination, enumeration was estimated in five stomacher bags. The results were studied by analysis of variance (ANOVA).

3. RESULTS

The significance test of model parameters for *S. typhimurium* inactivation was based on

the analysis of variance shown in *table II*. This table summarizes the results of adjusting a general linear statistical model relating the enumeration of surviving cells after high-pressure treatment to the four selected variables. Since the *P*-value was less than 0.01, a statistically significant relationship existed between efficiency and the predictor variables at a 99 % confidence level.

Table III tests the individual statistical significance of each variable in the model. The highest *P*-value was 0.23, relating to the interaction between time and temperature. Consequently, this interaction was considered as negligible for future treatments. R-squared statistical analysis indicated that the model accounted for 98.43 % of the variability in the number of cells surviving after treatment. Except for the duration \times temperature interaction, all the variables and interactions shown were significant.

3.1. Effects of different variables

The results for analysis of variance indicate that all four variables were statistically significant in accounting for the inactivation of *S. typhimurium* by the high-pressure treatments.

3.1.1. Effect of pH

There was a statistically significant relationship between the inactivation of *S. typhimurium* and the effect of pH at the 99 % confidence level. The means for all enumerations obtained by treatments were 2.46 Log_{10} CFU/mL at pH 5.6 and 3.52 Log_{10} CFU/mL at pH 7. The difference between these means represents an increased treatment efficiency of more than 1 Log_{10} . This variable had a negative effect on the inactivation of *S. typhimurium* since a pH increase in our experimental conditions indicated that high-pressure treatment was less efficient.

Table I. Combinations of the experimental design.

Runs	PH	Duration (min)	Temp. (°C)	Pressure (MPa)
1	5.6	3	4	300
2	5.6	3	4	250
3	5.6	3	40	400
4	5.6	3	40	350
5	5.6	3	20	350
6	5.6	3	20	250
7	5.6	3	20	200
8	5.6	20	4	400
9	5.6	20	4	350
10	5.6	20	40	400
11	5.6	20	40	250
12	5.6	20	20	300
13	5.6	20	20	200
14	5.6	10	4	350
15	5.6	10	4	250
16	5.6	10	4	200
17	5.6	10	40	300
18	5.6	10	40	200
19	5.6	10	20	400
20	5.6	10	20	300
21	7	3	4	400
22	7	3	4	350
23	7	3	4	200
24	7	3	40	300
25	7	3	40	250
26	7	3	20	400
27	7	3	20	300
28	7	20	4	300
29	7	20	4	250
30	7	20	40	350
31	7	20	40	200
32	7	20	20	400
33	7	20	20	250
34	7	10	4	400
35	7	10	4	300
36	7	10	40	400
37	7	10	40	250
38	7	10	20	350
39	7	10	20	250
40	7	10	20	200

Table II. Significance of linear model analysed by ANOVA.

Source	Sum of squares	Df	Mean square	F-Ratio	Statistical Significance
Model	3 750.6	33	113.65	686.89	$P < 0.001$
Residual	60.55	366	0.165		
Total (corr.)	3 811.16	399			

Table III. Significance of factors and their interactions by ANOVA.

Source	Sum of squares	Df	Mean square	F-Ratio	Statistical Significance
PH	89.16	1	89.16	541.31	$P < 0.001$
Duration	233.30	2	116.65	708.21	$P < 0.001$
Temperature	30.45	2	15.23	92.45	$P < 0.001$
Pressure	2 113.14	4	528.28	3 207.31	$P < 0.001$
PH × duration	1.066	2	0.532	3.24	$P < 0.005$
PH × pressure	51.30	4	12.82	77.86	$P < 0.001$
Pressure × duration	93.59	8	11.69	71.03	$P < 0.001$
Pressure × temperature	44.45	8	5.55	33.73	$P < 0.001$
Duration × temperature	0.933	4	0.23	1.42	N.S.
PH × Temperature	13.64	2	6.82	41.41	$P < 0.001$
Residual	59.62	362	0.164		
Total (corrected)	3 811.16	399			

All F-ratios are based on the residual mean square error.
R-squared = 98.43 %.

3.1.2. Effect of temperature

Treatment temperature was also found to be very significant in reducing the bacterial population. The treatments at 20 °C were least efficient (mean Log_{10} CFU/mL: 3.43) as compared to 4 °C (2.87 Log_{10} CFU/mL) and 40 °C (2.67 Log_{10} CFU/mL), which differed by only 0.2 Log_{10} CFU/mL. The effect of temperature was more complex than that of pH. It seemed to be negative between 4 and 20 °C, because the treatments were more efficient at 4 than 20 °C, and positive between 20 and 40 °C, in which case a tem-

perature rise corresponded to a greater inactivation of the *S. typhimurium* population.

3.1.3. Effect of duration

The statistical significance of duration also showed a confidence level greater than 99 %. The mean counts for surviving cells after 3-min treatments was 4.18 Log_{10} CFU/mL as compared to 2.04 Log_{10} CFU/mL after 20 min. The gain in efficiency was quite significant (2.14 Log_{10} CFU/mL). Predictably, the effect of duration seems to have been positive: the reduction of the bac-

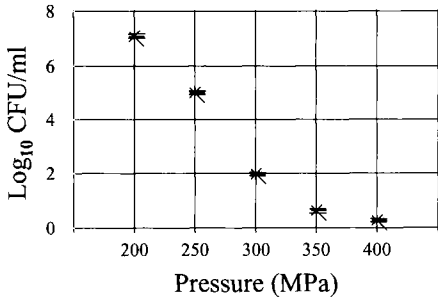


Figure 1. Effect of pressure as illustrated by means and 95.0 percent confidence intervals of all enumerations after treatment.

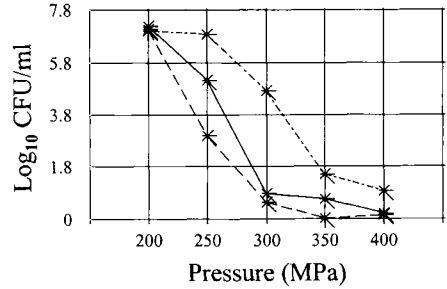


Figure 2. Effect of the interaction of pressure/duration on enumeration after treatment (---- 3 min, — 10 min, — — 20 min).

terial population was proportionally greater for longer periods of high pressure.

3.1.4. Effect of pressure

As expected, the effect of pressure level was most significant (*figure 1*), with a difference between results at low (200 MPa) and high (400 MPa) pressure levels of more than 6.8 Log₁₀ CFU/mL. The effect of pressure was thus very positive since treatment efficiency improved as pressure increased. Moreover, this figure suggests that the relationship between pressure increase and inactivation was non-linear.

3.2. Effects of interactions of two variables

The interaction between duration and temperature is not statistically significant and is not considered here. The most interesting results characterize and quantify the effects of interactions of the four variables on the inactivation of *S. typhimurium*.

3.2.1. Effect of interaction of duration and pressure

Figure 2 shows the three curves of pressure level effects for different treatment times (3, 10 and 20 min). These effects were

greater as treatment time increased (maximal for 20 min). However, for treatments at 200 and 400 MPa, the time period had no significance on inactivation in our experimental conditions. These curves also show similar results (0.77 Log₁₀ CFU/mL versus 0.85 Log₁₀ CFU/mL) for treatments at 300 MPa for 10 min versus some treatments at 400 MPa for 3 min, as indicated by the overlapping of the 95.0 % confidence intervals.

3.2.2. Effect of interaction of pH and temperature

Figure 3 shows the effect of pH at different temperatures (4, 20 and 40 °C). At pH 5.6, there was no significant difference

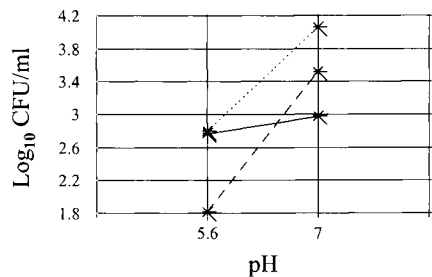


Figure 3. Effect of the interaction of pH/temperature on enumeration after treatment (---- 20 °C, — 4 °C, — — 40 °C).

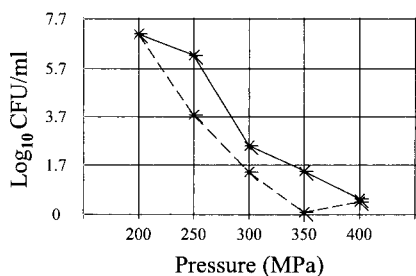


Figure 4. Effect of the interaction of pH/pressure on enumeration after treatment (— pH 5.6, — — pH 7.0).

between a treatment at 4 and at 20 °C. The most efficient treatments involved a combination of pH 5.6 and 40 °C. However, at neutral pH, treatments at 4 °C were most efficient. It is noteworthy that the gain in efficiency of more than 1.7 Log₁₀ CFU/mL was obtained when a treatment at 40 °C was carried out at pH 5.6 instead of pH 7.

3.2.3. Effect of interaction of pH and pressure

Figure 4 shows that pressure was more efficient when the treatment was carried out at pH 5.6, except for the lowest and highest pressures (200 and 400 MPa). As the interaction of pH and pressure was significant, analysis of variance indicated that the same result could be obtained with a treatment at 300 MPa in acid pH and at 350 MPa in neutral pH.

4. DISCUSSION

One of the most important observations in this series of experiments was the statistically significant relationship between inactivation of the *S. typhimurium* population and the four variables of high-pressure treatment (pH, time, temperature and pressure) at the 99 % confidence level. In fact, the application of high hydrostatic pressure treatments to the *S. typhimurium* population

always took these four variables into account. In addition, R-squared statistical analysis showed that the model accounts for 98.45 % of the variability in the inactivation of *S. typhimurium*, suggesting that either all the variables influencing inactivation were taken into account or that other possible variables showed no appreciable changes during the experiments. Analysis of variance indicates that the interactions were also all significant, except for the interaction of pH and time and of time and temperature (which had *P*-values greater than 0.01). This study enabled us to identify the interactions which influence the inactivation of *S. typhimurium* at high hydrostatic pressure. Several authors have investigated the effects of various individual variables (pH, temperature, *a_w*, antioxidant concentration, etc.) on the inactivation of microorganisms. However, the combined influence of these variables was not considered by these authors, so that it was impossible to determine the statistical significance of interactions [9, 10, 14].

The effect of pressure on bacterial inactivation has been well-documented [1, 3, 8, 18]. Shigehisa et al. [17] determined that inactivation of *S. typhimurium* or any other species is dependent on pressure level. Their study was carried out at 25 °C for 10 min, and pressure was the only variable which differed, indicating its positive effect on the inactivation of *S. typhimurium*. Our study confirms these results since a positive effect of pressure was noted regardless of the level of the other variables. Increasing the level of compression generally resulted in greater inactivation. However, figure 1 shows that there was no proportional relationship between the increase in pressure and the reduction of the bacterial population. This relation appeared to follow a non-linear pattern, becoming almost sigmoid. The inactivation curve for microorganisms subjected to high-pressure treatments is similar to that observed for heat treatment, with a shoulder representing a period during which the cells are able to resynthesize a vital com-

ponent. Death ensues only when the rate of destruction exceeds the rate of resynthesis. These curves presenting such a tail may be due to a low number of cells that are protected by dead cells or the products of their destruction [13]. However, Pascal's law, in which hydrostatic pressure is isostatic, seems to make this hypothesis unlikely.

Mackey et al. [10] determined that a reduction in the pH of the suspending medium causes a progressive increase in cell sensitivity to pressure. Our data suggest that high hydrostatic pressure, in combination with acid pH 5.6, is a viable processing method to inactivate *S. typhimurium* since the mean gain in efficiency between treatments at pH 5.6 or 7 was 2.46 Log₁₀ CFU/mL. With the application of treatment and acidity, a smaller intensity of pressure and exposure time might be required to inactivate *S. typhimurium*. The pure effect of pH was always negative in our experimental conditions since a rise in this variable corresponded to a decrease in the efficiency of high hydrostatic pressure treatment. Moreover, acid pH is not always the most efficient condition determining the significance of interactions of pH with other variables. An interesting hypothesis is that pressure treatment could restrict the pH range that the bacteria can tolerate, possibly because of the inhibition of ATPase-dependent transfer of protons and cations [9], or of their direct denaturation or the dislocation of bound ATPase in the membrane [19]. It is generally considered that the primary site of pressure damage for microorganisms is the cell membrane. It might be of interest, in another experimental design, to consider the composition of this membrane as a variable that could influence inactivation.

S. typhimurium is a mesophilic organism capable of growth between +5 and +47 °C (optimally between 35 and 37 °C) [7]. Over a limited temperature range, there is an Arrhenius-type relationship between microorganism growth and temperature. When the temperature is increased towards the maximum, growth rates fall more rapidly

than when temperature is reduced towards the minimum [13]. The effect of temperature measured during high-pressure treatment confirms this law. Our results show that the inactivation of *S. typhimurium* was more efficient at 4 or 40 than at 20 °C, probably because of the greater difficulty for microorganisms to tolerate a pressure increase when temperature approaches minimal or maximal growth level. Two factors could account for the effect of low temperature: the reaction rates for individual enzymes that act together to make the function of the organism much slower, or low temperatures which affect the cytoplasmic membrane by reducing the fluidity of membrane lipids and hence interfere with transport mechanisms [13]. Previous studies have considered the effect of high temperature combined with high-pressure treatment [2, 11], and our results show that this effect was significant from 40 °C. The same phenomenon as for pH was observed for temperature: for mean results a 40 °C temperature was more efficient than 4 or 20 °C, whereas in certain combinations some treatments at 4 °C were more efficient. Nevertheless, the difference between inactivation at 20 and 4 °C or 20 and 40 °C was never greater than 1 Log₁₀ CFU/mL. Thus, if it is impossible to carry out treatments on products at low or high temperature, or if it is easier or cheaper, the effects of the other variables could be used to compensate for this small loss in efficiency.

Inactivation due to pressure is a function of duration, although the relationship between the reduction (in Log₁₀ CFU/mL) and time is not linear. Generally, inactivation curves do not follow first-order kinetics but tend to be exponential, with a rapid initial decrease in cell numbers during the first 15 min of treatment followed by a 'tail' indicating that a small fraction of the population was more pressure-resistant [12, 20]. Our results confirm that treatment time is a very significant variable. The gain in efficiency between a treatment for 3 or 20 min represented more than 2 Log₁₀ CFU/mL,

whereas the increase in activation between 10 and 20 min of treatment was only 0.7 Log₁₀ CFU/mL. Thus, in a practical situation in which product throughput must be maximal, duration could be reduced to 10 min for a combination of treatments compensated by the effect of the other variables.

The experimental design used here indicates that some interactions of the studied variables could be significant, the knowledge of these interactions is particularly useful in optimizing treatment characteristics. For instance, the interaction of pressure and duration was estimated to be significant at a 99 % confidence level. As illustrated by *figure 2*, the effect of pressure was greater when treatment time increased. However, regardless of the treatment time, no efficiency gain is observed at 200 and 400 MPa. One possible reason is that pressure of 200 MPa is always not very efficient. On the contrary, pressure of 400 MPa is immediately very efficient (< 3 min in our experiment).

The study of pH interaction with temperature allowed us to determine that the most efficient treatments at pH 5.6 are performed at 40 °C, so that treatments at 4 °C are best at pH 7. This suggests that the pH of foods could condition the optimal treatment temperature. Moreover, this interaction shows that, for acid foods, treatments at 4 or 20 °C produce the same inactivation. Refrigerated treatment, though not necessary for food with an acid pH, is very important for food with a neutral pH. Naturally, after treatment, storage conditions and shelf-life may be determined according to the growth abilities of surviving microorganisms in the product.

The interaction of pH and pressure was also significant at a 99 % confidence level. *Figure 4* suggests that the effect of pressure was always greater when medium pH was 5.6. However, for 200 and 400 MPa, as for the interaction of pressure and treatment time, the pressure effect was not increased by acid pH. Even at pH 5.6, treatments at

200 MPa remained inefficient, so that treatments at 400 MPa were always very efficient even at pH 7.

In conclusion, our experimental conditions provide a statistically significant linear model for the inactivation of *S. typhimurium* relative to the four variables of high-pressure treatment (pressure, time, temperature and pH of the treatment medium) and three of their interactions (pressure/temperature, pH/temperature and pH/pressure). This study enabled us to select optimal treatment conditions for the inactivation of *S. typhimurium*, which could be consistent with the demands of a limited budget (product throughput, energy savings) or with technical problems (product pH, texture, temperature, etc.). This model will be tested further and validated for other pH, treatment times, pressures and temperatures and for different microorganisms and foods.

ACKNOWLEDGEMENTS

This work was supported by grants from the Region des Pays de la Loire. The authors are especially grateful to F. Jugiau and F. Rama for technical assistance.

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