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Modeling of the inactivation kinetics of *Escherichia coli*, *Saccharomyces cerevisiae* and pectin methylesterase in orange juice treated with ultrasonic-assisted supercritical carbon dioxide

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ABSTRACT

The combined effect of supercritical carbon dioxide (SC-CO2) and high power ultrasound (HPU) on the inactivation kinetics of *E. coli*, *S. cerevisiae* and pectin-methyl esterase (PME) in orange juice was studied in order to select models that can predict their inactivation behavior based on process parameters. Experiments were performed at different temperatures (31-41 °C, 225 bar) and pressures (100-350 bar, 36 °C). The inactivation rate of *E. coli*, *S. cerevisiae* and PME increased with pressure and temperature during SC-CO2+HPU treatments. The SC-CO2+HPU inactivation kinetics of *E. coli*, *S. cerevisiae* and PME were represented by models that included temperature, pressure and treatment time as variables, based on the Biphasic, the Peleg Type B, and the fractional models, respectively. The HPU-assisted SC-CO2 batch system permits the use of mild process conditions and treatment times that can be even shorter than those of continuous SC-CO2 systems.

Key words: supercritical inactivation, residual enzyme activity, inactivation kinetics, ultrasound, modeling, synergistic effect.
1. Introduction

Orange juice is a very popular product due to its high nutritional value, its bioactive components such as phenolics, vitamin C and carotenoids [1] and its well-liked sensory characteristics.

Cloud is a desirable attribute that positively affects turbidity, flavour and the characteristic colour of orange juice. Cloud loss has been primarily attributed to the activity of pectin methyl-esterase (PME), a cell-wall bound pectic enzyme released into the juice during extraction [2]. Acid-tolerant bacteria, yeasts, and moulds also play an important role in causing the quality deterioration of citrus products during storage and distribution [3].

In order to prevent cloud loss and to ensure juices with low microbial levels, preservation techniques must be applied. SC-CO₂ has been reported to inactivate different undesirable enzymes [4-6] and many microorganisms [3, 7-9] in liquid foods without exposing them to the adverse effects of heat, thereby retaining their fresh-like nutritional and sensory qualities [10]. Balaban et al. [2] studied the inactivation of PME in orange juice with a batch SC-CO₂ system. These authors achieved the total inactivation of PME after 145 min at 269 bar and 56 ºC. Fabroni et al. [11] used a continuous high-pressure carbon dioxide pilot-plant system to reduce the PME activity of blood orange juice. They showed a reduction of 25-35 % in the PME activity after treatments at between 130 and 230 bar at 36 ºC for 15 min.

Kincal et al. [3] reported that a continuous SC-CO₂ treatment (210 bars, 34.5 ºC, 10 min) caused at least a 5 log-cycle reduction of pathogens (Escherichia coli O157:H7, Salmonella typhimurium, and Listeria monocytogenes) inoculated into orange juice. Ortuño et al. [12] reported that by using a batch-mode SC-CO₂ at 350 bar and 36 ºC for 25 min, a reduction of 1 log-cycle of Escherichia coli DH1 (E. coli) was obtained in orange juice. Batch-mode equipment requires a much longer inactivation time if compared with that of continuous SC-CO₂ systems. In fact, one of the main inconveniences to the industrial application of batch SC-CO₂ systems is the long treatment time required, a fact which hinders its adoption for use in the food industry [13].

In a continuous system, the agitation caused by the flow of the mixture of treated liquid and SC-CO₂ allows a faster dissolution of CO₂, and therefore its better contact with
cells and enzymes, when compared to batch systems [10]. However, even in continuous systems, the process times needed for the SC-CO\(_2\) inactivation of PME in orange juice are too long to obtain an acceptable enzymatic reduction.

In order to enhance the efficiency of SC-CO\(_2\) microbial and enzyme inactivation processes, a technique based on the combination of SC-CO\(_2\) with high-power ultrasound (HPU) has been developed [14]. This simultaneous application has been shown to accelerate the death of *E. coli* and *Saccharomyces cerevisiae* (*S. cerevisiae*) inoculated into a culture medium, compared with the use of only SC-CO\(_2\) [12, 15]. These studies have shown that the effect of increasing the treatment pressure or temperature in an SC-CO\(_2\)+HPU process conducted on culture media did not significantly enhance the already-rapid inactivation level.

Only two studies have shown that the application of SC-CO\(_2\)+HPU in orange juice completely inactivated the population of *E. coli* and *S. cerevisiae* after 5 min (350 bar, 36 °C) and 1.5 min (225 bar, 36 °C) of treatment, respectively. No microbial reduction was observed in orange juice under the same process conditions (pressure, temperature and time) when using only SC-CO\(_2\) [12, 15].

The use of mathematical modeling is an important tool that allows the effect of different inactivation treatments and process parameters on microbial loads and enzyme concentrations to be analysed, minimizing the number of experiments to be carried out. To describe microbial inactivation using SC-CO\(_2\), different models have been proposed: the Weibull, Gompertz and Logistic models [7-9, 15, 16]. Also, PME inactivation was described by first-order kinetics [2], fractional conversion models, and the Weibull model [6].

At present, the effect of pressure and temperature on the SC-CO\(_2\)+HPU microbial inactivation in juices addressed in the present study has not been evaluated and could differ from that found in culture media reported in the literature [12, 15]. Moreover, the effect of this novel combined treatment on the inactivation of enzymes cannot be found elsewhere in the literature.

Therefore, the objective of this work was to study the combined effect of SC-CO\(_2\) and HPU on the inactivation kinetics of *E. coli*, *S. cerevisiae* and PME in orange juice, and to select models that can best describe and predict their inactivation behavior based on the process parameters.
2. Material and methods

2.1. Orange juice

Valencia Navel oranges (*Citrus sinensis*) were purchased from a local market and kept at 4°C for 2 days until juice extraction. Orange juice was obtained by washing, peeling and extracting the fruit (Ultra Juicer, Robot Coupe J80, USA). The orange juice (pH = 3.8; °Brix = 11.6) was sealed in plastic containers and stored at -18 °C until required.

2.2. Microorganisms and growth conditions

The microbial strains used in this study were *Escherichia coli* DH1, (chromosomal genotype: endA1 gyrA9, thi-1, hsdR179(rK-,mK+), supE44, relA1), and *Saccharomyces cerevisiae* T73, which is a natural strain isolated from wine fermentation in Alicante (Spain) [17] and is commercialised as Lalvin T73 (Lallemand Inc., Montreal, Canada). A single colony of *E. coli* or *S. cerevisiae* was grown overnight in Luria Bertani Broth (LB Broth, Sigma-Aldrich, USA) at 37°C, or in Yeast Peptone Dextrose Broth (YPD Broth, Sigma- Aldrich, USA) at 30°C, respectively, using an incubation chamber (J.P. SELECTA, Model 3000957, Barcelona, Spain) and an orbital shaker at 120 rpm (J.P. SELECTA, Rotabit Model 3000974, Barcelona, Spain). For each experiment with *E. coli* or *S. cerevisiae*, a subculture was prepared by inoculating 50 µL from the starter culture into 50 mL sterilized medium and incubating at 37 °C-24 h or at 30 °C-24 h, respectively, to obtain cells in the early stationary phase. Growth curves were determined in advance by both plating and measuring the absorbance at 625 nm (data not shown).

2.3. Inoculated juice

For each experiment, a container of orange juice was thawed at 4 °C for 12 h. The juice was inoculated by the addition of 5 mL of either *E. coli* or *S. cerevisiae* cells in the early stationary phase (see section 2.2) to 50 mL of orange juice to reach a cell concentration of $10^7$ CFU/mL for *S. cerevisiae* and $10^8$ CFU/mL for *E. coli*.

2.4. Supercritical fluid equipment and processing

2.4.1. Apparatus

The supercritical fluid lab-scale batch system was specially designed and built by our research group. It includes a CO2-tank, a N2-tank, a chiller reservoir kept at -18 °C; a pump and a thermostatic bath to keep the treatment vessel at the desired temperature. The system includes ultrasound equipment [14] embedded in the supercritical fluid
vessel. The ultrasound equipment consists of a high power piezoelectric transducer, an insulation system and a power generator unit (40 W ± 5 W). The transducer is inserted inside the inactivation vessel and includes two commercial ceramics (35 mm external diameter; 12.5 mm internal diameter; 5 mm thickness; resonance frequency of 30 kHz) and a sonotrode, which was specially constructed to concentrate the highest amount of acoustic energy on the application point. The equipment is described in detail in Ortuño et al. [15].

2.4.2. Supercritical fluid processing.

Fifty-five mL of inoculated orange juice for microbial inactivation, and 55 mL of non-inoculated orange juice for enzyme inactivation, was subjected to the SC-CO$_2$+HPU treatment under different process conditions. To determine the effect of pressure, samples were treated by SC-CO$_2$+HPU at 36 ºC and 100, 225 and 350 bar. To determine the effect of temperature, samples were exposed to SC-CO$_2$+HPU at 225 bar and 31, 36 and 41 ºC. The temperature and pressure ranges chosen were higher than the critical point for CO$_2$ and lower than lethal levels for both microorganisms. According to previous studies of the inactivation of these microorganisms using SC-CO$_2$+HPU, higher temperatures or pressures were not necessary to reach acceptable levels of inactivation [12, 15]. The experimental process has previously been described by Ortuño et al. [15] in detail. All experiments were run in triplicate.

2.5. Enumeration of viable microorganisms.

The viability of *E. coli* and *S. cerevisiae* in the orange juice samples was determined by the plate count method. Each sample was serially diluted with sterilised distilled water. 100 µL of the appropriate dilution were plated in triplicate on LB Agar or YPD Agar plates and incubated for 24 h at 37 ºC or 30 ºC, for *E. coli* or *S. cerevisiae* respectively, before counting. Microbial cells in the initial non-treated sample (control sample) were counted following the same procedure. The results were expressed as log$_{10}$ (N/N$_0$) versus time, where N$_0$ is the initial number of cells in the control sample and N is the number of cells in the sample after the different times of treatment. The data presented are the means of triplicate experiments. The results shown are the arithmetic mean and the standard deviation of log$_{10}$ (N/N$_0$) for at least three plates.

2.6. PME activity measurements.
The PME activity of orange juice was determined at pH 7 and 25 °C using the Castaldo et al. [18] method, with modifications. The reaction mixture consisted of orange juice and a substrate solution that was prepared by dissolving 10 g of pectin powder (Sigma Chemical Co., St. Louis, MO) in 1 L of 0.15 M NaCl. The NaCl solution was heated to 50-55 °C and added to the blender while pectin powder was sprinkled on the surface and blended. The pectin solution was stored at 4 °C until required.

The pH of the pectin solution was adjusted to 7 prior to each analysis and 5 mL of orange juice were added to 50 mL of pectin solution. The pH was quickly adjusted to 7 (0.5 M NaOH for gross adjustment, 0.05 M NaOH for fine adjustment). The pH was maintained at 7 by means of the addition of 0.05 M NaOH. The consumption of NaOH was recorded during a period of about 30 min. The dVNaOH/dt slope was determined in the linear part of the titration curve. The PME activity of the orange juice sample, A, was calculated by Eq. (1) and expressed as microequivalents per min and mL of juice.

\[ A = \frac{dV_{NaOH}}{dt} \frac{N_{NaOH}}{V_{sample}} \]  

Eq. (1)

where \( V_{NaOH} \) and \( N_{NaOH} \) are the volume and molarity of the NaOH solution used for the titration, respectively, and \( V_{sample} \) is the volume of the orange juice added to the substrate solution (mL).

Each sample was analyzed in triplicate. The data were normalized to percentage of activity relative to the untreated orange juice and the PME residual activity (RA) was calculated using Eq. (2).

\[ \text{PME residual activity} = \frac{\text{Specific activity PME after treatment}}{\text{Specific activity PME control sample}} \times 100 \]  

Eq. (2)

2.7. **Modeling of the microbial and enzyme inactivation kinetics**

The modeling of microbial inactivation using SC-CO\(_2\) [7-9] and HPU [19] processing has been studied for different microorganisms. Six different models which had previously been used in the literature [8, 20-23] to fit inactivation kinetics for other non-thermal techniques were selected to describe the inactivation kinetics of microorganisms using SC-CO\(_2\)+HPU (Table 1).
Two models, used to fit the residual activity curves of PME treated with non-thermal techniques, have been selected in this study to fit the inactivation curves of PME treated with SC-CO$_2$+HPU (Table 1).

2.8. Statistical analysis of the inactivation kinetics

The Statgraphics Plus (Statistical Graphics Corp. 5.1, Warrenton, USA) statistics package was used to perform multifactorial ANOVA, and LSD (Least Significant Differences) were identified to evaluate the effect of pressure, temperature and time on the inactivation rate of microorganisms and on the residual PME activity of treated orange juice.

The kinetic constants of the models were calculated by minimizing the sum of the square differences between experimental and model-predicted data using the Solver Microsoft Excel$^\text{TM}$ tool. The root mean square error (RMSE, Eq. 3) and the coefficient of determination ($R^2$, Eq. 4) were used to evaluate the goodness of fit of the model and the accuracy of estimation. RMSE is a measure of the standard error in the estimation, whereas $R^2$ is used as a measure of explained variance [24].

\[
\text{RMSE} = \sqrt{\frac{1}{z} \sum_{k=1}^{z} (y_k - y_k^*)^2}
\]

\[R^2 = 1 - \frac{S_{yx}}{S_y^2}\]

where $y$ and $y^*$ are the experimental data and the estimated values, respectively, calculated as $\log_{10}(N/N_0)$ or $\log_{10}(A/A_0)$ for microorganisms or enzymes, respectively; $z$ is the number of experimental values and $S_y$ and $S_{yx}$ are the total standard deviation and the standard deviation of the estimation, respectively.

3. Results and discussion

3.1. Combined effect of HPU and SC-CO$_2$ on E. coli inactivation.

Figure 1A shows the inactivation curves of E. coli in orange juice treated with a combined SC-CO$_2$+HPU process. The survivor numbers began to decrease immediately and no lag-phase was observed for any temperature or pressure studied. A reduction of 4.12, 4.62 and 6.15 log-cycles was obtained after 1 min of treatment, at 31, 36 and 41 $^\circ$C, respectively. There were no significant differences ($p>0.05$) between the
inactivation at 31 and 36 °C; however, when the temperature was increased to 41 °C, a significantly (p<0.05) faster inactivation was observed. Although the inactivation rate decreased after the first minute in every case, 7 min was needed to attain total inactivation (7-8 log-cycles) at 31 and 36 °C and only 3 min at 41 °C.

Regarding the effect of pressure on the inactivation of *E. coli*, reductions of 2.5, 4.6 and 5.4 log-cycles were reached after 1 min of treatment at 36 °C and 100, 225 and 350 bar, respectively (Figure 1B). After the first minute, the population decrease was slower and after 7 min of treatment reductions of 5.8, 7.2 and 7.9 log-cycles at 100, 225 and 350 bar, respectively, were reached. On average, the inactivation rate significantly increased (p<0.05) as the pressure rose from 100 to 225 bar, and from 225 to 350 bar.

The inactivation of *E. coli* has been explored in previous studies using both techniques (SC-CO₂ and HPU) individually. Liao et al. [25] studied the inactivation of *E. coli* with a batch SC-CO₂ system in cloudy apple juice at different temperatures and pressures. After 75 min, the microbial reduction increased from 5 to 7 log-cycles as the temperature rose from 32 to 42 °C (300 bar), respectively; and from 5.5 to 7.5 log-cycles as the pressure increased from 100 to 300 bar (42 °C). Kincai et al. [3] tested a continuous high-pressure CO₂ system for the inactivation of *E. coli* inoculated in orange juice. These authors reached a reduction of 4 log-cycles (10⁵ CFU/mL initial population) using 34.5 °C and 380 bar after a residence time of 10 min. Thus, it can be concluded that, in continuous systems, the treatment time is drastically reduced compared to batch systems due to the agitation of the medium which enhances the solubilization of the SC-CO₂ and the extraction of cellular components. However, the HPU-assisted batch supercritical system used in the present study attained similar inactivation levels in shorter process times than in continuous systems. This fact could be due to the high energy agitation of the ultrasonic waves and to the cavitation phenomenon.

In this regard, using SC-CO₂+HPU, the acceleration of the solubilization rate of SC-CO₂ into the liquid and the increase in the mass transfer due to the vigorous agitation produced by the ultrasonic field would permit the rapid saturation of CO₂ in the medium, which might accelerate the inactivation mechanisms (a decrease of the medium pH, an increase in membrane fluidity and permeability, the diffusion of CO₂ into the cells, cell membrane rupture, the alteration of intracellular equilibrium, the inactivation of key enzymes, and the extraction of critical intracellular materials) [26,
Moreover, the cell wall damage caused by cavitation could play an important role in both the penetration of SC-CO₂ and the extraction of intracellular compounds, accelerating the death of the microbial cells [15].

Contrary to the results observed in previous studies into the inactivation of *E. coli* in LB Broth medium [12], where the effect of increasing pressure and temperature did not enhance the already-rapid inactivation rate, the present study using orange juice showed that increases in both pressure and temperature led to a rise in the inactivation rate. It is known that the inactivation rate is affected by the composition of the suspending medium [12, 26]. There are approximately 70% more sugars in the orange juice (11.6 °Brix) than in LB Broth. They bind water from the medium and there is a smaller amount of free water in which CO₂ could be dissolved than in LB Broth. Despite the intense ultrasound agitation, the orange juice was not as quickly saturated with CO₂ as LB Broth, due to the lower CO₂ solubility as a consequence of the high sugar content. Therefore, increasing pressure or temperature could facilitate the solubilization of CO₂ into the orange juice. This is the first step in the inactivation mechanisms of SC-CO₂+HPU, from which other mechanisms follow. It is also known that the viscosity of the medium directly affects the phenomenon of cavitation. To generate cavitation bubbles, the cohesive forces of the liquid have to be overcome by the negative pressure. The cohesive forces increase as the liquid becomes more viscous; therefore, it is more difficult to obtain cavitation [28]. The orange juice is more viscous than the LB broth. Therefore, cavitation could be less intense and its inactivation mechanisms against microorganisms less severe.

The nature of the medium influenced the effect of HPU and variations between different temperatures and pressures were observed. Therefore, it is important to determine the effect of the combination of treatment medium and process temperature / pressure on the inactivation of microorganisms, to find optimum SC-CO₂+HPU process conditions. For that purpose, the modeling process is of great importance.

### 3.2. Modeling of *E. coli* inactivation kinetics

Table 2 shows the statistical parameters for the fit of the kinetic models to the inactivation data of *E. coli* in orange juice treated by SC-CO₂ and HPU. R² and RMSE values (Table 2) indicate that, overall, a good fit was obtained with the six models for the different process conditions considered, with R² > 0.9 for most of the conditions studied except for the Gompertz model (R²_avg = 0.887; RMSE_avg = 0.549). The standard
deviation of the differences between the values which were actually observed and those
estimated by the model was below 0.5 log-cycles. The Biphasic model provided the best
fit ($R^2_{avg} = 0.967$) for all the process conditions used, with an accuracy of prediction of
0.286 log-cycles. In this model, to relate $f$, $D_{sens}$ and $D_{res}$ (see Table 1) to pressure and
temperature, we assumed that these parameters were described by a log-logistic model
[22], with simultaneous pressure and temperature dependences (Eqs. (5-7)).

\begin{align*}
\text{Eq. (5)} & \quad f(T, P) = \ln(1 + \exp(a_f (T - Tc) + b_f (P - Pc))) \\
\text{Eq. (6)} & \quad D_{sens}(T, P) = \ln(1 + \exp(a_{Ds} (T - Tc) + b_{Ds} (P - Pc))) \\
\text{Eq. (7)} & \quad D_{res}(T, P) = \ln(1 + \exp(a_{Dr} (T - Tc) + b_{Dr} (P - Pc)))
\end{align*}

where $a_f$, $b_f$, $a_{Ds}$, $b_{Ds}$, $a_{Dr}$, $b_{Dr}$, $Tc$ and $Pc$ are the characteristic constants of the
microorganism. Substituting Eqs. (5-7) in the Biphasic model (Table 1), a general
expression of the Biphasic model is obtained that can be used to predict the inactivation
kinetics of *E. coli* in orange juice at different pressures and temperatures.

The characteristic constants of the microorganism were calculated by minimizing the
sum of square differences between all the experimental data and all the predicted data
obtained from every pressure and temperature condition studied, using the Excel Solver
tool. The values of the coefficients $a_f$, $b_f$, $a_{Ds}$, $b_{Ds}$, $a_{Dr}$, $b_{Dr}$, $Tc$ and $Pc$ were: -0.442, -
0.021, -0.045, -0.003, 0.057, 0.005, 39.296 and -272.474, respectively. The predicted
survival curves of *E. coli* in orange juice, using the described Biphasic general model,
can be seen in Figure 1. The $R^2_{avg} = 0.960$, is comparable to that provided by the
individual fits to each temperature and pressure combination (Table 1: $R^2_{avg} = 0.967$).
The average prediction error only increased from 0.286 log-cycles to 0.391 log-cycles.

**Figure 2** shows the comparison between experimental and predicted log reductions with
low and randomly distributed prediction errors around the fit of the model.

### 3.3. Combined effect of HPU and SC-CO₂ on *S. cerevisiae* inactivation

At different temperatures and pressures (Figure 3), the viability of *S. cerevisiae* began to
decrease immediately and no lag-phase was observed for any condition studied. Figure
3A shows the inactivation for the three temperatures studied. On average, the
inactivation rate at 31 °C was significantly slower ($p<0.05$) than at 36 and 41 °C,
between which no significant ($p>0.05$) differences were observed. After 6 min at 31 °C,
an inactivation of 4 log-cycles was obtained, however for the other two temperatures,
the total microbial inactivation (6.5-7 log-cycles) was reached in less than 3 min.
Regarding the inactivation of *S. cerevisiae* at different pressures (Figure 3B), the three survival curves showed a faster inactivation rate for the first minute, then a progressively slower decrease of the population was observed and total inactivation was obtained after 4, 1.5 and 2 min using 100, 225 and 350 bar, respectively. On average, the inactivation levels obtained at 100 bar were significantly lower (p<0.05) than those at 225 and 350 bar, between which no significant differences (p>0.05) were obtained.

The inactivation levels of *S. cerevisiae* inoculated in orange juice with SC-CO$_2$+HPU increased with pressure and temperature, although temperatures and pressures higher than 36 ºC and 225 bar, respectively, were not necessary to attain the total inactivation after 1-2 min of treatment.

The inactivation of *S. cerevisiae* by means of SC-CO$_2$ or HPU alone has previously been studied. Li et al. [29] reduced the population of *S. cerevisiae* inoculated in bean sprout extract with a batch high pressure CO$_2$ system. The microbial reduction increased from 2.5 to 4.5 logs as the temperature rose from 25 to 35 ºC (100 bar, 120 min); and from 2.5 to 5 logs as the pressure went up from 100 to 300 bar (25 ºC, 120 min). Shimoda et al. [30] studied the inactivation of *S. cerevisiae* with a continuous CO$_2$ system in phosphate buffer with an initial concentration of 10$^8$-10$^9$ CFU/mL. After 15 min of residence time at 35 ºC, 60 bar and 20 g CO$_2$/100 g sample, no survivors were found. Similarly to *E. coli*, the required times for the inactivation of *S. cerevisiae* with SC-CO$_2$+HPU are much shorter than in batch systems and are comparable or better than in continuous systems.

Different components, such as sugars, etc., lessen the effect of SC-CO$_2$+HPU during *S. cerevisiae* inactivation treatments in orange juice, compared to that in culture medium reported by Ortuño et al. [15]. In the latter, pressure and temperature increases were not needed for inactivation, since even low process parameters resulted in total inactivation. In the present study, the higher sugar content of orange juice resulted in temperature and pressure having a positive effect on the inactivation levels.

The application of HPU had a different effect against different microorganisms. It is known that Gram-positive cells are more resistant than Gram-negative ones due to their thicker cell wall [31]. It is also known that *S. cerevisiae* has a thicker cell wall, which makes it similar to Gram-positive bacteria [32]. Comparing the results of the present study between *E. coli* and *S. cerevisiae*, at 31 ºC and 225 bar, a reduction of 7 and 4 logs was attained respectively, after 6 min of treatment. These results would support the
connection between wall thickness and inactivation resistance [9]. Therefore, under the
same process conditions, a slower \textit{S. cerevisiae} inactivation was obtained. However, \textit{E. coli} showed more resistance to \textit{SC-CO}$_2$+HPU treatments than \textit{S. cerevisiae} for all the other pressure and temperature conditions. This fact could be related to the cavitation phenomenon and the microorganism’s size. The \textit{S. cerevisiae} cells, 8-10 µm [33] in size, are much bigger than \textit{E. coli} cells, 1.2-2 µm [34]; therefore, there is more likelihood that the cavitation bubbles might affect the cell structure of \textit{S. cerevisiae} than that of \textit{E. coli}.

The nature of the medium influenced the effect of HPU and, in addition to permitting observable effects of increasing temperatures and pressures, it also allowed observable differences between microorganisms.

3.4. \textit{Modeling of inactivation kinetics of S. cerevisiae}

Table 3 shows the statistical parameters for the fit of the kinetic models to the inactivation data of \textit{S. cerevisiae} in orange juice treated by SC-CO$_2$ and HPU. For all the models $R^2_{\text{avg}} > 0.94$ and the standard deviation of the differences between the values which were actually observed and those estimated by the model was below 0.5 log-cycles, with the exception of the Log-linear model ($R^2_{\text{avg}} = 0.768$; RMSE $\text{avg} = 0.306$). The best fit was obtained by the Peleg Type B model ($R^2_{\text{avg}} = 0.983$; RMSE $\text{avg} = 0.188$). A general equation was sought to describe the inactivation kinetics of \textit{S. cerevisiae} obtained with SC-CO$_2$+HPU at any pressure and temperature over the range of these variables considered in the present study. The parameters of the Peleg Type B model, $a_1$, $a_2$ and $r$, were defined using a log-logistic equation that included [22] a simultaneous pressure and temperature dependence (Eqs. (8-10)).

$$a_1(T, P) = \ln (1 + \exp (a_{a1} (T - Tc) + b_{a1} (P - Pc))) \quad \text{Eq. (8)}$$

$$a_2(T, P) = \ln (1 + \exp (a_{a2} (T - Tc) + b_{a2} (P - Pc))) \quad \text{Eq. (9)}$$

$$r(T, P) = \ln (1 + \exp (a_r (T - Tc) + b_r (P - Pc))) \quad \text{Eq. (10)}$$

where $a_{a1}$, $b_{a1}$, $a_{a2}$, $b_{a2}$, $a_r$, $b_r$, $Tc$ and $Pc$ are the characteristic constants of the microorganism.

Substituting Eqs. (8-10) in the Peleg Type B model, a general expression of the model was obtained and used to predict the inactivation kinetics of \textit{S. cerevisiae} in orange juice (Figure 3). The different characteristic constants of the \textit{S. cerevisiae} inactivation model were calculated by minimizing the sum of square differences between all the
experimental data and all the predicted data considered for every pressure and temperature condition studied, using the Excel Solver tool. The values of the coefficients, $a_{a1}$, $b_{a1}$, $a_{a2}$, $b_{a2}$, $a_r$, $b_r$, $T_c$ and $P_c$, were: 9.788, 0.355, -0.157, -0.007, 1.929, 0.070, 3.523 and 973.078, respectively.

The value of $R^2 = 0.894$, indicate that the Type B model satisfactorily described the survival curves of $S. cerevisiae$ (Figure 3). As expected, the statistical parameters of the general model showed a worse fit than the initial individual fits for each survival curve obtained at each temperature and pressure (Table 3: $R^2_{avg} = 0.983$). The error in the estimation increased from 0.188 log-cycles to 0.687 log-cycles. However, according to the $R^2$ and RMSE values, the proposed model appropriately described the inactivation kinetics of $S. cerevisiae$ under SC-CO$_2$+HPU treatment as a function of temperature, pressure and time of treatment, over the practical range of 100-350 bar and 31-41ºC. Figure 4 shows the correlation between the experimental and predicted log reduction values. For low microbial reductions, between 0 and 3 logs, the modified Type B model predicted higher values. The highest deviation value occurred at 350 bar, 36 ºC and 0.33 min of treatment time and is equal to 2.10 log.

From these results, it could be concluded that the survival models that have previously been used to describe microbial inactivation by means of other non-thermal technologies, such as SC-CO$_2$ or HPU alone, have appropriately predicted the SC-CO$_2$+HPU inactivation kinetics of $E. coli$ and $S. cerevisiae$.

3.5. Combined effect of HPU and SC-CO$_2$ on pectin methyl-esterase inactivation.

Figure 5 shows the inactivation of orange juice PME after three SC-CO$_2$+HPU treatments. The RA of PME decreased as the treatment time increased (Figure 5A), and the higher the temperature, the greater the RA decrease. The effect of temperature was noticeable from the beginning of the process; after 2 min, the RA was 83.63, 81.01 and 50.46 % at 31, 36 and 41 ºC, respectively. No significant differences ($p>0.05$) were observed in the RA values at 31 and 36 ºC, which decreased until reaching an average 47.5 % after 10 min of treatment. At 41 ºC, however, a significantly faster ($p<0.05$) inactivation was observed when compared to what occurred at 31 and 36 ºC. The lowest value of RA after 10 min of treatment was 10.65 %.

The effect of pressure was not as pronounced as that of temperature (Figure 5B). After 2 min of treatment, no significant differences ($p>0.05$) were found between the pressures
studied: on average, 80 % RA was attained. No significant differences (p>0.05) were found between 100 and 225 bar: on average, the RA reached 54.2 % after 10 min of treatment. The highest level of pressure studied, 350 bar, produced significantly different (p<0.05) results compared to 100 and 225 bar. At 350 bar, after 8 and 10 min of treatment, the % of RA was 32.38 and 15.90 %, respectively.

The inactivation of PME by means of SC-CO₂ or HPU has previously been explored. Balaban et al. [2] studied the degree of inactivation of PME in orange juice with a batch SC-CO₂ system. Similarly to what occurred in this study, these authors decreased the % RA as the temperature and pressure increased; furthermore, although the inactivation degree reached at 44 ºC and 269 bar after 50 min, 30 %, was similar to the 32% obtained in the present study at 36 ºC and 350 bar after 8 min of treatment, when using the SC-CO₂+HPU system, lower temperatures and much shorter process times were necessary. Fabroni et al. [11] investigated the inactivation of PME in blood orange juice with a continuous SC-CO₂ system. They obtained an RA of 33.19% and 40.88 %, using 230 and 130 bar (36 ºC, 15 min), respectively. Similar values of RA have been obtained in this study in shorter process times at lower temperatures: an RA of 46 % was attained after 10 min of SC-CO₂+HPU treatment at 225 bar and 31 ºC.

Therefore, it may be concluded that in a batch SC-CO₂+HPU system at lower pressures and temperatures, shorter process times can be used compared to batch and continuous SC-CO₂ systems, which would contribute to the preservation of the nutritional value and desirable sensory characteristics of orange juice.

The mechanisms associated with the inactivation of enzymes are those linked to the denaturation of proteins. Enzymes are folded three-dimensionally, determined by covalent, hydrophobic and ionic intra-molecular forces [35]. The inactivation of enzymes is associated with the fragmentation or modification of their secondary and tertiary structure; therefore, any mechanism that might affect the structure of enzymes can cause their denaturation.

The inactivation of enzymes exposed to SC-CO₂ treatments can be explained by different mechanisms, such as the lowering of the pH, the inhibitory effect of molecular CO₂ on enzyme activity and the fact that SC-CO₂ causes conformational changes [36]. Treatments with high pressure CO₂ are accompanied by a lowering of the pH because of the formation of carbonic acid from the dissolution of CO₂ in water and under a lower pH environment, protein bound arginine can easily interact with CO₂, forming a
bicarbonate complex [35]. Therefore, in addition to its pH-lowering effect, CO₂ may
directly bind to the enzyme and cause a loss of activity. Moreover, the inactivation of
enzymes exposed to SC-CO₂ treatment can be explained by the fact that SC-CO₂ causes
conformational changes in the secondary and tertiary structure. Ishikawa et al. [37]
reported that several enzymes, such as lipase, alkaline protease, acid protease and gluco-
amylase, were inactivated and their α-helix structures were decomposed after SC-CO₂
treatment.

In the present study, PME was inactivated more quickly in orange juice by applying SC-
CO₂ and HPU simultaneously, despite using lower pressures and temperatures and
shorter process times than with the single SC-CO₂ or ultrasound treatments reported in
other works. The synergistic effect of SC-CO₂+HPU accelerates the solubilization rate
of SC-CO₂ into the liquid and the increase in the mass transfer due to the vigorous
agitation produced by the ultrasonic field results in the quick saturation of CO₂ in the
medium, which accelerates the inactivation mechanisms. The cavitation generated by
HPU could contribute to the change in the conformation of the enzyme, accelerating its
inactivation.

Comparing the SC-CO₂+HPU inactivation of *E. coli*, *S. cerevisiae* and PME, the
enzyme needed longer process times to be inactivated and its total inactivation was not
attained in any of the process conditions. This could be attributed to the different nature
and size of microorganisms and enzymes.

3.6. *Modeling of the pectin methyl-esterase inactivation kinetics*

The data obtained for each pressure and temperature condition in the inactivation of
PME was fitted to two previously described mathematical models: the fractional
conversion model and the Weibull model. Table 4 shows the statistical parameters for
the fit of the kinetic models to the inactivation data of PME in orange juice treated by
SC-CO₂ and HPU. On average, both models adequately fitted the inactivation kinetics,
$R^2_{avg} > 0.9$; $\text{RMSE}_{avg} < 0.07$. The best fit was provided by the fractional model ($R^2_{avg} =
0.95$; $\text{RMSE}_{avg} = 0.067$).

In order to obtain an estimation of the pectin-methyl esterase inactivation at any
pressure and temperature, the equation developed by Polydera et al. [21] was used to
select and modify the fractional model (Eq. 11), including the dependence of parameter
$k$ (Fractional model, Table 1) on pressure and temperature.
where \( w \) is a kinetic parameter, \( k_{P,T_{ref}} \) the inactivation rate at \( T_{ref} \) (304 K), \( E_{aP} \) is the activation energy at \( P_{ref} \) (100 bar), \( z \) is a kinetic parameter, \( T_{ref} \) is the reference temperature (304 K), \( V_{aT_{ref}} \) is the activation volume at \( T_{ref} \), \( R \) the universal gas constant (8.314 J mol\(^{-1}\) K\(^{-1}\)). \( P_{ref} \) and \( T_{ref} \) were selected as the lowest values of each range studied.

The different characteristic constants of the modified model were calculated by minimizing the sum of square differences between all the experimental data and all the predicted data considered for every pressure and temperature condition studied, using the Excel Solver tool. The value of the coefficients were: \( w = 2.196 \times 10^{-7} \) bar\(^{-1}\), \( k_{P,T_{ref}} = 0.201 \) min\(^{-1}\), \( E_{aP} = 85.873 \) kJ mol\(^{-1}\), \( z = 0.704 \) mL min\(^{-1}\)K\(^{-1}\) and \( V_{aT_{ref}} = 3.124 \) mL mol\(^{-1}\).

The statistical parameters obtained, \( R^2 = 0.931; \) RMSE = 0.085, were comparable with the individually obtained fit for each pressure and temperature condition studied (\( R^2_{avg} = 0.95; \) RMSE\(_{avg} = 0.067 \)). Figure 5 shows the modeling of the inactivation kinetics of PME in orange juice by SC-CO\(_2\)+HPU.

Figure 6 shows the correlation between the experimental and predicted values obtained by means of Eq. (11). The model properly predicted the experimental RA between 0 and 50% and for values higher than 80%; the estimation was slightly poorer from 50 to 80%. The figure reveals that the highest deviation value occurs at 350 bar, 36 °C and 8 min of treatment time. All the other treatment conditions fitted using Eq. (11) provided low deviation values. The proposed model provided a satisfactory correlation between experimental and predicted values of % RA in the practical range of 100-350 bar and at 31-41 °C for SC-CO\(_2\)+HPU treatments. Therefore, it has been demonstrated that the fractional model that provided good results for the modeling of PME inactivation with SC-CO\(_2\), also provided good results when HPU is simultaneously applied in an SC-CO\(_2\) treatment.

4. Conclusions

The application of HPU enhanced the SC-CO\(_2\) inactivation mechanisms and reduced the treatment time needed to achieve a required level of inactivation. HPU leads to a vigorous agitation that would accelerate the SC-CO\(_2\) inactivation mechanisms. The
cavitation generated by HPU could damage the microorganism’s cell wall and could also change the conformation of the enzymes, accelerating their inactivation.

A rise in pressure or temperature increased the inactivation rate of *E. coli*, *S. cerevisiae* and PME, and the nature of the medium influenced how increasing the pressure and temperature affected the inactivation rate.

HPU had a different effect on the SC-CO₂ inactivation of different microorganisms. The lower resistance showed by *S. cerevisiae* could be related to the fact that they are bigger than *E. coli* cells. The cavitation bubbles might produce a greater effect on the cell structure of *S. cerevisiae* than on that of *E. coli*. The SC-CO₂+HPU inactivation of PME required longer process times than for microorganisms, and total inactivation was not achieved for any condition.

Models were developed to describe the inactivation kinetics of microorganisms and enzymes at different pressures and temperatures.

It is recommended that more research be conducted to elucidate the effects of the viscosity and water-binding of the treatment media on the SC-CO₂-HPU inactivation treatments as well as to study the effect of applying HPU in a continuous system on the microbial inactivation.

**Acknowledgments**

The authors acknowledge the financial support from project CSD2007-00016 (CONSOLIDER-INGENIO 2010) funded by the Spanish Ministry of Science and Innovation and from project PROMETEO/2010/062 financed by the Generalitat Valenciana. The authors acknowledge the Universitat Politècnica de València for the FPI grant given to Carmen Ortuño Cases and Dr. Emilia Matallana and Dr. Paula Alepuz for the generous gift of *S. cerevisiae* T73 and *E.coli* DH1 strains, respectively.

**References**


Graphical Abstract
Highlights

- HPU enhanced the SC-CO$_2$ inactivation of microorganisms and enzymes in orange juice.
- The effect of HPU depended on the type of microorganism and the nature of the medium.
- The effect of increasing pressure or temperature depended on the nature of the medium.
- The combined SC-CO$_2$ and HPU process permits the use of mild process conditions.
- SC-CO$_2$+HPU allows using shorter process times for a given inactivation level.
Figure Captions

Figure 1. Experimental data (discrete points) and modeling (M) of the inactivation kinetics of *E. coli* in orange juice treated by SC-CO$_2$ and HPU at different temperatures (A, 225 bar) and different pressures (B, 36 ºC). **M**: modified Biphasic model.

Figure 2. Predicted (modified Biphasic model) against experimental *E. coli* inactivation data during SC-CO$_2$+HPU processing at various pressures (100-350 bar) and temperatures (31-41 ºC).

Figure 3. Experimental data (discrete points) and modeling (M) of the inactivation kinetics of *S. cerevisiae* in orange juice treated by SC-CO$_2$+HPU at different temperatures (A, 225 bar) and different pressures (B, 36 ºC). **M**: modified Peleg Type B model.

Figure 4. Predicted (modified Peleg Type B model) against experimental *S. cerevisiae* inactivation data during SC-CO$_2$+HPU processing at various pressures (100-350 bar) and temperatures (31-41 ºC).

Figure 5. Experimental data (discrete points) and modeling (M) of the inactivation kinetics of pectin methyl-esterase in orange juice treated by SC-CO$_2$+HPU at different temperatures (A, 225 bar) and different pressures (B, 36 ºC). **M**: modified Fractional model.

Figure 6. Predicted (modified Fractional model) against experimental % RA of PME data during SC-CO$_2$+HPU processing at various pressures (100-350 bar) and temperatures (31-41 ºC).
Figure 3

A

B

Time (min)

log (N/N₀)

100 bar

31 ºC

31 ºC-M

36 ºC

36 ºC-M

41 ºC

41 ºC-M

100 bar

225 bar

225 bar-M

350 bar

350 bar-M
Figure 4

Predicted log$_{10} \frac{N}{N_0}$ vs. Observed log$_{10} \frac{N}{N_0}$

- 31°C-225bar
- 36°C-225bar
- 41°C-225bar
- 36°C-100bar
- 36°C-350bar
Figure 5

A

B

% Residual PME activity vs. Time (min)

31ºC-225 bar
31ºC-225 bar-M
36ºC-225 bar
36ºC-225 bar-M
41ºC-225 bar
41ºC-225 bar-M

100 bar-36ºC
100 bar-36ºC-M
225 bar-36ºC
225 bar-36ºC-M
350 bar-36ºC
350 bar-36ºC-M
Figure 6
Table 1. Models used to fit the microbial and enzyme inactivation kinetics.

### Modelling of the microbial inactivation kinetics

<table>
<thead>
<tr>
<th>Model</th>
<th>Equation</th>
<th>Parameters</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weibull</td>
<td>( \log_{10} \left( \frac{N}{N_0} \right) = -b \ t^n )</td>
<td>b, n</td>
<td>Corradini &amp; Peleg, 2012</td>
</tr>
<tr>
<td>Gompertz</td>
<td>( \log_{10} \left( \frac{N}{N_0} \right) = Ce^{-\lambda t} - Ce^{-\mu t} )</td>
<td>A, B, C</td>
<td>Linton et al., 1996</td>
</tr>
<tr>
<td>Biphasic</td>
<td>( \log_{10} \left( \frac{N}{N_0} \right) = \log \left( (1-f)10^{D_{sens}} + f10^{D_{res}} \right) )</td>
<td>f, D_{sens}, D_{res}</td>
<td>Lee et al., 2009</td>
</tr>
<tr>
<td>Logistic</td>
<td>( \log_{10} \left( \frac{N}{N_0} \right) = \frac{Q}{1 + e^{-\sigma \log \left( t \right)/\tau}} )</td>
<td>Q, σ, τ</td>
<td>Lee et al., 2009</td>
</tr>
<tr>
<td>Peleg Type A</td>
<td>( \log_{10} \left( \frac{N}{N_0} \right) = \frac{a_1 t}{(1 + a_2 t) (a_3 t - t)} )</td>
<td>a_1, a_2, a_3</td>
<td>Peleg, 2006</td>
</tr>
<tr>
<td>Peleg Type B</td>
<td>( \log_{10} \left( \frac{N}{N_0} \right) = \frac{b_1 f}{b_2 + t^r} )</td>
<td>b_1, b_2, r,</td>
<td>Peleg, 2006</td>
</tr>
</tbody>
</table>

### Modelling of the inactivation kinetics of pectin methyl-esterase

<table>
<thead>
<tr>
<th>Model</th>
<th>Equation</th>
<th>Parameters</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fractional</td>
<td>( \frac{A - A_t}{A_0 - A_t} = e^{kr} )</td>
<td>k</td>
<td>Polydera et al., 2004</td>
</tr>
<tr>
<td>Weibull</td>
<td>( \log_{10} \left( \frac{A}{A_0} \right) = -b t^n )</td>
<td>b, n</td>
<td>Corradini &amp; Peleg, 2012</td>
</tr>
</tbody>
</table>

\( N_0 \): the initial number of microorganisms at time 0; \( N \): the corresponding number after a time \( t \).
\( A_0 \): the PME activity of the untreated orange juice; \( A \): the PME activity of the treated orange juice after time \( t \); \( A \): the PME activity at the end of the treatment.
b: non-linear rate parameter; \( n \) is the shape factor.
A, B and C: different regions of the survival curve: the initial shoulder (A), the maximum death rate (B) and the overall change in the survivor number (C).
\((1-f)\) and \( f \): the fraction of treatment-sensitive and treatment-resistant populations, respectively; \( D_{sens} \) and \( D_{res} \) are the decimal reduction times of the two populations (min)
Q: the upper asymptote-lower asymptote; \( \sigma \): the maximum inactivation rate; \( \tau \): the log time needed to reach the maximum inactivation rate
a_1, a_2, a_3, b_1, b_2, r: model parameters
k: the inactivation rate parameter
Table 2. Statistical parameters for the fit of the kinetic models to the inactivation data of *E. coli* in orange juice treated by SC-CO$_2$ and HPU at three temperatures (31, 36 and 41 °C, at constant P = 225 bar) and three pressures (100, 225 and 350 bar, at constant T = 36 °C).

<table>
<thead>
<tr>
<th>Treatment conditions</th>
<th>Statistics</th>
<th>Weibull</th>
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<th>Biphasic</th>
<th>Log-linear</th>
<th>Type A</th>
<th>Type B</th>
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</thead>
<tbody>
<tr>
<td>225 bar 31 °C</td>
<td>$R^2$</td>
<td>0.916</td>
<td>0.752</td>
<td>0.943</td>
<td>0.881</td>
<td>0.961</td>
<td>0.902</td>
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<tr>
<td></td>
<td>RMSE</td>
<td>0.587</td>
<td>0.932</td>
<td>0.446</td>
<td>0.590</td>
<td>0.367</td>
<td>0.587</td>
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<tr>
<td>225 bar 36 °C</td>
<td>$R^2$</td>
<td>0.932</td>
<td>0.818</td>
<td>0.967</td>
<td>0.904</td>
<td>0.947</td>
<td>0.833</td>
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<td>RMSE</td>
<td>0.493</td>
<td>0.743</td>
<td>0.317</td>
<td>0.494</td>
<td>0.402</td>
<td>0.712</td>
</tr>
<tr>
<td>225 bar 41 °C</td>
<td>$R^2$</td>
<td>0.978</td>
<td>0.987</td>
<td>0.999</td>
<td>0.934</td>
<td>0.989</td>
<td>0.993</td>
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<td>RMSE</td>
<td>0.366</td>
<td>0.226</td>
<td>0.015</td>
<td>0.363</td>
<td>0.214</td>
<td>0.168</td>
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<td>100 bar 36 °C</td>
<td>$R^2$</td>
<td>0.936</td>
<td>0.972</td>
<td>0.957</td>
<td>0.906</td>
<td>0.973</td>
<td>0.965</td>
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<td>0.490</td>
<td>0.296</td>
<td>0.368</td>
<td>0.485</td>
<td>0.291</td>
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</tr>
<tr>
<td>350 bar 36 °C</td>
<td>$R^2$</td>
<td>0.957</td>
<td>0.906</td>
<td>0.914</td>
<td>0.940</td>
<td>0.963</td>
<td>0.950</td>
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<tr>
<td></td>
<td>RMSE</td>
<td>0.429</td>
<td>0.586</td>
<td>0.560</td>
<td>0.429</td>
<td>0.370</td>
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<tr>
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<td>$R^2_{avg}$</td>
<td>0.944</td>
<td>0.887</td>
<td>0.967</td>
<td>0.906</td>
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<td>RMSE$_{avg}$</td>
<td>0.473</td>
<td>0.549</td>
<td>0.286</td>
<td>0.482</td>
<td>0.318</td>
<td>0.449</td>
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Table 3. Statistical parameters for the fit of the kinetic models to the inactivation data of *S. cerevisiae* in orange juice treated by SC-CO$_2$ and HPU at three temperatures (31, 36 and 41 ºC, at constant P = 225 bar) and three pressures (100, 225 and 350 bar, at constant T = 36 ºC).

<table>
<thead>
<tr>
<th>Treatment conditions</th>
<th>Statistics</th>
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<th>Gompertz</th>
<th>Biphasic</th>
<th>Log-linear</th>
<th>Type A</th>
<th>Type B</th>
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<tr>
<td>225 bar 31 ºC</td>
<td>$R^2$</td>
<td>0.983</td>
<td>0.942</td>
<td>0.969</td>
<td>0.976</td>
<td>0.975</td>
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<td>0.158</td>
<td>0.264</td>
<td>0.193</td>
<td>0.158</td>
<td>0.178</td>
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<td>225 bar 36 ºC</td>
<td>$R^2$</td>
<td>0.976</td>
<td>0.967</td>
<td>0.993</td>
<td>0.954</td>
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<td>225 bar 41 ºC</td>
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<td>0.431</td>
<td>0.253</td>
<td>0.234</td>
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<tr>
<td>100 bar 36 ºC</td>
<td>$R^2$</td>
<td>0.967</td>
<td>0.975</td>
<td>0.985</td>
<td>0.947</td>
<td>0.975</td>
<td>0.959</td>
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<tr>
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<td>0.362</td>
<td>0.285</td>
<td>0.218</td>
<td>0.357</td>
<td>0.281</td>
<td>0.361</td>
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<tr>
<td>350 bar 36 ºC</td>
<td>$R^2$</td>
<td>0.847</td>
<td>0.849</td>
<td>0.925</td>
<td>0.589</td>
<td>0.840</td>
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<tr>
<td></td>
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<td>0.784</td>
<td>0.550</td>
<td>1.290</td>
<td>0.806</td>
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$R^2_{avg}$ 0.945 0.942 0.973 0.768 0.947 0.983

$RMSE_{avg}$ 0.452 0.273 0.168 0.306 0.242 0.188
Table 4. Statistical parameters for the fit of the kinetic models to the inactivation data of pectin methyl-esterase in orange juice treated by SC-CO$_2$ and HPU at three temperatures (31, 36 and 41 °C, at constant P = 225 bar) and three pressures (100, 225 and 350 bar, at constant T = 36 °C).

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<td>RMSE</td>
<td>0.023</td>
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<td>$R^2$</td>
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<td>RMSE</td>
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<td>225 bar 41 °C</td>
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<td>RMSE</td>
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<td>100 bar 36 °C</td>
<td>$R^2$</td>
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<td>RMSE</td>
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<td>$R^2$</td>
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<tr>
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<th>$R^2_{\text{Avg}}$</th>
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<tr>
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<td>RMSE$_{\text{Avg}}$</td>
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