ABSTRACT

Non-thermal food preservation techniques have been developed by the food industry in response to an increasing consumer's demand for natural, fresh and free of chemical preservatives food. Supercritical carbon dioxide (SC-CO_2) inactivation technology represents a promising non-thermal processing method, as it promotes minimum impact on the nutritional and organoleptic food properties. However, in some cases high pressures or temperatures and too long treatment times are required to guarantee the food's safety and stability. In order to obtain the required lethality at shorter processing times or with lower treatment intensity, a combination of SC-CO_2 with high power ultrasound (HPU) or with high hydrostatic pressure (HHP) have been developed and used in the present work for microbial/enzyme inactivation purposes.

The main aim of this Thesis was to evaluate non-thermal preservation techniques, based on the combination of SC-CO_2 and HPU, and on the combination of SC-CO_2 and HHP. Regarding the combination of SC-CO_2 with HPU, the influence of the culture growth stage, the process conditions, the nature of the medium and the use or not of HPU on the inactivation kinetics of microorganisms (*Escherichia coli* (*E. coli*) and *Saccharomyces cerevisiae* (*S. cerevisiae*)) or enzymes (pectin-methyl esterase (PME)) was studied. Mathematical models and microscopy techniques have been used in order to describe the inactivation kinetics and to study the inactivation mechanisms involved in the treatments, respectively. In regard to the combination of SC-CO_2 and HHP, the effect of different levels of added carbon dioxide in the package on the efficiency of HHP treatment to inactivate PME, peroxidase (POD) and polyphenol oxidase (PPO) in feijoa puree was evaluated.

The influence of the culture growth stage of *E. coli* and *S. cerevisiae* inoculated in culture medium, LB and YPD Broth, respectively, on their inactivation kinetics using SC-CO_2 (350 bar, 35 ºC) was studied. Cultures of *E. coli* and *S. cerevisiae* were grown to four different growth stages from the early exponential phase to the stationary phase, and then treated with SC-CO_2 at 350 bar and 35 ºC. The combined SC-CO_2+HPU inactivation process was compared to the SC-CO_2 treatment in order to evaluate the effect of HPU on the SC-CO_2 inactivation kinetics of *E. coli* and *S. cerevisiae* in the early stationary

phase inoculated in culture medium, and to determine the effect of different temperatures (31-41 ºC, 225 bar) and pressures (100-350 bar, 36 ºC). In order to elucidate the inactivation mechanisms associated to the combined technology (SC-CO_2+HPU) a morphological study was carried out. The differences between untreated, SC-CO_2 (350 bar, 36 ºC, 5 min) and SC-CO_2+HPU (350 bar, 36 ºC, 5 min, 40 W) treated *E. coli* and *S. cerevisiae* cells, were determined using light microscopy (LM) and transmission electron microscopy (TEM). Apple and orange juice were selected to study the inactivation of these microorganisms with SC-CO_2+HPU in real matrices; additionally, the inactivation of the enzyme pectin-methyl esterase (PME) in orange juice was addressed. Experiments with juices were performed at different temperatures (31-41 ºC, 225 bar) and pressures (100-350 bar, 36 ºC). The chosen temperature and pressure ranges were higher than the critical one for CO_2 and lower than lethal for both microorganisms. Both *E. coli* and *S. cerevisiae* have been selected in the present study because they are habitual components of the microbiota involved in food spoilage and are commonly used as indicators of food contamination.

The combination of SC-CO_2 with HHP was assessed to determine the effect of different levels of CO_2 in the package (only HHP (HHP); carbonation and HHP (HHPcarb); carbonation + addition of 8.5 mL CO_2/g puree into the headspace of the package and HHP (HHPcarb+CO_2)) on the efficiency of HHP inactivation treatment on PME, POD and PPO in feijoa puree at different pressures (300, 450 and 600 MPa, for 5 min).

Results showed that the resistance of both microorganisms to the SC-CO_2 inactivation treatment increased progressively as the growth phase advanced, which could be due to the natural protective systems that become activated as cells approach the stationary phase. The inactivation kinetics of *E. coli* and *S. cerevisiae* were fitted to the Weibull Model (R^2 = 0.93; RMSE = 0.59) and to the Gompertz function (R^2 = 0.96; RMSE = 0.53), respectively, which were adapted considering the growth stage as one of the model parameters.

Using only SC-CO_2, the inactivation rate of both microorganisms inoculated in culture media increased progressively as the pressure and temperature rose. The required time to reach the total inactivation of *E. coli* (8 log-cycles) was reduced from 60 to 25 min as pressure increased from 100 to 350 bar (36 ºC),

and from 75 to 40 min as temperature increased from 31 to 41 ºC (225 bar). The total inactivation of *S. cerevisiae* (7 log-cycles) was attained only at 350 bar, 36 ºC and after 140 min. In general, higher pressures and temperatures enhance the SC-CO_2 solubilization into the medium and increase the fluidity of the cell membrane, respectively, making the contact and penetration of CO_2 into the cells easier and facilitating the decrease of intracellular pH and the extraction of vital cell constituents. However, when HPU was applied during the SC-CO_2 treatments in growth media, a drastic inactivation effect was observed and a total reduction of about 10^7-10^8 log-cycles was attained after only 1-2 min. Using SC-CO_2+HPU the effect of increasing pressure or temperature did not significantly influenced the inactivation level due to HPU leads a vigorous agitation that would accelerate the SC-CO_2 inactivation mechanisms and mask the effect of these process variables. Moreover, the cavitation generated by HPU could damage the microorganism's cell wall, accelerating its inactivation. The study of a possible synergistic effect revealed that the combination of SC-CO_2 and HPU had a greater effect on the microbial inactivation than the addition of their individual effects. For *E. coli*, a reduction of 0.3, 0.9 and 8 log-cycles was attained after 5 min of SC-CO_2, HPU and SC-CO_2+HPU treatments, respectively; for *S. cerevisiae* a reduction of 6.83 log-cycles was achieved after 2 min of SC-CO_2+HPU treatment, while for the same period of time, no inactivation was observed when using only HPU or SC-CO_2.

In all the experiments carried out, *S. cerevisiae* showed higher resistance to the SC-CO_2 treatments than *E. coli*, which could be linked to the fact that *S. cerevisiae* has a thicker and more resistant cell wall than *E. coli*, 124.8 nm, compared to 17.7 nm, respectively. However, combining SC-CO_2 and HPU, the vigorous agitation and cavitation of the medium masked the different resistances showed by both microorganisms in the SC-CO_2 treatments.

LM and TEM images provided evidences that 5 min of SC-CO_2 treatment could generate uneven distribution of the cytoplasm content and slight modifications in the cell envelope, which was not lethal neither for *E. coli* nor for *S. cerevisiae* cells. Moreover, the greatest differences between both microorganisms appeared in the cell envelope: minor alterations were observed in *S. cerevisiae* and no disruption of cell wall was appreciated, while the cell envelope of *E. coli* cells was observed with a high degree of dissolution, loss of

cohesiveness, protuberances, and some disintegrated areas. On the other hand, 5 min of SC-CO_2+HPU treatment resulted in the total inactivation of both microorganisms. LM and TEM images revealed greater proportions of empty regions inside of SC-CO_2+HPU-treated cells, indicating clearly a drastic reduction of the cytoplasm content. The cell envelope of *E. coli* cells were totally disrupted, while the cell wall of *S. cerevisiae* cells lost partially their layered structure and some broken walls could be observed. Therefore, the inactivation mechanisms associated to SC-CO_2+HPU could be related to the cavitation phenomenon, generated by HPU, which drastically damage the cell envelope increasing both the rupture of the cellular membrane and the disintegration of the intracellular content. The damages generated by the SC-CO_2+HPU treatment were strong enough to avoid a possible regrowth of cells during post-treatment storage (6 weeks at 4 ºC).

On average, the SC-CO_2+HPU inactivation of both microorganisms was slower in apple juice (5.3 min) than in orange juice (4.6 min); and in both juices slower than in culture media (1.5 min). This fact could be linked to the sugar content and the CO_2 solubilization. The sugar binds water from the medium, thus the free water where the CO_2 can be dissolved was lower in apple juice (15.6 ºBrix) than in orange juice (11.6 ºBrix); and lower in both juices than in LB (2 ºBrix) or YPD (5 ºBrix) Broth. In addition, the SC-CO_2+HPU inactivation of both microorganisms inoculated in juices was accelerated by increasing pressure and temperature. This fact could be related to the composition of juices, which were not so quickly saturated with CO_2 in the SC-CO_2+HPU treatments like in the experiments conducted in culture media, therefore an increase of pressure or temperature could facilitate the solubilization of CO_2.

Contrarily to the results obtained using culture media, where no difference between *E. coli* and *S. cerevisiae* was found, *E. coli* inoculated in juices showed more resistance to the SC-CO_2+HPU treatments than *S. cerevisiae*. On average, to reach the total inactivation, the treatment time required was 6.6 and 3.3 min for *E. coli* and *S. cerevisiae*, respectively. In juices, the vigorous solubilization of CO_2 generated by HPU could be hindered by the higher sugar content, thus the inactivation mechanisms would be mainly driven by the cavitation phenomenon and the size of the microorganisms. The size of *S. cerevisiae* cells is much bigger than *E. coli* ones, therefore, the probability that the implosion of the cavitation

bubbles might affect the cell structure could be larger for *S. cerevisiae* than for *E. coli*.

On the other hand, the SC-CO_2+HPU inactivation of PME increased with pressure and temperature, although its total inactivation was not attained in any of the studied conditions. The inactivation of enzymes exposed to SC-CO_2 treatments can be explained by the lowering of the pH, the inhibitory effect of molecular CO_2 on enzyme activity and the fact that SC-CO_2 causes conformational changes. The enzyme PME was more resistant to SC-CO_2+HPU than *E. coli* or *S. cerevisiae* in orange juice (at 36 ºC, 225 bar and after 2 min, a reduction of 18.9 %, 62.4 % and 88.1 % was attained, respectively), which could be attributed to the different nature and size of microorganisms and enzymes.

The Peleg Type A model (R^2 = 0.936; RMSE = 0.561) and the Weibull model (R^2 = 0.923; RMSE = 0.561), were adapted to describe the SC-CO_2+HPU inactivation kinetics of *E. coli* and *S. cerevisiae*, respectively, in apple juice, including pressure and temperature as model parameters. The Biphasic model (R^2 = 0.960; RMSE = 0.391), the Peleg Type B (R^2 = 0.894; RMSE = 0.687) and the fractional model (R^2 = 0.931; RMSE = 0.085), were adapted to describe the SC-CO_2+HPU inactivation kinetics of *E. coli*, *S. cerevisiae* and PME, respectively, in orange juice, including pressure and temperature as model parameters.

Results revealed that the residual activity of PME, PPO and POD decreased as increasing pressure, since pressure causes structural rearrangements that can change the three-dimensional structure of enzymes. The HHPcarb+CO_2 treatment increased the inactivation level of the three enzymes, compared to HHPcarb and HHP treated samples, at any pressure condition selected. This fact could be explained because the amount of dissolved CO_2 should be higher, causing a larger pH lowering with the consequent wider denaturation of enzymes. Moreover, the CO_2 dissolved into the puree during the HHP treatment, could generate a significant and sudden bubbling during the fast depressurization of the process that could contribute to conformational changes responsible for the inactivation of enzymes.

Finally, it can be concluded that the combination SC-CO_2 with HPU or HHP significantly improved the inactivation mechanisms of microorganisms and

enzymes. The application of HPU enhanced the SC-CO_2 inactivation mechanisms accelerating the CO_2 solubilization into the medium, which is the first step in the SC-CO_2 treatments; and generating cavitation which damage the cell wall, facilitating both the penetration of SC-CO_2 into the cells and the extraction of intracellular compounds, which accelerates the death of the microbial cells. Moreover, the combination of SC-CO_2 with HHP demonstrated an improvement of the HHP inactivation of enzymes. Using these combined techniques, reasonable industrial processing times and mild process conditions could be selected, which could result into a cost reduction and lead to the minimization of the impact on the nutritional and organoleptic properties of the treated products.

It is recommended that more research is conducted to elucidate the cell/enzyme inactivation mechanisms using SC-CO_2+HPU and SC-CO_2+HHP. Additional studies on the effect of these non-thermal combined technologies on physico-chemical properties and consumer acceptance of different treated food matrices are also of interest.