Pasteurization of apple juice contaminated with *Escherichia coli* by a combined UV-mild temperature treatment.

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ABSTRACT

The bactericidal efficacy of UV treatments to fruit juices is limited because of their low UV transmittance; therefore it is necessary to design combined processes to improve their lethality. This investigation was carried out to determinate the lethal effect of UV-C treatments at mild temperatures (UV-H treatments) on the UV-resistant *Escherichia coli* strain STCC 4201 suspended in apple juice. A synergistic effect was observed and the optimum temperature for the combined process was established. Subsequently the effect of the optimized treatment on the lethality of a *E. coli* cocktail (STCC 4201, STCC 471, ATCC 27325, ATCC 25922, and O157:H7 Chapman strain) and on freshly squeezed apple juice quality were evaluated.

A UV treatment of 20.33 J/mL reached 0.61±0.01, 0.83±0.07, 1.38±0.04, 1.97±0.06, 3.72±0.14, 5.67±0.61, and more than 6 Log_{10} cycles of inactivation at 25.0, 40.0, 50.0, 52.5, 55.0, 57.5, and 60.0 ºC, respectively. The optimum conditions for exploiting the synergistic effects were UV doses of 27.10 J/mL, temperature of 55.0 ºC, and 3.58 minutes of treatment time. This treatment guaranteed more of 5 Log_{10} reductions of the cocktail of five strains of *E. coli* without affecting pH, ºBrix, and acidity of freshly squeezed apple juice. The UV-H treatment did not increase the loss of ascorbic acid compared to the same UV treatment at room temperature, but approximately doubled the inactivation of polifenoloxidase.

Keywords: pasteurization, apple juice, *Escherichia coli*, ultraviolet irradiation, heat treatments.

1. Introduction

Apple juice is one of the most popular juices in Europe, the United States, and Japan due to its pleasant organoleptic qualities (Komthong et al., 2007). Historically, food safety for apple juice has relied upon the product's inherent acidity (pH 3.3-4), refrigeration, natural antimicrobial compounds, and the addition of chemical preservatives (Basaran et al., 2004). However, apple juice products have received increasing attention since they were implicated in a disease outbreak caused by *Escherichia coli* O157:H7 in the early 1980s in Canada, and the frequency of outbreaks increased over the next two decades (Vojdani et al., 2008). In addition, numerous studies have demonstrated the ability of *E. coli* O157:H7 to survive in apple juice despite its low pH and the use of refrigeration or preservatives (Duffy et al., 2001). In response, the U.S. Food and Drug Administration (U.S. FDA) published a rule to improve the safety of juice products. Juice manufacturers must develop a hazard analysis critical control points (HACCP) plan for the production of juice products. Part of this regulation includes the requirement of either implementing decontamination treatment to achieve 5 Log_{10} cycles of the pertinent pathogen in the finished product, or putting a warning label on the bottle (U.S. FDA, 2001).

Heat treatment is the most commonly used strategy to eliminate microorganisms and enzymes and extend shelf-life of apple juice products. However, this process may have adverse effects on the sensory and nutritional quality of food (Choi, 2005). Since current consumers demand minimally processed foods with characteristics as similar as possible to fresh products, non-thermal technologies have received increasing attention in recent years (Raso & Barbosa-Cánovas, 2003). Among the non-thermal technologies developed in the last few decades, ultraviolet (UV) light irradiation is one of the most promising.

Short-wave UV radiation (200–280 nm)—UV-C light—is considered germicidal against a wide variety of microorganisms, its maximum lethal effect being between 250

and 270 nm (Bintsis et al., 2000). UV germicidal properties are due to DNA's absorption of the UV light, which results in cross-linking between the neighboring pyrimidine nucleoside bases (thymine and cytosine) in the same DNA strand, thereby causing cell death (Sizer & Balasubramaniam, 1999). In the food industry, UV irradiation is currently widely used to disinfect water and waste-water systems as an alternative to chlorine-based applications. In the last few years, the application of UV radiation has been focused on the treatment of liquid food, especially fruit juices, due to its multiples advantages: its potential for inactivating spoilage and pathogenic microorganisms minimizing the loss of nutritional and sensorial quality, the non-existence of known toxic effects and insignificant non-toxic residues formed during the treatment (Guerrero-Beltrán & Barbosa-Cánovas, 2004), and very little energy consumed compared to other non-thermal pasteurization processes (Geveke, 2005).

However, applying conventional UV treatment to fruit juices is limited because of their low UV transmittance. Color compounds and the presence of greater amounts of suspended and soluble solids reduce penetration of UV light into juices to about 1 mm for absorption of 90% of the light (Sizer & Balasubramaniam, 1999). Previous literature indicates that UV irradiation may result in significant reduction of microbial pathogens in fruit juices. However, achieving the FDA requirement solely through the use of UV light would only be possible for clarified juice with very low levels of background microflora processed at extremely slow flow rates or multiple passes that are probably impractical for use in commercial settings (Wright et al., 2000). To overcome this limitation, turbulent flow reactors were designed to optimize the effect of UV radiation (Koutchma et al., 2007; Geveke, 2008; Franz et al., 2009). Another alternative is to combine UV light with milder conventional preservation methods in a so-called "hurdle" approach (Leistner, 1992). In fact, the U.S. FDA has approved UV irradiation as a suitable

method for preserving fruit juices only if the turbulent flow conditions can be ensured throughout the treatment process. However, this requirement is not necessary when UV radiation is not the only hurdle (U.S. FDA, 2000). UV-radiation-based technologies have been applied in combination with other non-thermal hurdles such as pulsed electric fields (PEF) (Gachovska et al., 2008; Walkling-Ribeiro et al., 2008) and ultrasounds (US) (Char et al., 2010; Muñoz et al., 2011). In most cases, observed microbial reductions after successive application of both technologies indicated an additive lethal effect. Some authors have suggested the combination of UV light and heat at sublethal temperatures (Wright et al., 2000; Franz et al., 2009), because beneficial effects have been observed with other non-thermal technologies (Raso & Barbosa-Cánovas, 2003). Surprisingly, there are very little data in the literature about the lethal effect of UV light at midrange temperatures. Previously, improvement of UV lethal effect at temperatures of 40-50 ºC has been reported in liquid egg white (Geveke, 2008) and in apple juice (Ukuku & Geveke, 2010). More recently, we have demonstrated that the lethal effects of UV light slightly increased up to 40-50 ºC, but dramatically increased between 50 and 60 ºC (Gayán et al., 2011). However, this investigation was carried out in buffers.

The objectives of this work were 1) to study the bactericidal effect of UV light at different temperatures (up to 60 ºC) on a UV resistant *E. coli* strain suspended in apple juice; 2) to optimize the combined process for pasteurization of apple juice (5 Log_{10} reductions); 3) to validate the optimized process with a cocktail of five strains of *E. coli* in natural apple juice; and 4) to evaluate its effects on selected quality parameters.

2. Materials and Methods

2.1. Bacterial culture and media

The strains of *E. coli* STCC 4201 and STCC 471 (clinical isolated) were provided by the Spanish Type Culture Collection (STCC). The strains of *E. coli* ATCC 27325 (isolated from human faeces) and ATCC 25922 (clinical isolated) were provided by the American Type Culture Collection (ATCC). The strain *E. coli* O157:H7 (isolated from bovine rectal faeces) used in this investigation is a VTEC- (Phage type 34) isolated by Dr. Chapman (Chapman et al., 1993). The bacterial cultures were maintained frozen at -80 ºC in cryovials. Stationary-phase cultures were prepared by inoculating 10 mL of tryptone soy broth (Biolife, Milan, Italy) supplemented with 0.6% (w/v) yeast extract (Biolife, Milan, Italy) (TSBYE) with a loopful of growth from tryptone soy agar (Biolife) supplemented with 0.6% (w/v) yeast extract (TSAYE). The cultures were incubated at 35 ºC for 6 h in a shaking incubator. Fifty μL of the cultures were inoculated into 50 mL of fresh TSBYE and incubated for 24 h under the same conditions, which resulted in stationary-phase cultures containing approximately 2×10^9 CFU/mL.

2.2. UV equipment and treatments

UV treatments were carried out in equipment previously described (Gayán et al., 2011). The whole system consisted of 8 individual annular thin film flow-through reactors connected in series. Each reactor include a low pressure UV lamp (TUV 8WT5, Philips, U.S.A.) with a length of 282.3 mm and 8 W of total power, emitting 85% of energy at a wavelength of 254 nm, fixed at the axis of an outer glass tube (25 mm of inner diameter and 60 mL of total volume) and enclosed by a quartz tube (20 mm of outer diameter) to prevent direct contact of the lamp with the treatment medium. In the annular gap (2.5 mm) a stainless steel coil spring was installed to improve the turbulence of the flow. Outside and inside coil diameters of the spring were 23 and 25 mm, respectively, and its length and pitch were 270 mm and 10 mm, respectively. The whole equipment includes a feed tank, a peristaltic pump (Ismatec, mod. ISM 10785, Glattbrugg, Switzerland), a

heating/cooling coil exchanger, 8 UV reactors connected in series, and 8 sampling valves. The circuit and reactors were submerged in a 90 L water bath (T ± 1.5 ºC) heated by the circulating water of a peripheral thermostatic bath (Huber, mod. Kattebad K12, Offenburg, Germany). Two thermocouples (Almeco, mod. ZA 020-FS, Bernburg, Germany) fitted to the input of the first and the outlet of the last reactor allowed control of the treatment temperature.

We calculated the applied dose by dividing the volume of the reactor (V) by the flow rate (Q). As in non-ideal reactors the calculation of UV dose by the theoretical retention time could lead to important errors because of the existence of a residence time distribution (RTD) among different fractions of the flow, we used a

Treatment medium was added with the bacterial suspension to achieve 10^7-10^8 CFU/mL and pumped (8.5 L/h) through the heat exchanger to the reactors. When the treatment conditions were stabilized, samples were withdrawn through the sampling valves at the outlet of each reactor, and 0.1 mL or 1 mL was immediately pour plated in the recovery media. Apple juice (García Carrion S.A., Spain) used as treatment medium (absorption coefficient=25.54 cm^{-1}, turbidity=3.34 NTU, pH=3.27) was purchased from a local market in Zaragoza, Spain. Freshly squeezed Fuji apple juice, used for validation experiments, was prepared by automatic liquidizer apple fruits (Moulinex, JU 2000 Vitae, Barcelona, Spain), and filtered through a sterile stainless filter with net square hole of 1 mm^2 (absorption coefficient=28.54 cm^{-1}, turbidity=1,523 NTU, pH=3.75).

2.3. Heat treatments

Heat treatments were carried out in a specially designed resistometer (Condón et al., 1993). Briefly, this instrument consists of a 350 mL vessel provided with an electrical heater for thermostation, an agitation device to ensure inoculums distribution and temperature homogeneity, and ports for injecting the microbial suspension and for

extraction of samples. Once the preset temperature had attained stability (T±0.05 °C), 0.2 mL of an adequately diluted microbial cell suspension were inoculated into the corresponding treatment medium. Before inoculation, bacterial cells were preadapted to the heating media by suspending 0.1 mL of grown culture into 0.9 mL of apple juice and incubating for 15 min at 25 ºC. Previous experiments demonstrated that longer incubation times did not modify *E. coli* heat resistance (data not shown). After inoculation, 0.2 mL samples were collected at different heating times and immediately pour plated.

2.4. Incubation of treated samples and survival counting

Tryptone Soy Agar (Biolife) supplemented with 0.6% of yeast extract (TSAYE) was used as a non-selective recovery medium, and plates were incubated for 24 h at 35 ºC. After incubation, colony forming units (CFU) were counted with an improved Image Analyzer Automatic Colony Counter (Protos, Synoptics, Cambridge, UK), as described elsewhere (Condón et al., 1996).

2.5. Curve fitting, resistance parameters, and synergistic effect calculation

Survival curves to UV treatments were obtained by plotting the logarithm of the survival fraction versus treatment doses, expressed in J/mL, and to heat versus treatment times in min. To fit survival curves and calculate resistance parameters, the Geeraerd and Van Impe inactivation model-fitting tool (GInaFiT) was used (Geeraerd et al., 2005). Because our survival curves did not show tails but rather shoulders, the log-linear regression plus shoulder model (Geeraerd et al., 2000) was used. This model describes the survival curves through two parameters: the shoulder length (*Sl*) or dose before the exponential inactivation begins and the inactivation rate (*Kmax*), defined as the slope of the exponential portion of the survival curve. Therefore, the traditional decimal reduction time value (*D*) can be calculated from the *Kmax* parameter by the equation: $D =$

2.303/*Kmax*. For comparison purposes, the GInaFiT also provides the parameter 4*D*, defined as the treatment dose necessary to inactivate 99.99% of the microbial population.

To determine whether an additive or synergistic effect existed between UV light and heat, theoretical 4*D* values for the UV-H combined treatment ($Theoretical\ 4D_{UV-H}$) were calculated and compared with *4D* obtained experimentally ($Experimental\ 4D_{UV-H}$). Theoretical $4D_{UV\text{-}H}$ values represent the time to achieve 99.99% reductions of the microbial inactivation if the two processes act simultaneously but independently, and therefore lethal effects were additive. These values were calculated with the equation proposed by Raso et al. (1998):

$$Theoretical\ 4D_{UV-H} = \frac{(4D_H\ x\ 4D_{UV})}{(4D_H + 4D_{UV})} \quad (1)$$

where $4D_H$, and $4D_{UV}$ values were obtained from the fit of the inactivation curves for the thermal and UV light treatments, respectively.

The magnitude of the synergistic effect for each temperature was calculated comparing the theoretical $4D_{UV\text{-}H}$ values with those obtained experimentally via the following equation:

$$\%Synergism = \frac{Theoretical\ 4D_{UV-H} - Experimental\ 4D_{UV-H}}{Theoretical\ 4D_{UV-H}} x100 \quad (2)$$

2.6. Analytical measurements

2.6.1. Absorption coefficient, pH and ºBrix

Absorbance of media was measured at 254 nm using a Unicam UV500 spectrophotometer (Unicam Limited, Cambridge, UK). Sample solutions were diluted and evaluated using quartz cuvettes (Hellma, Müllheim, Germany) with path lengths of 1, 2, and 10 mm. The absorption coefficient of the sample solution was determined from the slope of the absorbance versus path length correcting the dilution factor. Turbidity was measured using a HI 83749 nephelometer (Hanna Instrument, Szeged, Hungary), the

pH by a Basic 20 pH meter (Crison Instrument, Barcelona, Spain), and ºBrix with a PR-101 refractometer (Atago, Tokyo, Japan).

2.6.2. Polyphenol oxidase activity

Polyphenoloxidase (*PPO*) activity was evaluated at 25 ºC measuring the increase in absorbance at 420 nm using 4-methylcatechol as a substrate. The reaction was carried out in a 1 cm light path quartz cell. The reaction mixture consisted in 1 mL of McIlvain buffer pH 6.6, 1 mL cathecol (0.2 M), and 0.5 mL of apple juice. The linear portion obtained by plotting the reaction time versus absorbance was used to compute enzime activity units (*PPOU*). One unit of *PPO* was defined as the amount of enzyme that caused the increase of 1 absorbance unit at 420 nm in a minute (Ülker-Yerlitürk, 2008)

2.6.3. Ascorbic Acid Retention

Ascorbic acid (*AA*) content was measured using AOAC's official titrimetric method employing 2, 6-dichloindophenol titration procedure (AOAC, 1990b). In brief, 10 mL of apple juice were mixed with 50 mL of 5% acetic acid (Panreac, Barcelona, Spain) as a stabilizing agent and diluted to 100 mL. The mixture was titrated with 2, 6-dichloindophenol solution until a faint pink color appeared and persisted for 15 s. The *AA* content of the samples was calculated by interpolation in a calibration curve previously obtained with several solutions of pure *AA* (AnalaR Normapur, Leuven, Belgium) in 5% acetic acid solution. *AA* retention of treated samples was calculated using the equation:

$$Relative\ AA\ (\%) = \frac{(AA\ treated\ sample)}{(AA\ untreated\ sample)}\ x\ 100 \quad (3)$$

2.6.4. Acidity determination

The acidity (*A%*) of treated and untreated apple was determined by titrating with 0.1 N NaOH and phenolphthalein as an indicator, which is the official method described by AOAC (1990a). Two mL of apple juice sample were titrated with NaOH solution until the point of neutrality, when the indicator changed from colorless to pink and persisted

for 15 s. Results were expressed as malic acid concentration in 100 mL of apple juice, so that 1mL 0.1 N NaOH is equivalent to 0.0067 g malic acid:

$$A\% = \frac{mL\ NaOH\ x\ 0.0067\ x\ 100\ mL\ apple\ juice}{2\ mL\ sample} \quad (4)$$

2.7. Statistical analyses

Statistical analyses, *t*-test (p=0.05), and ANOVA tests (p=0.05) followed by Tukey's test were carried out using the GraphPad PRISM 4.1 software (GraphPad Software, Inc., San Diego, CA, USA), and differences were considered significant for $p \leq 0.05$. All microbial resistance determinations as well as analytical assays were performed at least three times on different working days. The error bars in the figures correspond to the mean standard deviation.

3. Results and Discussion

This investigation has determined the lethal effect of UV treatments at middle temperatures on UV-resistant *E. coli* STCC 4201 suspended in sterilized apple juice. It has also established the optimum temperature for the combined process and evaluated the effect of the optimized treatment on a cocktail of five strains of *E. coli* (STCC 4201, STCC 471, ATCC 27325, ATCC 25922, and O157:H7 Chapman strain) and on the quality of freshly squeezed apple juice.

3.1. UV and thermal resistance of *E. coli* in commercial apple juice

The *E. coli* strain STCC 4201 was used to evaluate the lethal effect of UV and heat combined treatment (UV-H) in commercial apple juice because it is the most resistant strain to UV of five strains previously studied (Gayán et al., 2011). Figure 1 shows survival curves to UV-H of *E. coli* STCC 4201 in commercial apple juice at different temperatures. Survival curves to UV light at 25.0 ºC and to heat at each temperature have also been included for comparison. Even when we applied the

maximum dose obtainable in one pass (27.10 J/mL), UV treatment at room temperature hardly decreases 0.96±0.16 Log_{10} cycles of the *E. coli* population. This was due to the high UV absorption coefficient of apple juice (25.54 cm^{-1}). Different works in the literature have been conducted to analyze the effect of UV light on *E. coli* population in apple juice (Wright et al., 2000; Basaran et al., 2004; Franz et al., 2009; Müller et al., 2011). However, it is difficult to compare such results based on dosage (Müller et al., 2011), because conformation and geometry of UV equipment, flow pattern, and optical properties of the liquid (turbidity and absorption coefficient) play an important role in UV germicidal efficacy (Koutchma et al., 2004).

We previously reported that UV lethal effect can be synergistically improved at midrange temperature in our UV equipment by working with buffers (Gayán et al.,2011). Therefore, a combination of UV light and heat was exploited to design a combined process that allowed increased microbial inactivation rate in apple juice. As shown in Figure 1, UV inactivation of *E. coli* was dramatically increased with temperature: A treatment of 20.33 J/mL reached 0.61±0.01, 0.83±0.07, 1.38±0.04, 1.97±0.06, 3.72±0.14, 5.67±0.61, and more than 6.22 (below the detection limit) Log_{10} cycles of inactivation at 25.0, 40.0, 50.0, 52.5, 55.0, 57.5, and 60.0 ºC, respectively. Although the combination of UV light and middle temperatures for juice processing has been proposed by some authors (Wright et al., 2000; Franz et al., 2009) is only one study that reported the heat effect in UV lethal efficacy with fruit juice. Ukuku & Geveke (2010) developed a combined treatment of UV light and radio frequency electric field for inactivation of *E. coli* in apple juice, but only up to 40 ºC. The authors found that UV inactivation at room temperature was 5.8 Log_{10} and 6.2 Log_{10} reductions at 40 ºC. Our results agree with these data. As shown in Figure 1, a UV treatment of 27.10 J/mL at 40.0 ºC increased 0.4 Log_{10} cycles

299 of the inactivation found at 25.0 ºC. But our results also demonstrated that the efficacy of
300 the combined process was much greater above 50.0 ºC.

301 It was difficult to quantify the magnitude of the contribution of heat and UV light
302 to the whole lethal effect because some survival curves showed a shoulder (Figure 1).
303 Several authors have described UV microbial inactivation as a one-hit process
304 characterized by first-order kinetics, assuming that the death of microorganisms is due to
305 a single event (the reaction of one UV photon) (Franz et al., 2009; Oteiza et al., 2010).
306 Nevertheless, many published survival curves show shoulders, tails, or both (Quintero-
307 Ramos et al., 2004; Unluturk et al., 2010). Shoulders have been related to damage and
308 repair phenomena (Harm, 1980). DNA repair systems may repair damage up to certain
309 UV dosages, resulting in shoulders, according to the multi-hit theory or the multi-target
310 theory. In a previous work, Webb & Brows (1976) observed large shoulders in wild *E.*
311 *coli*, whereas strains with deficient damage-repair mechanisms exhibit inactivation
312 kinetics without shoulder. Moreover, we demonstrated that the shoulder of survival
313 curves of this strain increased when a photoreactivation step was included before culture
314 of survivors (Gayán et al., 2011). Whatever the cause of shoulders, their presence
315 complicates quantitative comparison of the microbial UV resistance.

316 To describe UV non-linear inactivation kinetics, several models, such as Weibull,
317 Gompertz, modified Chick-Watson, Hom, biphasic linear, and log-logistic, have been
318 proposed (Ngadi et al., 2003; Quintero-Ramos et al., 2004; Unluturk et al., 2010).
319 However, nowadays there is not agreement about the most adequate model to fit these
320 deviations. For our purpose, the log-linear regression plus shoulder model (Geeraerd et
321 al., 2000) was used because it allowed us to describe accurately and independently the
322 length of the shoulders and the log-linear rate of inactivation. Table 1 includes the
323 averages and the standard deviations of parameters obtained by fitting Geeraerd et al.'s

model (*Kmax* and *Sl*) to UV-H survival curves of *E. coli* in commercial apple juice at different temperatures, as well as *4D* parameter for comparison. The coefficient of determination (R^2) and the root mean square error (*RMSE*) values have also been included to illustrate the goodness of fit. As is shown, the shoulder length (*Sl*) of UV-H survival curves decreased with temperature, disappearing at 60.0 ºC. On the contrary, the slope of the survival curves (*Kmax*) increased with temperature.

Table 1 also includes the heat resistance parameters of *E. coli* STCC 4201 in apple juice obtained by fitting survival curves to the Geeraerd et al.'s model. Data in the table demonstrated that heat survival curves showed long shoulders. Other authors have also found shoulders in survival curves when fruit juices were used as heating media (Arroyo et al., 2009; Espina et al., 2010). As was observed in UV treatments, shoulder length decreased and *Kmax* values increased with temperature. A similar behavior has been observed for *Cronobacter sakazakii* heated in powdered rehydrated milk (Arroyo et al., 2011).

Plotting the Log_{10} *4D* values shown in Table 1 for heat treatments at different temperatures obtained the thermal death time curve (TDT) that, as was expected, showed an exponential course following the equation:

$$\text{Log } 4D = -0.1903T + 11.95 \qquad (R^2 = 0.999)$$

The equation allowed us to calculate a *z* value (degrees of temperature increase necessary to reduce *4D* value 10 times) of 5.26 ºC. This value was similar to the *z* values obtained by Enache et al. (2011) (4.9 ºC-6.4 ºC) and by Espina et al. (2010) (5.4 ºC) with stationary phase *E. coli* cells in apple juice, as well as with those reported by Splittstoesser et al. (1996) (4.8 ºC) and Mazzotta (2001) (5.6 ºC).

Figure 2 shows the homologous TDT curve to the combined UV-H process. Unlike in heat treatments, there was no exponential relationship between $4D_{UV\text{-}H}$ values

and temperature, and UV-TDT curve showed a concave downward profile. Whereas increasing temperature from 25.0 to 40.0 ºC decreased the $4D_{UV\text{-}H}$ value 1.1 fold, from 40.0 to 55.0 ºC it decreased 3.65 folds. This allowed us to conclude that up to 40.0 ºC, the temperature hardly affected UV lethality, and above this threshold value, microbial inactivation would result from the combination of both technologies.

3.2. Modeling synergistic lethal effect

Figure 1 allowed comparing inactivation curves of *E. coli* STCC 4201 to the combined UV-H treatment, to heat treatments at the same temperature, and to UV light at room temperature. As observed, *E. coli* inactivation by heat for 3.58 min at 50.0 ºC, 52.5 ºC, and 55.0 ºC was negligible (0.05, 0.16, 0.91±0.02 Log_{10} cycles, respectively). Since UV light inactivation at room temperature for the same time was 0.96±0.16 Log_{10}, the lethal effect of the combined treatment was rather higher than the sum of the lethality of individual technologies (2.10±0.20, 2.61±0.09, and 5.30±0.35 Log_{10} cycles, respectively). Therefore, a synergistic effect was deduced. At temperatures above 55.0 ºC, lethality of the UV-H combined treatment was further increased (Figure 1) as was the heat inactivation rate, which decreased the synergistic effect, and at 60.0 ºC, survival curves to heat and UV-H tended to overlap. These results suggested that there was an optimum temperature at which a maximized synergistic effect can be achieved, and above this temperature, synergism would decrease.

To quantify the magnitude of the synergistic effect at each temperature, we calculated the theoretical UV-TDT curve by assuming that heat and UV inactivation were simultaneous but independent processes; in other words, we assumed that the effects of the combined treatment were additive. These calculations were carried out with the equation of the heat TDT curve and with the equation 1 proposed by Raso et al. (1998).

373 The theoretical UV-TDT curve was also included in Figure 2 (dotted line). The area
374 between theoretical and experimental curves illustrates the evolution of the synergy.
375 To determine the optimum temperature for the combined process, the % of the
376 synergistic effect at each temperature was calculated from 4*D* theoretical and
377 experimental values with equation 2. The results of the calculations are shown in Figure
378 3. The % of synergy increased with temperature up to 55.0 ºC, decreasing at higher
379 temperatures. The diminution of the synergistic effect was probably due to the higher
380 thermal dependence of the bacterial heat inactivation compared to UV inactivation.
381 Similar behavior has been observed by combining ultrasonic waves under pressure and
382 middle temperatures (Álvarez et al., 2003). From data in Figure 3, we concluded that the
383 optimum temperature for the inactivation of *E. coli* in apple juice by UV-H treatments
384 was 55.0 ºC.

385 **3.3. Effect of the optimized UV-H treatment on freshly squeezed apple**
386 **juice**

387 Bacterial UV resistance may change widely with the microbial strain (Basaran et
388 al., 2004; Oteiza et al., 2010; Alonzo A et al, 2012). Therefore, use of a single strain of
389 particular specie to establish treatment is risky (Oteiza et al., 2010). To reduce this
390 problem, the U.S. Environmental Protection Agency (U.S. EPA) specifically
391 recommended the testing of five strains in a cocktail for each pathogen when a new
392 process has to be validated (EPA, 1997). Therefore, the lethal effect of the UV-H
393 treatment was validated with a cocktail of five *E. coli* strains (*E. coli* STCC 4201, STCC
394 471, ATCC 27325, ATCC 25922 and Chapman O157:H7). The experiment was carried
395 out by inoculating freshly squeezed apple juice with the cocktail up to reach 10^7 UFC/mL
396 of each strain, and pasteurizing with the optimized UV-H treatment (27.10 J/mL, 55.0 ºC
397 for 3.58 min).

Figure 4 shows the effect of UV radiation at 55.0 °C on the five strain cocktail inoculated in freshly squeezed apple juice. Also, the survival curve of *E. coli* STCC 4201 in commercial apple juice was included for comparison. As observed, at high UV doses, the cocktail showed a lower survival that of the most resistant *E. coli* strain in commercial apple juice, although no significant differences (p>0.05) were found in resistant parameters (*Sl* and *Kmax*) (data not shown). Oteiza et al. (2010) observed in orange juice that the slopes of the decline curves of the inoculated *E. coli* cocktail at high UV doses were lower than the slope of the log-linear equation calculated for the individual most resistant-strain. Char et al. (2010) and Alonzo A et al. (2012) reported that the inactivation rate by UV light of the composited *E. coli* strains were closed to the mean inactivation rates of individual inocula in apple juice. These results cannot be explained from a biological point of view. However, it is important to highlight that small differences in the optical properties, such as absorption coefficient and turbidity, strongly change the lethal effect of UV light (Koutchma et al., 2004). Commercial and freshly squeezed apple juice used in this investigation showed slightly different absorption coefficient (25.34 and 28.59 cm^{-1}, respectively) and turbidities (3.34 and 1,523 NTU, respectively). It is well known that suspended particles can negatively impact UV efficacy due to additional absorbance, scattering, and/or blocking of UV light (Liltved and Cripps, 1999). However, particles can artificially increase the measured absorption coefficient, so that higher inactivation rates may be detected than those predicted (Koutchma et al., 2004). This can explain why UV-H treatment was more efficient for the inactivation of *E. coli* cocktail in freshly squeezed apple juice. Nevertheless, our results convincingly demonstrated the ability of the equipment to decrease *E. coli* microorganisms more than 5 Log_{10} reductions, as demanded by FDA (2001).

422 The loss of juice quality and nutritional properties during processing has become
423 an important issue due to increased consumer demand for like-fresh food products.
424 Therefore, freshly squeezed apple juices treated with the combined UV-H process at 55.0
425 ºC were analyzed for changes in their physic-chemical properties (pH, ºBrix, %*A*), *AA*
426 retention and *PPO* activity. Table 2 depicts analytical results of untreated samples, used
427 as control, and of UV-H treated apple juice. Data on the effect of the thermal treatment at
428 the same temperature and of the UV treatment (27.10 J/mL) have also been included for
429 comparison. In this experiment, thermal treatments were performed in the UV equipment
430 tempered at 55.0 ºC with lamps switched off. Results in the table demonstrated that the
431 pH, ºBrix, and %*A* parameters kept constant their original value (p>0.05) after 3.58
432 minutes of UV, heat, and UV-H treatments. Our results agree with others previously
433 published with apple juices subjected to UV light at room temperature (Noci et al., 2008;
434 Falguera et al., 2011).
435 Although apples are not a significant source of *AA*, most commercially available
436 apple juices are fortified with vitamin C to enhance their nutritional appeal and/or to serve
437 as an antioxidant to minimize losses in color, flavor, and nutrients during processing and
438 storage (Tikekar, 2011). Therefore, it was worthwhile to evaluate *AA* retention after UV
439 light processing. *AA* degradation after the combined UV-H treatment (17.62±6.21%) did
440 not significantly differ (p<0.05) from this obtained after UV at room temperature
441 treatment (24.95±11.36%) and was higher than that from thermal treated juice
442 (5.61±6.15%). Furthermore, the effect of air oxidation of *AA* was measured by passing
443 apple juice through the installation at 25.0 ºC with UV light lamps switched off. We
444 demonstrated that *AA* destruction due to air oxidation was negligible (data not shown).
445 These results indicated that *AA* loss was mainly due to UV light. UV light is known to
446 generate free radicals through a wide variety of photochemical reactions that can oxidize

447 vitamins (Koutchma, 2009). Although it is well known that UV induced oxidation of *AA*
448 in animal and plant tissues (Rohan et al., 2011), there are few studies on *AA* losses in UV-
449 treated juice products. Falguera et al. (2011) indicated that *AA* of juices had very different
450 behaviors depending on the apple variety, reporting 4% loss in Fuji apple juice after 120
451 minutes of UV irradiation. Tran and Farid (2004) and Torkamani and Niakousari (2011)
452 found 18% of *AA* loss in orange juice after a UV treatment of 73.8 mJ/cm^2 and found that
453 *AA* destruction was exponentially related with UV dose. Other authors have found higher
454 *AA* loses. Adzahan (2006) applying a UV treatment to apple cider with the CiderSure^{TM}
455 system (14.3 mJ/cm^2) found 30% reduction, and Koutchma (2009) reported
456 approximately 50% *AA* degradation in enriched apple juice after 3 passes through a
457 CiderSure 1500 UV system (16.48 J/mL). Overall, our results demonstrated that middle
458 temperatures did not contributed to the % loss of *AA* by the combined process, which
459 represents an important advantage.

460 The optimized UV-H treatment reduced by 39.39% the initial *PPO* activity of the
461 juice. We do not found statistical significant differences ($p<0.05$) among the % loss of
462 *PPO* activity by heat (22.72%) and UV at room temperature treatments (13.87%). These
463 results demonstrated that enzymatic activity was affected by both, UV light and middle
464 temperatures. Also other authors (Manzocco et al., 2009; Falguera et al., 2011; Falguera
465 et al., 2012) have demonstrated the ability of UV light to reduce the activity of the *PPO*
466 enzyme. Seiji and Iwashita (1965) achieved an inactivation of 74.6% with a dose of
467 $11.7x10^6$ erg/cm^2, and Falguera et al. (2012) demonstrated the possibility of complete and
468 irreversible inactivation of *PPO* after 100 min of treatment with a mercury vapor lamp of
469 400 W. Similarly, Manzzoco et al. (2009) achieved destruction of *PPO* under the
470 detection limit after 90 min of treatment with an UV irradiance of 21.9 W/m^2.
471 Unfortunately, this treatment also affected other quality parameters. Overall, our results

demonstrated that the combined process UV-H is more efficient for the *PPO* inactivation than its homologous treatment at room temperature. From kinetic data of Gui et al. (2006) it can be deduced that the browning rate of our UV-H treated apple juice will be a half of untreated juice. If a greater inhibition is wanted, the acidification or addition of inhibitory chemicals will be necessary.

4. Conclusion

Results obtained in this investigation indicate that bactericidal effect of UV light on *E. coli* suspended in apple juice synergistically increases with temperature up to a threshold value. The optimum conditions to exploit the synergistic effects were UV doses of 27.10 J/mL, temperature of 55.0 ºC, and 3.58 minutes of treatment time. This treatment guaranteed more of 5 Log_{10} reductions of a cocktail of five strains of *E. coli* without affecting pH, ºBrix, and acidity of freshly squeezed apple juice. The UV-H treatment did not increased the loss of ascorbic acid compared to the same UV treatment at room temperature, but approximately doubled the inactivation of polifenoloxidase.

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Table 1. UV and heat resistance parameters- *Sl* (shoulder length), *Kmax* (inactivation rate), and 4*D* (time to inactivate 99.99% of the population) obtained from the fitting of Geeraerd et al.´s model to the survival curves of *E. coli* STCC 4201 in commercial apple juice at different temperatures. Estimated Standard Deviations (SD) of the means are in brackets.

Temperature (°C)	**UV-Dose (J/mL)**	***Sl* (SD)**	***Kmax* (SD)**	**Time for *4D* reductions (SD)**	***R^2***	***RMSE***
25.0	27.10	1.15 (0.58)	0.57 (0.15)	11.49 (1.31)	0.979	0.036
40.0	27.10	1.29 (0.80)	0.50 (0.06)	10.46 (0.20)	0.970	0.049
50.0	27.10	0.90 (0.53)	1.67 (0.45)	7.14 (1.72)	0.987	0.098
52.5	27.10	0.72 (0.24)	2.23 (0.09)	5.37 (0.17)	0.975	0.187
55.0	27.10	0.24 (0.22)	3.46 (0.68)	2.86 (0.25)	0.988	0.201
57.5	27.10	0.18 (0.03)	5.21 (0.73)	1.84 (0.04)	0.989	0.194
60.0	27.10	0	10.41 (0.84)	0.88 (0.03)	0.974	0.374
56.0	0	2.23 (0.56)	2.34 (0.31)	6.25 (0.15)	0.996	0.133
58.0	0	0.74 (0.09)	4.99 (0.15)	2.61 (0.05)	0.999	0.103
60.0	0	0.44 (0.04)	15.42 (0.58)	1.05 (0.02)	0.993	0.144
62.0	0	0.24 (0.03)	41.37 (4.42)	0.46 (0.15)	0.994	0.173

Table 2. Physicoquemical properties (pH, ºBrix, *%A*), *AA* content and *PPO* activity of untreated and treated freshly squeezed apple juice by UV light at room temperature, (UV; 27.10 J/mL), the combined UV-H treatment at 55.0 ºC (UV-H; 27.10 J/mL, 3.58 min), and heat treatment at 55.0 ºC (H; 3.58 min). Estimated Standard Deviations (SD) of the means are in brackets.

		pH	ºBrix	*%A*	*AA* (mg /100ml)	*% AA* loss	*PPO*	% *PPO* inactivation
UV	Untreated	3.99 (0.03)	13.83 (0.12)	0.127 (0.019)	9.26 (0.23)		0.260 (0.025)	
	Treated	3.91 (0.06)	13.50 (0.20)	0.119 (0.081)	6.91 (0.68)^{*b}	24.95 (11.36)^{b}	0.224 (0.024)*	13.87 (4.32)^{c}
H	Untreated	3.51 (0.59)	12.87 (0.16)	0.142 (0.020)	9.81 (0.27)		0.264 (0.032)	
	Treated	3.70 (0.03)	12.43 (0.40)	0.127 (0.019)	9.26 (0.68)^{*a}	5.61 (6.15)^{a}	0.204 (0.024)*	22.72 (6.24)^{c}
UV-H	Untreated	3.51 (0.59)	12.87 (0.06)	0.142 (0.020)	9,81 (0,27)		0.264 (0.032)	
	Treated	3.77 (0.05)	12.43 (0.15)	0.127 (0.018)	8.08 (0.68)^{*b}	17.62 (6.21)^{b}	0.160 (0.014)*	39.39 (7.55)^{ab}

* There are significant differences ($p \leq 0.05$) among mean values of quality parameters of untreated and treated samples.

a, b, and c indicate significant differences between mean values of quality parameters of UV, heat, and combined UV-H treated samples, respectively.

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712 **Figure captions**

713 **Figure 1.** Survival curves of *E. coli* STCC 4201 treated by UV light at room temperature

714 (■), heat (●), and the combined UV-H process (▲) in commercial apple juice at

715 temperatures of 40.0, 50.0, 52.5, 55.0, 57.5, 60.0 ºC.

716 **Figure 2.** Relationship between temperature and $4D_{UV-H}$ values of *E. coli* STCC 4201 in

717 apple juice: (solid line) experimental $4D_{UV-H}$ values; (doted line) theoretical $4D_{UV-H}$ values

718 calculated with equation 1.

719 **Figure 3.** Evolution of synergistic effect of the combined UV-H treatment at different

720 temperatures, calculated with equation 2.

721 **Figure 4.** Survival curves of *E. coli* STCC 4201(●) in commercial apple juice and of *E.*

722 *coli* cocktail (▲) in freshly squeezed apple juice treated by UV light at 55.0 ºC.

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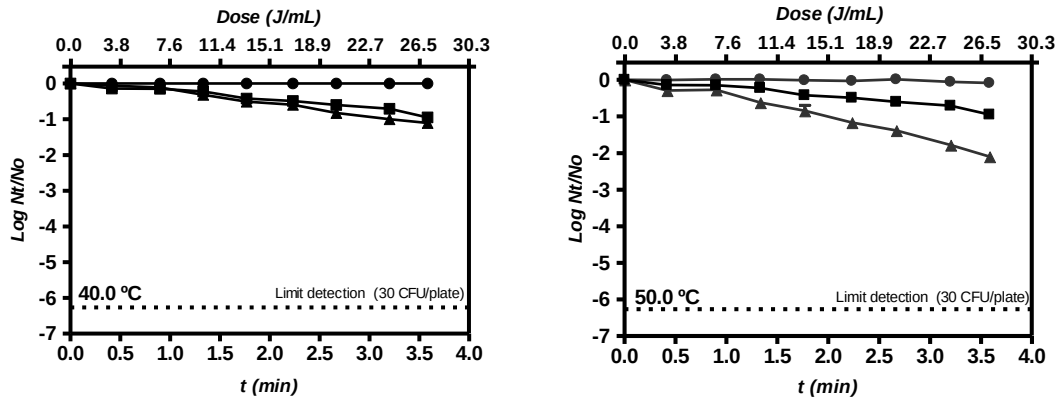
737 **Figure 1**



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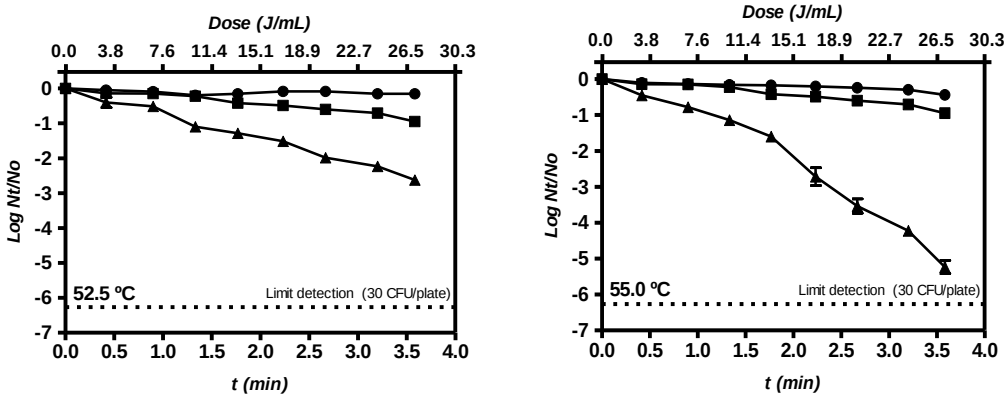

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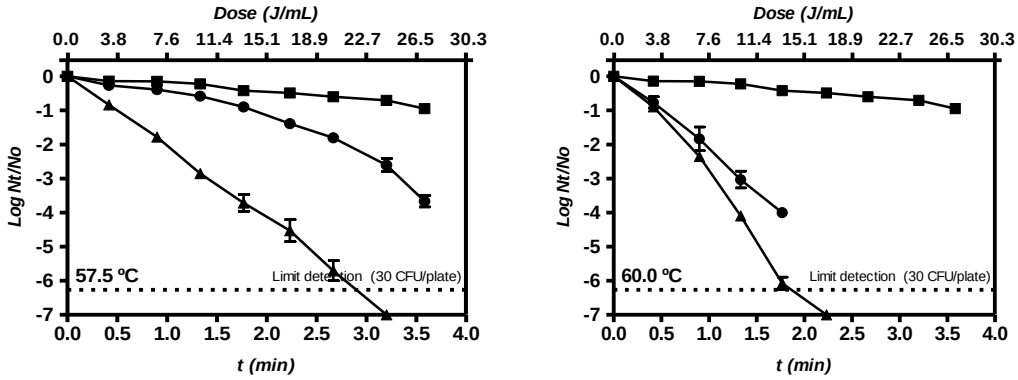

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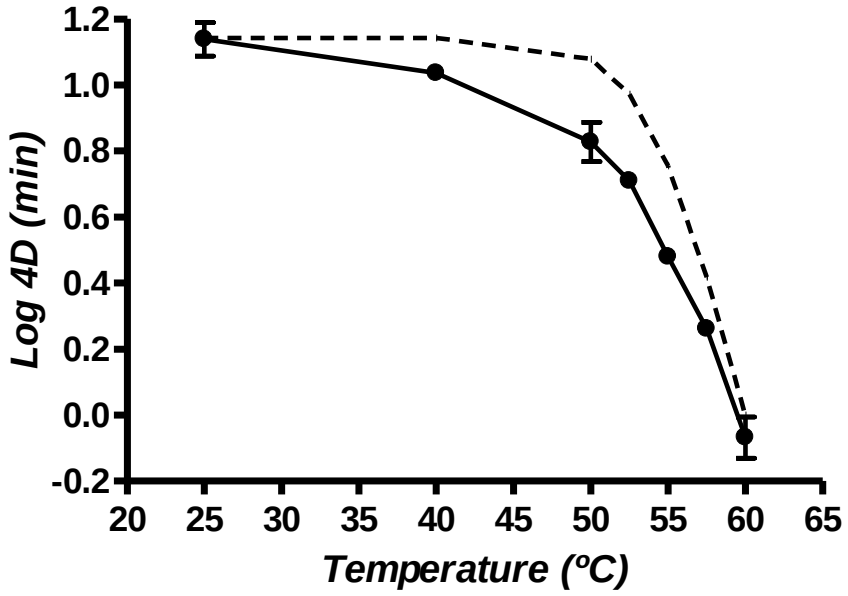
745 **Figure 2**



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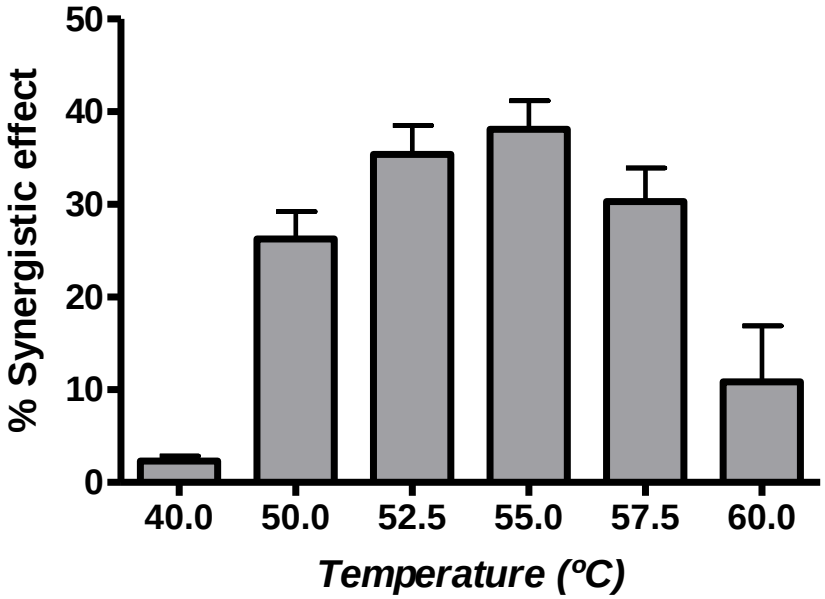
757 **Figure 3**


% Synergistic effect
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52.5
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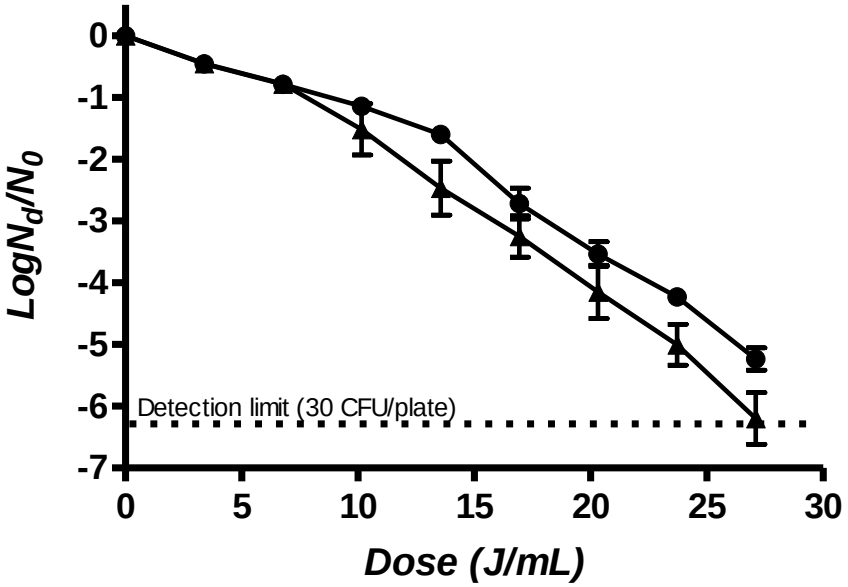
Temperature (ºC)

Figure 4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Detection limit (30 CFU/plate)
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LogN_d/N_0
Dose (J/mL)