Inactivation of bacterial spores by UV-C light

E. Gayán, I. Álvarez, and S. Condón*

Food Technology, Faculty of Veterinary of Zaragoza, Spain.

Running Title: UV-C inactivation at mild temperatures

Keywords: ultraviolet light, sterilization, combined processes, non-thermal technologies, bacterial spores.

* Corresponding author: Prof. Santiago Condón. Mailing address: Tecnología de los Alimentos, Facultad de Veterinaria, Universidad de Zaragoza, C/ Miguel Servet 177, CP 50013, Zaragoza, Spain. Phone: 0034 976 76 26 38. Fax: 0034 976 76 15 90. E-mail: scondon@unizar.es

ABSTRACT

This investigation evaluates the ability of UV light for inactivating bacterial spores of relevance in food preservation, the effect of the absorptivity of the treatment medium on the UV lethality, and the effect of the simultaneous or sequential combination of UV light and mild heating. A UV dose of 23.72 J/mL reached 2.25, 2.93, 3.24, 3.85, and 4.05 Log_{10} reductions of *B. coagulans*, *B. cereus, A. acidocaldarius*, *B. licheniformis*, and *G. stearothermophilus* spores, respectively. The shoulder phase of UV survival curves of the most resistant *B. coagulans* increased linearly with the absorptivity of the treatment medium, whereas the inactivation rate decreased exponentially. The UV inactivation of *B. coagulans* increased synergistically when increasing the treatment temperature from 25 to 60 ºC. A previous heat treatment (60 ºC for 3.58 min) did not affect UV sensitivity of *B. coagulans*, but prior UV exposure (27.10 J/mL) sensitized bacterial spores to subsequent heat treatments.

Keywords: UV light, combined processes, bacterial spores, absorption coefficient,…

Industrial relevance: This investigation demonstrated the ability of UV light for inactivating bacterial spores of interest for food preservation suggesting that UV technology, contrary to other non-thermal technologies, is a real alternative to current heat sterilization treatments. For UV sterilization of liquid foods with high absorption coefficient, the efficacy of UV light can be enhanced by its combination with heat.

1. Introduction.

Current consumer´s demand for minimal processed food has led to considerable efforts for development alternative non-thermal processes for microbial inactivation. Most of these new technologies, such as Pulsed Electric Fields (PEF), High Hydrostatic Pressure (HHP), and Ultrasounds (US) are able to inactivate efficiently bacterial vegetative forms but fails against bacterial spores. Therefore, their application in the food industry is focused on pasteurization processes to control pathogenic microorganisms (NACMCF, 2006), but are unsuitable to replace thermal sterilization treatments. Ultraviolet (UV) irradiation is another non-thermal technology that has been traditionally used for the disinfection of air, water, and surfaces (Bintsis, Litopoulou-Tzanetaki, & Robinson, 2000). However, currently it has gained considerable interest for liquid food pasteurization because it is easy to use and lethal to most types of microorganisms, it does not generate chemical residues, and it is a dry cold process that can be effective at low cost in comparison with other pasteurization methods (Guerrero-Beltrán & Barbosa-Cánovas, 2004; Tran & Farid, 2004). Contrary to other non-thermal technologies UV treatments are potentially capable of inactivating bacterial spores (Hijnen, Beerendonk, & Medema, 2006).

When bacterial spores are treated with UV-C radiation (200–280 nm), which is considered the most germicidal wavelength-range, DNA absorbs photons inducing the formation of bipyrimidine dimmers, especially spore photoproducts (SP) (Moeller et al., 2007), which transiently block DNA transcription and replication leading to cell death (Friedberg et al., 2006). Most data in literature have been obtained with *Bacillus subtilis*, one of the challenge microorganisms used in biodosimetry assays for evaluating water treatment plant performance (USEPA, 2003). However, there is little additional information about the UV inactivation of different bacterial spores of interest

for food preservation. The effect of important environmental factors on the UV resistance of bacterial spores, such as the absorption coefficient of the treatment media, has not been investigated so far.

The main drawback of UV processing is its low penetration capacity in liquid foods with high absorption coefficient (Koutchma, Forney, & Moraru, 2009). It is foreseeable that this limitation is greater the higher the UV resistance of the target microorganism is. Most authors agree that bacterial spores are more UV tolerant than vegetative cells (Setlow, 2006), which is attributed to altered DNA conformation (A-form) caused by the presence of small acid-soluble proteins (SASP) binding to the DNA; to an efficient DNA repair mechanisms specific for SP; to the accumulation of high percentage of dipicolinic acid in the dormant spore core; and to the presence of a thick spore protein coating. Overall it is expected that the inactivation of bacterial spores in media of high absorptivity will be difficult.

UV light can be combined with other novel processing techniques or milder conventional preservation methods in a so-called 'hurdle' approach to guarantee acceptable inactivation of microorganisms of concern (Walkling-Ribeiro et al., 2008; Maktabie, Watson, & Parton, 2011). To improve the UV lethal effect, UV light has been combined with chemical agents, mild heating, or other novel technologies. In fact, it is well known that the sporicidal effect of UV-C light increases in combination with hydrogen peroxide and ozone (Gardner & Shama, 1998; Jung, Oh, & Kang, 2008), and this has been the basis for the design of sterilization processes of food packaging materials. Unfortunately this strategy cannot be used for food preservation. Also infrared radiation and conductive heating increase the lethal effect of UV light (Hamanaka et al., 2011), which probably is due to thermal effects. Recently it has been demonstrated that the lethal effect of UV light on *Escherichia coli* and *Salmonella enterica* subsp. *enterica* serovar *Typhimurium* increases synergistically with

temperature between 40 and 60 ºC (Gayán, Monfort, Álvarez, & Condón, 2011; Gayán, Serrano, Raso, Álvarez, & Condón, 2012). To the best of our knowledge there is no data in the literature of the effect of the combination of UV light and mild heat treatments (UV-H treatments) on the inactivation of bacterial spores.

The goal of this study was to determine the ability of UV light for inactivating different spore-forming bacteria (*Bacillus coagulans*, *Bacillus lilcheniformis*, *Geobacillus stearothermophilus*, *Bacillus cereus*, and *Alicyclobacillus acidocaldarius*) of relevance for food preservation. The effect of applied dose and absorptivity of the treatment medium on UV lethality was studied with the most UV resistant species. Finally, the lethal effect of the simultaneous or sequential combination of UV light with mild heating was also explored.

2. Material and methods.

2.1. Bacterial spore production.

The strains used in this investigation: *Bacillus coagulans* (STCC 4522) *Bacillus licheniformis* (STCC 4523), *Geobacillus stearothermophilus* (STCC 12980), *Bacillus cereus* (STCC 9818) and *Alicyclobacillus acidocaldarius* (STCC 5137) were provided by the Spanish Type Culture Collection (STCC). The bacterial spores were maintained frozen at -80 ºC in cryovials. To obtain the vegetative cultures, a loopful of growth in nutrient agar (Biolife, Milano, Italy) with 0.6% (w/v) of yeast extract (Biolife) (NAYE) added was inoculated into a 50 mL-flask of nutrient broth (Biolife) with 0.6% (w/v) of yeast extract (Biolife) (NBYE) added and incubated at the required temperatures in a shaking incubator for 24 h. Sporulations were carried out in Petri dishes of NAYE with 3 ppm (w/v) of manganese sulphate (Carlo Erba, Milan, Italy) added. Agar surface was inoculated with a 24 h culture in liquid media and incubated at the corresponding temperature for 5 days. After incubation spores were collected by flooding the agar

surface with sterile distilled water. After harvesting, spores were washed five times by centrifugation at 2500*g* for 20 min at 4 ºC (Jouan, mod. CR 4.11, Saint-Herblain, France), and re-suspended in sterile distilled water. The spore suspensions were stored at 0-5 ºC until used. Incubation temperatures for growth and sporulation were: 35 ºC for *B. coagulans*, *B licheniformis* and *B. cereus*; and 55 ºC for *G. stearothermophilus* and *A. acidocaldarius.*

2.2.UV equipment and treatments.

UV treatments were carried out in the equipment previously described by Gayán et al. (2011). Briefly, it consisted of eight individual annular thin film flow-through reactors connected sequentially. Each reactor consisted of a low pressure UV-C lamp (TUV 8WT5, Philips, USA), with 8 W of input power, enclosed by a quartz sleeve to prevent the direct contact with the treatment medium, and fixed at the axis of an outer glass at which end was placed a sampling valve. The whole system was 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). The equipment included a peristaltic pump (Ismatec, mod. ISM 10785, Glattbrugg, Switzerland) and a heating/cooling coil exchanger prior to the entrance of the first UV reactor and placed inside the bath water. Two thermocouples (Almeco, mod. ZA 020-FS, Bernburg, Germany) fitted to the input of the first and the outlet of the last reactor allowed the control of the treatment medium temperature.

Treatment medium was added with the spore suspensions to achieve 10^4-10^6 CFU/mL and pumped through the installation at 8.5 L/h. When the treatment conditions were stabilized, samples were withdrawn through the sampling valve of each reactor and 0.1 mL or 1 mL were immediately pour plated in the recovery media. McIlvaine citrate-phosphate buffer pH 7.0 with different absorption coefficients (from 3.9 to 20.0 cm^{-1};

144 turbidity < 5 NTU) were prepared by adding different quantities of tartrazine (Sigma-
145 Aldrich, St. Louis, USA). Absorbance of treatment media was measured at 254 nm
146 using a Unicam UV500 spectrophotometer (Unicam Limited, Cambridge, UK). Sample
147 solutions were diluted and evaluated using quartz cuvettes (Hellma, Müllheim,
148 Germany) with path lengths of 1, 2, and 10 mm. The absorption coefficient of each
149 sample was determined from the slope of the absorbance versus path length, correcting
150 for the dilution factor. Turbidity was measured using a HI 83749 nephelometer (Hanna
151 Instrument, Szeged, Hungary).
152
153 **2.3.Heat treatments.**
154 Heat treatments were carried out in a specially designed resistometer described by
155 Condón, Arrizubieta, and Sala (1993). Basically this instrument consists of a 350 mL
156 vessel provided with an electrical heater for thermostation, an agitation device to ensure
157 inoculums distribution and temperature homogeneity, a pressurization system, and ports
158 for injecting the microbial suspension and for sampling. Once the preset temperature
159 had attained stability (T ± 0.05 °C), 0.2 mL of an adequately diluted microbial cell
160 suspension were inoculated into the corresponding treatment medium. After inoculation,
161 0.2 mL samples were collected at different heating times and immediately pour plated.
162
163 **2.4.Incubation of treated samples and survival counting.**
164 Bacterial spores were recovered in NAYE and plates were incubated for 48 h at the
165 corresponding temperature for each microorganism. Longer incubation times did not
166 change the profile of survival curves (data not shown). After incubation, colony forming
167 units (CFU) were counted with an improved Image Analyzer Automatic Colony
168 Counter (Protos, Synoptics, Cambridge, UK), as described elsewhere (Condón , Oria, &
169 Sala, 1987).

170 **2.5.Curve fitting and statistical analysis.**

171 Survival curves to UV treatments were obtained by plotting the logarithm of the
172 survival fraction versus treatment doses expressed in energy consumption unit (J/mL)
173 according to Gayán et al. (2011), and to heat versus time in min. To fit survival curves
174 and calculate resistance parameters, the Geeraerd and Van Impe inactivation model-
175 fitting tool (GInaFiT) was used (Geeraerd, Valdramidis, & Van Impe, 2005). As UV
176 survival curves obtained in this investigation did not show tails but rather shoulders, the
177 log-linear regression plus shoulder model (Geeraerd, Herremans, & Van Impe, 2000)
178 was used with the equation:

179 $$N_d = N_0 e^{-K_{max} d}\left(\frac{e^{K_{max} Sl}}{1 + (e^{K_{max} Sl} - 1)e^{-K_{max} d}}\right) \quad (1)$$

180 where N_d represents the number of survivors, N_0 the initial count, d the applied dose, Sl
181 the shoulder length, and K_{max} the first-order inactivation constant. This model describes
182 the survival curves through two parameters: the shoulder length (Sl) or dose before the
183 exponential inactivation begins, and the inactivation rate (K_{max}), defined as the slope of
184 the exponential portion of the survival curve. For comparison purposes, the GInaFiT
185 software also provides the parameter 4D, defined as the treatment dose necessary to
186 inactivate 99.99% of the microbial population. Heat survival curves which showed a
187 log-liner profile were fitted by the Bigelow and Esty (1920) model (Eq. 2) also
188 incorporated in the GInaFiT software by the equation:

189 $$N_t = N_0 e^{-K_{max} t} \quad (2)$$

190 All microbial resistance determinations were performed at least three times on different
191 working days. The error bars in the figures correspond to the mean standard deviation.
192 Statistical analyses, *t*-test (p = 0.05) and ANOVA tests (p = 0.05), were carried out

193 using the GraphPad PRISM 5.0 software (GraphPad Software, Inc., San Diego, USA),
194 and differences were considered significant for $p \leq 0.05$.
195
196 **1. Results.**
197 Survival curves of the five spore suspensions to UV light were assessed in buffer with
198 an absorption coefficient of 11.1 cm^{-1} (Figure 1). As observed, UV susceptibility of
199 bacterial spores varied between species: a dose of 23.72 J/mL reached 2.25 ± 0.07, 2.93
200 ± 0.09, 3.24 ± 0.38, 3.85 ± 0.5, and 4.05 ± 0.30 Log_{10} cycles of inactivation of *B.*
201 *coagulans*, *B. cereus*, *A. acidocaldarius*, *B. licheniformis*, and *G. stearothermophilus*
202 spores, respectively. UV survival curves showed a significant shoulder, followed by an
203 exponential order of death. Survival curves were fitted with the Geeraerd et al.´s
204 equation (Eq. 1) and resistance parameters (*Sl* and K_{max}), and 4*D* values for comparison,
205 were compiled in Table 1. Table 1 also includes the coefficient of determination (R^2)
206 and the root mean square error (*RMSE*) to illustrate the goodness of the fit. No
207 statistically significant differences ($p > 0.05$) were found among K_{max} values, whereas
208 the shoulder length widely changed among species: *B. coagulans* exhibited the highest
209 *Sl*, whereas the most sensitive *G. stearothermophilus* showed a *Sl* the half of the former.
210 To study the effect of the absorptivity of the treatment medium on UV lethality at room
211 temperature, survival curves of the most resistant spores, *B. coagulans*, were obtained in
212 buffers with absorption coefficient ranging from 3.9 to 20.0 cm^{-1}. UV resistance
213 parameters were calculated from the fit of experimental data with the Geeraerd et al.´s
214 equation and data have also been included in Table 1. As observed, the lethal effect of
215 UV light decreased by increasing the absorption coefficient. The lower UV
216 effectiveness was due to both an increase of the shoulder length and a decrease of the
217 inactivation rate. Plotting the Log_{10} K_{max} against the absorption coefficient (α) (Figure
218 2), an exponential relationship was deduced (Log_{10} K_{max} = -0.060 α + 0.208; R^2 =

219 0.996), from which was calculated that K_{max} decreased 10-fold by increasing the
220 absorption coefficient by 16.8 ± 0.8 cm^{-1}. On the contrary, a linear relationship (Sl =
221 0.909 α – 1.896; R^2 = 0.986) was found between shoulder length and absorptivity
222 (Figure 3).
223 To evaluate the lethal effect of the UV-H combined treatment, *B. coagulans* spores were
224 treated by UV light at 60 ºC in buffers with different absorption coefficients (8.8-17.0
225 cm^{-1}). Obtained survival curves are plotted in Figure 4 and their kinetics parameters are
226 included in Table 1. The UV inactivation applying a dose of 27.10 J/mL at 60 ºC in
227 buffers of 17.0, 13.9, 11.1, and 8.8 cm^{-1} increased 0.82, 1.19, 1.87, and more than 2
228 Log_{10} cycles, respectively, compared to treatments at 25 ºC (Figure 4). Inactivation UV-
229 H parameters (Table 1) showed that shoulder phases were shorter and inactivation rates
230 were higher than those obtained at 25 ºC. The relationship between the absorption
231 coefficient and K_{max} values (Log_{10} K_{max} = -0.065 α + 0.423; R^2 = 0.999) is illustrated in
232 Figure 2, and with the Sl (Sl = 0.245 α + 3.125; R^2 = 0.996) is in Figure 3. The statistical
233 analysis demonstrated that the effect of the absorptivity on K_{max} was the same ($p > 0.05$)
234 at both treatment temperatures. On the contrary, the effect of the absorptivity on Sl was
235 significantly higher at 20 ºC than at 60 ºC. As the lethal effect of heat at 60 ºC for 3.58
236 min (exposure time for a dose of 27.10 J/mL) was negligible -less than 0.08 ± 0.02
237 Log_{10} cycles of inactivation, a synergistic lethal effect was deduced from the
238 simultaneous UV-H combination.
239 To explore the mechanism responsible for enhanced inactivation of *B. coagulans* with
240 the combined process, both technologies were applied sequentially in the two possible
241 orders: a heat treatment at 60 °C for 3.58 min followed by a UV treatment of 27.10
242 J/mL and a UV treatment of the same intensity followed by thermal sterilization at
243 different temperatures, using buffer with an absorption coefficient of 17.0 cm^{-1}. The
244 combination of heat followed by a UV treatment did not change significantly ($p > 0.05$)

245 the UV inactivation (1.26 Log_{10} cycles). On contrary, the UV treatment prior to thermal
246 treatments improved heat lethality. Figure 5 shows heat survival curves of *B. coagulans*
247 at 110, 105, 100 ºC, as well as those preceded by the UV treatment. Heat inactivation
248 curves of *B. coagulans* at 110 and 105 ºC showed a log-linear profile and the first-order
249 kinetics was applied (Eq. 2). However, survival curves at 100 ºC displayed an initial
250 shoulder and were described by the Geeraerd et al.´s equation in time term. The decimal
251 reduction time value (D_T time necessary to reduce the 90% of the microbial population
252 at a treatment temperature T) of heat treatments at 110, 105, 100 ºC were reduced 0.13,
253 0.59, and 5.31 min, respectively, after applying a UV irradiation treatment. However,
254 the z value (number of degrees needed to decrease D values ten-fold) from conventional
255 heat treatments (z = 6.53 ºC) did not change significantly ($p > 0.05$) when they were
256 preceded by the UV treatment (z = 6.71 ºC).
257
258
259 **2. Discussion.**
260 In this investigation, the UV-C resistance of bacterial spores of different species of
261 interest in food preservation has been tested in a continuous device. The effect of the
262 most important environmental factor –the absorptivity of the treatment medium- on the
263 UV inactivation kinetics of the most resistant species has been studied, and the lethality
264 of a combined process of UV and heat, applied simultaneously or successively, has been
265 explored.
266 UV survival curves of all species studied showed shoulders (Figure 1). Deviations from
267 linearity have been attributed to non-uniform dose distribution inside UV reactors
268 (Koutchma, Keller, Chirtel, & Parisi, 2004). However, this is not the case in our device,
269 as we previously demonstrated (Gayán et al., 2011; 2012). This profile has also been
270 related in vegetative cells with damage and repair mechanisms (López-Malo & Palau,

2005). UV light acts by causing mutated bases that compromise cell functionality, but bacteria have developed DNA repair mechanisms to restore DNA structure and functionality (Friedberg et al., 2006). This phenomenon is reflected in the shape of the inactivation curves. After the initial shoulder, the maximum DNA repair capability is surpassed and survivors exponentially decline (López-Malo & Palau, 2005). It is not expectable that DNA repair mechanisms acts immediately after UV treatments because bacterial spores are metabolically inactive due to the low water activity of the protoplast. However, spore repair systems could operate during spore germination resulting in a lag phase before the inactivation began (Mamane-Gravetz & Linden, 2005). A very interesting conclusion can be deduced from our data: K_{max} values were the same for all species tested and differences in UV resistance were due to changes in *Sl*. Therefore, the different UV spore resistance of *G. stearothermophilus* < *B. licheniformis* < *B. cereus* < *A. acidocaldarius* < *B. coagulans* probably was due to different efficacies of their repair mechanisms. Other authors have found that *B. subtilis* was more UV sensitive than *B. cereus* (Rossitto et al., 2012) and *G. stearothermophilus* (Chaine, Levy, Lacour, Riedel, & Carlin, 2012). These disagreements could be due to UV resistance variability among strains or to methodological differences. Since the design of UV equipment, processing parameters, and optical properties of the liquid play an important role in UV germicidal efficacy (Keyser, Muller, Cilliers, Nel, & Gouws, 2008; Koutchma et al., 2009), the comparisons of UV resistance data obtained by different authors must be done with caution. Results obtained in this investigation have been carried out in the same conditions and they can be directly compared. From data in Table 1 it can be deduced that the UV resistance (4*D* values) of the most sensitive *G. stearothermophilus* was 30% lower than of the most UV resistant *B. coagulans*. This is a very small difference as compared with the very high heat resistance variations. Table 2 includes the decimal reduction times at 111 ºC ($D_{111ºC}$)

297 and *z* values of the strains used in this investigation. As observed, D_{111^oC} values can vary
298 338-fold. On the other hand, the most UV sensitive species (*B. stearothermophilus*) is
299 the most heat resistant one. These facts demonstrated that the mechanisms of spore
300 inactivation by both technologies are quite different. Furthermore, and from a practical
301 point of view, results indicate the advantage of UV treatments over current heat
302 sterilization: whereas the stability ensured by heat treatments depends very much on the
303 microbial species contaminating the raw material, that ensured by UV treatments would
304 be very similar, even if the contaminating bacterial spore changed.
305 Setlow (2001) estimated that the UV resistance of bacterial spores of *Bacillus* species
306 was around 5-50 times higher than of vegetative cells. Results obtained in our
307 laboratory with the same methodology demonstrated that the UV resistance of spores
308 was also greater than that of vegetative cells previously investigated but in a lower
309 extent. For instance, the estimated dose necessary to inactivate the 99.99% of the initial
310 population of the most resistant spore, *B. coagulans*, was about 2-fold higher than that
311 of the most UV tolerant strains of *E. coli* (Gayán et al., 2011) and *Salmonella*
312 Typhimurium (Gayán, et al., 2012). The difference in UV resistance between bacterial
313 spores and vegetative cells is very scarce as compared with the difference in heat
314 resistance (D_T multiply approximately 10^{12}-fold). The higher heat resistance of bacterial
315 spores has been correlated with the low water activity of the protoplast (Setlow, 2006),
316 and it has been demonstrated that low water activities does not protect bacterial cells to
317 UV light (Gayán et al., 2011; 2012). This could explain our results.
318 From data in Table 1, it was deduced that higher absorption coefficients increased
319 shoulder length (*Sl*) and decreased the inactivation rate (K_{max}). These results are
320 consistent with the Beer-Lambert-Bougerts Law which states that the amount of light
321 that penetrates through a solution decreases when the absorbance of the solution
322 increases. Plotting K_{max} against the absorption coefficient showed that there was an

exponential relationship between both variables (Figure 2). Working with vegetative cells in the same experimental conditions, Gayán et al. (2011; 2012) also found an exponential relationship. The regression line that relates Log_{10} K_{max} and the absorption coefficient allowed concluding that the inactivation rate (K_{max}) of *B. coagulans* decreased ten times by increasing the absorption coefficient 16.8 ± 0.8 cm^{-1}. This value does not significantly differ ($p > 0.05$) from that obtained for *E. coli* (15.9 ± 1.0 cm^{-1}) (Gayán et al., 2011) and *Salmonella* Typhimurium (18.9 ± 2.8 cm^{-1}) (Gayán et al., 2012). These results indicated that the effect of the absorptivity had a physical basis and was not related with the biological behaviour of microorganisms. Contrary to the inactivation rate, shoulder length (*Sl*) increased linearly with the absorptivity (Figure 3). At the moment, there are no data in the literature which similar behavior to be compared.

The difficulty of UV light treatment in achieving the necessary microbial reduction due to the low penetration capacity of UV photons in media with high absorptivity has prompted to develop hurdle strategies combining UV light with other novel processing techniques or milder conventional preservation methods. It has been demonstrated that the combination of heat at mild temperatures with other non-thermal technologies such PEF and US at low intensity results in an equivalent or even higher degree of microbial inactivation (Raso, Pagan, Condón, & Sala, 1998; Heinz, Toepfl, & Knorr, 2003). However, the knowledge of the combination of UV light and mild heat treatments are scarce. Gayán et al. (2011; 2012) demonstrated that the lethality of UV light on vegetative cells increased synergistically with temperature in the range of 40 to 60 ºC. Nevertheless, there is no data in the literature on the effect of the combined process on bacterial spores. Our results demonstrated that the UV inactivation (27.10 J/mL) of the most resistant *B. coagulans* spores increased from ten to a hundred times by increasing the temperature from 25 to 60 ºC. The magnitude of the effect was the same in the range

of 55 and 65 ºC (data not shown). The comparison of UV resistance parameters (Table 1) obtained at both temperatures demonstrated that the shoulder length decreased and the inactivation rate increased with temperature. It should be noted that the effect of the media absorptivity on K_{max} values (Figure 2) was the same at both temperatures, but the differences in shoulder length increased with the absorption coefficient (Figure 3). Therefore, the synergism of the combined UV-H treatment was greater in media with higher absorptivity, that is, when it might be more useful for UV food sterilization.

To explore the mechanism of the synergistic lethal effect of the combined UV-H treatment, we applied both technologies sequentially in two possible orders. Results demonstrated that a previous treatment at 60 ºC for 3.58 min does not sensitized *B. coagulans* to a subsequent UV treatment (27.10 J/mL). This fact discarded the possibility that the sublethal heat treatment could activate dormant spores potentiating the germination, and thereby sensitizing them to UV light, as it has been suggested for the combination of high pressures and mild temperatures (Lovdal, Hovda, Granum, & Rosnes, 2011). In contrast, the application of a prior UV treatment (27.10 J/mL) sensitized bacterial spores to a subsequent heat treatment (Figure 5). The magnitude of the sensitizing effect was the same at 100, 105 and 110 ºC (31.71, 30.72, and 38.01 %, respectively) which means that it was independent of the treatment temperature. In other words, the UV treatment did not affect *z* values. This behavior was similar to that observed for vegetative cells. The sequential application of heat (55 ºC for 3.58 min) and UV light (27.10 J/mL) on *Salmonella* Typhimurim showed an additive lethal effect, whereas in the inverse order, the thermotolerance of *Salmonella* was decreased synergistically (Gayán et al., 2012). Also the magnitude of the synergistic lethal effect on *Salmonella* was independent of the treatment temperature and did not affect the *z* value. Therefore, the mechanism of synergistic lethal effect of the combined treatment on bacterial spore inactivation could be related with that of vegetative bacteria. Gayán et

al (2012) suggested that the mechanism of the synergistic effect of UV light and heat on *Salmonella* was related to a sensitization of cell envelopes or the cell's inability to repair these structures. Also the highest synergistic benefits of the UV light in combination with chemical oxidant compounds to inactivate spores has been observed when both agents were applied simultaneously compared with the two sequential orders, suggesting that the oxidant agent could destroy the surface component of the cell and assist the UV transmission into inner cell components and DNA damage (Jung et al., 2008). Overall, this is an interesting aspect that deserves further investigation.

From this investigation it can be concluded that, unlike most non-thermal technologies, UV light is a real alternative to current heat sterilization treatments aimed at the inactivation of bacterial spores. Its main limitation is the absorption coefficient of the treatment medium, but this limitation is of similar importance in the pasteurization (inactivation of vegetative cells) and sterilization (inactivation of bacterial spores) processes. For UV sterilization of liquid foods with high absorption coefficients the efficacy of UV light can be enhanced by its combination with heat.

5. Acknowledgements.

This study has been carried out with financial support from the Ministerio de Ciencia e Innovación de España, EU-FEDER (CIT020000-2009-40) and the Departamento de Ciencia, Tecnología y Universidad del Gobierno de Aragón. E. G. gratefully acknowledges the financial support for her doctoral studies from the Ministerio de Educación y Ciencia de España.

References.

Arroyo, C., Gayán, E., Pagan, R, Condón, S. (2012). UV-C inactivation of *Cronobacter sakazakii*. *Foodborne Pathogens and Disease, 9,* 907-914.

Bigelow, W. D., & Esty, J. R. (1920). The thermal death point in relation to typical thermophylic organisms. *Journal of Infectious Diseases, 27*, 602-617.

Bintsis, T., Litopoulou-Tzanetaki, E., & Robinson, R. K. (2000). Existing and potential applications of ultraviolet light in the food industry - a critical review. *Journal of the Science of Food and Agriculture, 80*, 637-645.

Condón S, Oria, R, & Sala FJ. (1987). Heat resistance of microorganisms: An improved method for survival counting. *Journal of Microbiologycal Methods, 7*, 37-44.

Condón, S., Arrizubieta, M. J., & Sala, F. J. (1993). Microbial heat resistance determinations by the multipoint system with the thermoresistometer TR-SC Improvement of this methodology. *Journal of Microbiological Methods, 18*, 357-366.

Chaine, A., Levy, C., Lacour, B., Riedel, C., & Carlin, F. (2012). Decontamination of sugar syrup by pulsed light. *Journal of Food Protection, 75*, 913-917.

Friedberg, E. C., Walker, G. C., Siede, W., Wood, R. D., Schultz, R. A., & Ellenberger, T. (2006). *DNA repair and mutagenesis*. Washington: ASN Press.

Gardner, D. W. M., & Shama, G. (1998). The kinetics of *Bacillus subtilis* spore inactivation on filter paper by UV light and UV light in combination with hydrogen peroxide. *Journal of Applied Microbiology, 84*, 633-641.

Gayán, E., Monfort, S., Álvarez, I., & Condón, S. (2011). UV-C inactivation of *Escherichia coli* at different temperatures. *Innovative Food Science and Emerging Technologies, 12*, 531-541.

Gayán, E., Serrano, M. J., Raso, J., Álvarez, I., & Condón, S. (2012). Inactivation of *Salmonella enterica* by UV-C light and by its combinations with mild temperatures. *Applied and Environmental Microbiology, 78*, 8353-8361.

Geeraerd, A. H., Van Impe, J. F., & Herremans, C. H. (2000). Structural model requirements to describe microbial inactivation during a mild heat treatment. *International Journal of Food Microbiology, 59*, 185–209.

Geeraerd, A. H., Valdramidis, V. P., & Van Impe, J. F. (2005). GInaFiT, a freeware tool to assess non-log-linear microbial survivor curves. *International Journal of Food Microbiology, 102*, 95-105.

González, I., López, M., Martínez, S., Bernardo, A., & González, J. (1999). Thermal inactivation of *Bacillus cereus* spores formed at different temperatures. *International Journal of Food Microbiology, 51*, 81-84.

Guerrero-Beltrán, J. A., & Barbosa-Cánovas, G. V. (2004). Advantages and limitations on processing foods by UV light. *Food Science and Technology International 10*, 137-147.

Hamanaka, D., Yamada, H., Trivittayasil, V., Kadoyanagi, T., Tanaka, F., & Uchino, T. (2011). Effect of different heat treatments on *Bacillus subtilis* spores inactivated by ultraviolet irradiation. *Food Science and Technology Research, 17*, 289-293.

Heinz, V., Toepfl, S., & Knorr, D. (2003). Impact of temperature on lethality and energy efficiency of apple juice pasteurization by pulsed electric fields treatment. *Innovative Food Science and Emerging Technologies, 4*, 167-175.

Hijnen, W. A. M., Beerendonk, E. F., & Medema, G. J. (2006). Inactivation credit of UV radiation for viruses, bacteria and protozoan (oo)cysts in water: A review. *Water Research, 40,* 3-22.

Jung, Y. J., Oh, B. S., & Kang, J.-W. (2008). Synergistic effect of sequential or combined use of ozone and UV radiation for the disinfection of *Bacillus subtilis* spores. *Water Research, 42,* 1613-1621.

Keyser, M., Muller, I. A., Cilliers, F. P., Nel, W., & Gouws, P. A. (2008). Ultraviolet radiation as a non-thermal treatment for the inactivation of microorganisms in fruit juice. *Innovative Food Science and Emerging Technologies, 9,* 348-354.

Koutchma, T., Keller, S., Chirtel, S., & Parisi, B. (2004). Ultraviolet disinfection of juice products in laminar and turbulent flow reactors. *Innovative Food Science and Emerging Technologies, 5,* 179–189.

Koutchma, T., Forney, L. J., & Moraru, C. L. (2009). *Ultraviolet light in food technology*. Boca Raton: CRC Press.

López, M., González, I., Condón, S., & Bernardo, A. (1996). Effect of pH heating medium on the thermal resistance of *Bacillus stearothermophilus* spores. *International Journal of Food Microbiology, 28,* 405-410.

López-Malo, A., & Palou, E. (2005). Ultraviolet light and food preservation. In G. Barbosa-Canovas, M. S. Tapia, & M. P. Cano (Eds.), *Novel food processing technologies* (pp. 405–423). Boca Raton: CRC Press.

462 Lovdal, I. S., Hovda, M. B., Granum, P. E., & Rosnes, J. T. (2011). Promoting *Bacillus*
463 *cereus* spore germination for subsequent inactivation by mild heat treatment.
464 *Journal of Food Protection, 74*, 2079-2089.

465 Maktabi, S., Watson, I, & Partson, R. (2011). Synergistic effect of UV, laser and
466 microwave radiation or conventional heating on *E. coli* and on some spoilage
467 and pathogenic bacteria. *Innovative Food Science and Emerging Technologies*,
468 *12*, 129-134.

469 Mamane-Gravetz, H., & Linden, K. G. (2005). Relationship between physiochemical
470 properties, aggregation and u.v. inactivation of isolated indigenous spores in
471 water. *Journal of Applied Microbiology, 98*, 351-363.

472 Moeller, R., Douki, T., Cadet, J., Stackebrandt, E., Nicholson, W. L., Rettberg, P., et al.
473 (2007). UV-radiation-induced formation of DNA bipyrimidine photoproducts in
474 *Bacillus subtilis* endospores and their repair during germination. *International*
475 *Microbiology, 10*, 39-46.

476 NACMCF. (2006). Requisite scientific parameters for establishing the equivalence of
477 alternative methods of pasteurization. *Journal of Food Protection, 69*, 1190-
478 1216.

479 Palop, A., Raso, J, Pagan, R, Condón, S., Sala, F. J. (1996). Influence of pH on heat
480 resistance of *Bacillus licheniformis* in buffer and homogenised foods.
481 *International Journal of Food Microbiology, 29*, 1-10.

482 Palop, A., Raso, J., Pagan, R., Condón, S., & Sala, F. J. (1999). Influence of pH on heat
483 resistance of spores of *Bacillus coagulans* in buffer and homogenized foods.
484 *International Journal of Food Microbiology, 46*, 243-249.

485 Palop, A., Álvarez, I, Raso, J., Condón, S. (2000). Heat resistance of *Alicyclobacillus*
486 *acidocaldarius* in water, various buffers, and orange juice. *Journal of Food*
487 *Protection, 63,* 1377-1380.

488 Raso, Pagan, Condón, & Sala. (1998). Influence of temperature and pressure on the
489 lethality of ultrasound. *Applied and Environmental Microbiology, 64,* 465-471.

490 Rossitto, P. V., Cullor, J. S., Crook, J., Parko, J., Sechi, P., & Cenci-Goga, B. T. (2012).
491 Effects of UV irradiation in a continuous turbulent flow UV reactor on
492 microbiological and sensory characteristics of cow's milk. *Journal of Food*
493 *Protection, 75,* 2197-2207.

494 Setlow, P. (2001). Resistance of spores of *Bacillus* species to ultraviolet light.
495 *Environmental and Molecular Mutagenesis, 38,* 97-104.

496 Setlow, P. (2006). Spores of *Bacillus subtilis*: their resistance to and killing by radiation,
497 heat and chemicals. *Journal of Applied Microbiology, 101,* 514-525.

498 Tran, M. T. T., & Farid, M. (2004). Ultraviolet treatment of orange juice. *Innovative*
499 *Food Science and Emerging Technologies, 5,* 495-502.

500 USEPA. (2003). Ultraviolet disinfection guidance manual. Retrieved from
501 http://www.epa.gov/ogwdw/disinfection/lt2/pdfs/guide_lt2_uvguidance.pdf