Modelling microbial inactivation kinetics of combined UV-H treatments in apple juice

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ABSTRACT

In this investigation, temperature's effect on the ultraviolet (UV) inactivation kinetics of pathogens of concern in juices—*E. coli*, *Salmonella* Typhimurium, *L. monocytogenes,* and *S. aureus*—was studied to establish the target microorganism and process criteria for pasteurizing apple juice using combined shortwave UV light (UV-C) and mild heat (UV-H) treatments. For this purpose, mathematical models based on Geeraerd et al.´s model, which predict UV-H inactivation at different treatment temperatures, were developed for each microorganism. For comparisons, inactivation models for heat treatments were also performed in the same juice and for the same microorganisms. The UV inactivation notably improved at treatment temperatures between 50–60 ºC, but the thermodependence of the UV-H resistance differed among species. This behavior was related to the thermodependence of heat treatments for each bacterium so that the target microorganism for UV-H treatments was determined based on the most heat-resistant species at each treatment temperature. Thus, *E. coli* was the most UV-H-resistant microorganism between 44 ºC to 54 ºC, requiring a UV dose between 13.81 J/mL (12.71 min) and 5.20 J/mL (4.78 min) in order to achieve the 5 Log_{10} reduction that the U.S. Food and Drug Administration demands. Also, *L. monocytogenes* was the target microorganism at temperatures between 54 ºC and 60 ºC, requiring a UV dose from 5.20 J/mL (4.78 min) to 2.11 J/mL (1.93 min). The combination of UV and mild heat allowed for the UV doses and treatment times to be reduced from 49.6% to 89.1% in comparison with UV treatments at room temperatures.

1. Introduction

Fresh juice has been considered a safe product due to its inherent acidity, cold preservation, and the addition of chemical preservatives. However, unpasteurized juice has been implicated as a vehicle for food-borne outbreaks, which has forced the juice industry to implement a decontamination process for fresh juice (Vojdani, Beuchat, & Tauxe, 2008). Thus, the U.S. FDA requires juice producers to develop a hazard analysis critical control points (HACCP) plan that includes the reduction of 5 Log_{10} reductions of the pertinent pathogen in the finished product (U.S. Food and Drug Administration [FDA], 2001). *Escherichia coli* O157:H7, *Salmonella enterica* and *Listeria monocytogenes*, have been identified as pertinent bacterial pathogens for juice safety due to their historical association with outbreaks derived from unpasteurized juice consumption as well as the possibility of these pathogens to be involved in future outbreaks (Gabriel & Nakano, 2009).

Currently, the primary industrial intervention for improving the microbiological safety of juice is thermal pasteurization; however, heat treatment results in detrimental changes in the organoleptic and nutritional properties of the product. In order to retain the quality of fresh juice that consumers demand, considerable efforts have been directed toward the development of novel non-thermal processes. UV technology is one of the most attractive ones due to its multiple advantages. Among these is UV light's ability to inactivate a wide range of pathogenic and spoilage microorganisms in juices (Gabriel, 2012; Müller, Stahl, Graef, Franz, & Huch, 2011), thus minimizing the loss of nutritional and sensorial quality (Caminiti et al., 2012). The germicidal properties of UV light are due to deoxyribonucleic acid's (DNA's) absorption of UV photons, especially at 200–280 nm (UV-C), which results in cross-linking between two neighboring pyrimidine nucleoside bases of the same DNA strand, thus blocking DNA transcription

69 and replication, and eventually causing cell death (López-Malo & Palou, 2005).
70 Moreover, it does not generate chemical residues or toxic compounds (Guerrero-Beltrán
71 & Barbosa-Cánovas, 2004), and it requires very little energy consumption compared
72 with other non-thermal pasteurization processes (Geveke, 2005). In fact, the National
73 Advisory Committee on Microbiological Criteria for Foods (NACMCF) revised the
74 definition of "pasteurization" and included UV radiation as an alternative to heat for
75 pasteurization purposes (National Advisory Committee on Microbiological Criteria for
76 Foods [NACMCF], 2006).
77 Nevertheless, current possibilities of UV technology in the juice industry are still
78 limited. Color compounds and suspended particles in juice reduce UV light's
79 penetration depth into juices, thus hindering UV photons from reaching
80 microorganisms. Consequently, extremely high UV doses and therefore exposure times
81 are needed to achieve the U.S. FDA requirement, which are impractical for industrial
82 purposes (Wright, Sumner, Hackney, Pierson, & Zoecklein, 2000). One promising
83 alternative is to combine UV light with mild conventional preservation methods or with
84 other non-thermal technologies that allow for an equivalent level of or even higher
85 microbial inactivation to be achieved using lower UV doses. Previously, we
86 demonstrated that the lethal effect of UV-C treatments increased synergistically at
87 temperatures between 50 ºC and 60 ºC (Gayán, Monfort, Álvarez, & Condón, 2011;
88 Gayán, Serrano, Raso, Álvarez, & Condón, 2012a). Moreover, a combined UV and mild
89 heat (UV-H) treatment (27.10 J/L for 3.58 min at 55 ºC) was designed to inactivate 5
90 Log_{10} reductions of a cocktail of *E. coli* strains in apple juice (Gayán et al., 2012a).
91 In order to use UV-H technology for juice pasteurization, it is necessary to identify the
92 most resistant pathogenic microorganism of public health concern, to evaluate the effect
93 of the most important factors on the lethality of the process, to describe the inactivation

kinetics in a wide range of treatment conditions, and to develop a mathematical model that enables one to define the most suitable treatments for achieving 5 Log_{10} reductions of the reference microorganism(s) (process criteria). Several authors have reported the variability of UV resistance among different bacterial species and strains in juice (Gabriel & Nakano, 2009; Oteiza, Giannuzzi, & Zaritzky, 2010). However, no one has evaluated the thermo-dependence of UV inactivation of different pathogens, which may condition the target microorganism of interest for UV-H treatments at different processing conditions. Moreover, the knowledge of the effect of temperature on the UV inactivation kinetics of reference pathogens is needed in order to establish process criteria as well as to understand the mechanisms involved in microbial UV-H inactivation.

This investigation's objective is first to assess the influence of the treatment temperature on the UV inactivation of UV-tolerant strains of *E. coli* (STCC 4201), *Salmonella enterica* subsp. *enterica* serovar Typhimurium (STCC 878), and *L. monocytogenes* (STCC 5672) in apple juice (Gayán, Serrano, Álvarez, & Condón, 2012b). *Staphylococcus aureus* (STCC 4465) is included due to its possible implication in juice poisoning outbreaks (Baird-Parker, 2000) and its recent detection at considerable levels in fresh-squeezed juice (Piló et al., 2009; Sospedra, Rubert, Soriano, & Mañes, 2012). Subsequently, predictive equations for describing the UV-H inactivation of these microorganisms have been developed in order to finally identify possible target microorganisms for UV-H treatments at different treatment conditions and to establish process criteria.

2. Materials and methods

2.1. Bacterial culture and media

The strains of *E. coli* STCC 4201, *Salmonella* Typhimurium STCC 878, *L. monocytogenes* STCC 5672, and *S. aureus* STCC 4465 came from the Spanish Type Culture Collection (STCC). The bacterial cultures were maintained frozen at –80 ºC in cryovials. A broth subculture was prepared by inoculating 10 ml of tryptone soy broth (Biolife, Milan, Italy) supplemented with 0.6% (w/v) yeast extract (Biolife) (TSBYE) with a loopful of growth from tryptone soy agar (Biolife) supplemented with 0.6% (w/v) yeast extract (TSAYE). The subculture was incubated at 35 ºC for 6–12 h in a shaking incubator (150 rpm; Heidolph Instruments, Vibramax 100, Scwabach, Germany). With these subcultures, 250 ml flasks that contained 50 ml of TSBYE were inoculated to reach a concentration of 10^4 CFU/ml, and they were incubated for 24 h under the same conditions until the stationary growth phase was reached ($2–5\times10^9$ CFU/ml).

2.2. UV equipment and treatments

UV treatments were carried out, as Gayán et al. (2011) previously described, in a unit with 8 individual annular thin film flow-through reactors connected in series and equipped with a feed tank and a peristaltic pump (ISM 10785, Ismatec, Glattbrugg, Switzerland). Each reactor included a low-pressure mercury vapor lamp (8 W of input power; model TUV 8WT5, Philips, USA), which converted 30% of input power as UV-C radiation (Philips Electronics, 2012), thus emitting 85% of UV-C energy at 254 nm. The lamp was attached to the axis of an outer glass tube (25 mm of inner diameter), and it was enclosed using a quartz tube (20 mm of outer diameter) in order 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 so as to improve the flow's turbulence. Outside and inside coil diameters of the spring were 23 mm and 25 mm, respectively, and its length and pitch were 270 mm and 10 mm, respectively. A manual sampling valve was situated in the outlet of each reactor. The entire unit was submerged in a 90 l water bath (25.0–60.0 ºC) heated by the circulating water of a peripheral thermostatic bath (Kattebad K12, Huber, Offenburg, Germany). The equipment also included a heating/cooling coil exchanger before the inlet of the first reactor. Thermocouples (ZA 020-FS, Almeco, Bernburg, Germany) that were fitted to the inlet and outlet of the first and last reactor, respectively, allowed for treatment temperature control.

Apple juice (Antonio Muñoz y Cia, Murcia, Spain) used as a treatment medium (absorption coefficient = 24.0 ± 2.5 cm^{-1}, turbidity = 7.4 ± 2.5 NTU, pH = 3.4 ± 0.1) was purchased locally (Zaragoza, Spain). Juice's absorption coefficient was measured spectrophotometrically (254 nm; UV500, Unicam Limited, Cambridge, UK). Samples were diluted and evaluated using quartz cuvettes (Hellma, Müllheim, Germany), with path lengths of 1 mm, 2 mm, and 10 mm. The absorption coefficient of the diluted samples was determined from the slope of the absorbance versus the path length and correcting by the dilution factor. Turbidity was measured with a nephelometer (HI 83749, Hanna Instrument, Szeged, Hungary). pH was measured using a pH meter Basic 20 (Crison Instrument, Barcelona, Spain). Apple juice was inoculated with the bacterial suspension in order to achieve $1–5\times10^{7}$ CFU/ml and was pumped at 8.5 l/h through the heat exchanger and UV reactors. When the flow rate stabilized at its preset value, 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.

169 **2.3. Heat treatments**

170 Heat treatments were carried out in specially designed thermoresistometer TR-SC
171 (Condón, Arrizubieta, & Sala, 1993). Briefly, this instrument consisted of a 400 ml
172 vessel with an electrical heater for thermostation, an agitation device used to ensure
173 inoculum distribution and temperature homogeneity, a pressurization system, and ports
174 for injecting the microbial suspension and for extraction of samples. Once the preset
175 temperature had attained stability (T ± 0.05 °C), 0.2 ml of an adequately diluted
176 microbial cell suspension was inoculated into the vessel that contained350 ml of apple
177 juice. After inoculation, 0.2 ml samples were collected at different heating times and
178 were immediately pour-plated.

179

180 **2.4. Incubation of treated samples and survival counting**

181 TSAYE was used as a recovery medium, and plates were incubated at 35 ºC for 24 h
182 and 48 h for *L. monocytogenes*. After incubation, colony forming units (CFU) were
183 counted using an improved Image Analyzer Automatic Colony Counter (Protos,
184 Synoptics, Cambridge, UK), as described elsewhere (Condón, Palop, Raso, & Sala,
185 1996).

186

187 **2.5. Curve fitting and dose calculation**

188 Survival curves were obtained by plotting the logarithm of the survival fraction versus
189 UV dose (*d*) expressed in joules per milliliter and time (*t*) expressed in minutes for UV
190 and heat treatments, respectively. To compare UV-H treatments with thermal
191 treatments, UV-H survival curves were also expressed in treatment time. The UV dose
192 delivered to the treatment medium was estimated with a chemical dosimeter. To this
193 end, the iodide-iodate actinometer (quantum yield = 0.73 ± 0.02) was used following

194 the indications of Rahn, Stefan, Bolton, Goren, et al. (2003). The actinometer buffer
195 was pumped through the installation at 8.5 L/h, and the increase in absorbance (352 nm)
196 was determined at the outlet of each reactor (Gayán et al. 2011). From this data, the
197 photon flux (254 nm) that each volume fraction of the treatment medium received per
198 second was estimated according to Montalti, Credi, Prodi, and Gandolfi (2006). Thus,
199 considering the energy of a photon at 254 nm, the UV dose delivered in each reactor
200 was 0.49 J/mL.
201 To fit survival curves and to calculate resistance parameters, the GInaFiT inactivation
202 model-fitting tool was used (Geeraerd, Valdramidis, & Van Impe, 2005). Because most
203 survival curves did not show tails but rather showed shoulders, the log-linear regression
204 plus shoulder model from Geeraerd el al. (2000) was used (Equation 1). This model
205 describes the survival curves through two parameters: the shoulder length (*Sl*), defined
206 as dose or time before the exponential inactivation begins; and the inactivation rate
207 (K_{max}), defined as the slope of the exponential portion of the survival curve. N_0 and N_t
208 represent the initial number of the microbial population and the number of
209 microorganisms that survive at the end of the treatment time (t), respectively.

210 $$N_t = N_0 e^{-K_{max} Sl} \left(\frac{e^{K_{max} Sl}}{1+\left(e^{-K_{max} Sl}-1\right)e^{K_{max} t}} \right) \quad (1)$$

211 To describe the relationship between treatment temperature (T) and *Sl* and K_{max}
212 parameters, mathematical equations based on the Weibull distribution were chosen. For
213 *Sl*, the equation that Albert and Mafart (2005) (Equation 2) introduced was used as a
214 secondary model, whereas the thermo-dependence of K_{max} was described using the
215 mirror image of the Mafart, Couvert, Gaillard, and Leguerinel (2002) model (Equation
216 3):

217 $$Sl_T = (Sl_0 - Sl_{res}) 10^{-\left(\frac{t}{\delta}\right)^p} + Sl_{res} \quad (2)$$

$$K_{maxT} = -\left[K_{max_0} 10^{-\left(\frac{T}{\delta}\right)^p}\right] \quad (3)$$

where Sl_0 and K_{max0} are the shoulder length and the inactivation rate of the survival curves of UV treatments at room temperature, respectively; Sl_T and K_{maxT}, the shoulder length and the inactivation rate of UV-H treatments at temperature T, respectively; and Sl_{res}, the residual shoulder when the treatment temperature was increased. δ and p are, respectively, the scale and shape parameters. The δ value represents the temperature increase necessary to decrease 10-fold Sl or K_{max}. The p parameter ($p > 1$) accounts for the profile of the downward concavity of curves (Albert & Mafart, 2005; Mafart et al., 2002).

For heat survival curves, which showed an initial shoulder phase, Geeraerd et al. model's was also used as a primary model. To study the relationship between the inactivation model parameters and the treatment temperature, simple log-linear equations were used, considering as model parameters the slope and the intercept of the regression line.

In order to determine the goodness of fits of primary and secondary models as well as the accuracy of the final equations, the coefficient of determination (R^2), the root mean square error (RMSE), the bias (B_f), and the accuracy factors (A_f) were used (Baranyi, Pin, & Ross, 1999). The bias factor indicates by how much, on average, a model overpredicts ($B_f > 1$) or underpredicts ($B_f < 1$) the observed data. On the other hand, the accuracy factor indicates how much of the estimated data differ from the observed ones.

2.6. Statistical analyses

Statistical analyses, *t*-test and ANOVA tests, were carried out using the GraphPad PRISM 5.0 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

3.1. Microbial inactivation of combined UV-H treatments at different temperatures in apple juice

Figure 1 illustrates survival curves of *E. coli* (STCC 4201)*, Salmonella* Typhimurium (STCC 878), *L. monocytogenes* (STCC 5672), and *S. aureus* (STCC 4465) to UV treatment at room temperature (A) and to combined UV-H treatments at 50.0 ºC (B), 52.5 ºC (C), 55.0 ºC (D), 57.5 ºC (E), and 60.0 ºC (F) in apple juice. Most inactivation curves showed an initial lag phase followed by a logarithmic order of death, but no tailing was observed. To describe UV-H inactivation kinetics, the log-linear regression plus the shoulder model of Geeraerd et al. (2000) (Eq. 1) was used because it allowed for the length of the shoulders and the log-linear rate of inactivation to be described accurately and independently. Table 1 includes the averages and the standard deviations of the model parameters (K_{max} and *Sl*) in the time term obtained from the fitting of UV-H survival curves of all microorganisms tested at different temperatures. The coefficient of determination (R^2) and the root mean square error (RMSE) values are also included to illustrate the goodness of the fits.

As observed in Figure 1A, when the maximum UV dose possible with a single pass through our installation (3.92 J/mL) was applied, UV treatments at room temperature decreased 0.96 ± 0.16, 0.94 ± 0.06, 0.86 ± 0.44, 1.57 ± 0.07 Log_{10} cycles the initial population of *E. coli, Salmonella* Typhimurium, *L. monocytogenes*, and *S. aureus*, respectively. However, UV lethality significantly augmented when the treatment

268 temperature was increased to between 50.0 ºC and 60.0 ºC (Figure 1): The inactivation
269 of all microorganisms at 50.0 ºC slightly improved compared with the UV lethality at
270 room temperature, but above this temperature, it dramatically increased. For instance,
271 the inactivation of *L. monocytogenes* with a UV treatment of 2.45 J/mL (2.23 min)
272 augmented from 0.38 ± 0.33 Log_{10} cycles at 25.0 to 1.09 ± 0.51 Log_{10} cycles at 50.0 ºC.
273 The same treatment (2.45 J/mL) at 52.5 ºC, 55.0 ºC, 57.5 ºC, and 60.0 °C increased the
274 UV inactivation up to 1.47 ± 0.26, 2.46 ± 0.27, 5.20 ± 0.13, and more than 6 Log_{10}
275 cycles, respectively. The UV lethality improvement when the treatment temperature was
276 raised stemmed from the decrease of the shoulder phase (*Sl*) of UV-H survival curves
277 until it disappeared (Table 1). In addition, the slope of the survival curves (K_{max})
278 increased with temperature. Furthermore, the UV resistance variability between species
279 increased when the treatment temperature was increased. In the most extreme case,
280 when applying a dose of 0.98 J/mL (0.90 min), UV-H inactivation at 57.5 ºC ranged
281 from 1.01 ± 0.04 for *L. monocytogenes* to more than 6 Log_{10} cycles for *S. aureus*
282 (Figure 1E). Moreover, the kinetic profile of survival curves changed with temperature,
283 and this behavior was different for each microorganism. For instance, the shoulder
284 phase of UV-H survival curves of *S. aureus* and *Salmonella* Typhimurium disappeared
285 at around 57.5 ºC, while that of *E. coli* vanished at 60 ºC and that of *L. monocytogenes*
286 tended to become null even at the highest temperatures tested (Table 1). These results
287 indicate that the thermo-dependence of UV lethality is different for each
288 microorganism.
289 Subsequently, the secondary level of modelling used to describe the effect of
290 temperature on the kinetic parameters of Geeraerd et al.'s equation (primary model) was
291 carried out. Figure 2 shows the relationships between treatment temperature and *Sl* and
292 K_{max} parameters obtained from the fit of the primary model to UV-H survival curves of

293 *E. coli*, *Salmonella* Typhimurium, *S. aureus,* and *L. monocytogenes* in apple juice,
294 shown in Table 1. Mathematical equations based on the Weibull distribution, Albert and
295 Mafart's equation (Eq. 2) for *Sl* and Mafart et al.'s equation (Eq. 3) for K_{max}, were used
296 to describe the thermo-dependence of both parameters (secondary models). Table 2
297 compiles obtained parameters from the secondary models of *Sl* and K_{max} for each
298 microorganism (δ, *p*, Sl_0, Sl_{res}, and K_{max0}), previously defined in the section 2.5,
299 including the R^2 and RMSE values from the fits. Regarding the shoulder phase (Figure
300 2A), the relationship between the *Sl* and temperature displayed a sigmoid profile in all
301 microorganisms, firstly showing a lag phase and then dropping off to zero. The shoulder
302 phase of UV-H survival curves of *L. monocytogenes* was higher than that of the other
303 species at all treatment temperatures tested, especially at temperatures ranging from 25
304 ºC to 50.0 ºC, and above this value, differences were reduced until the shoulder length
305 became null. When the K_{max} parameter was evaluated against the temperature, concave
306 upward curves were observed (Figure 2B). Apparently, the inactivation rate of *E. coli*
307 and *Salmonella* Typhimurium was more sensitive to temperature changes than that of *S.*
308 *aureus* and *L. monocytogenes.* This behavior was also evidenced in the shape
309 parameters (*p*) of K_{max} secondary models, which determine the profile of the upward
310 concavity of the curves (Table 2). *p* values for *L. monocytogenes* and *S. aureus* were
311 significantly lower ($p \leq 0.05$) than those obtained for Gram-negative bacteria.
312 Tertiary models used to describe the microbial inactivation of *E. coli*, *Salmonella*
313 Typhimurium, *L. monocytogenes*, and *S. aureus* by UV-H treatments in apple juice
314 were developed by including in Geeraerd et al.'s equation (primary model; Eq. 1) the
315 secondary models for *Sl* and K_{max} values obtained (eqs. 2 and 3). Plots of the observed
316 and estimated data with the tertiary models for each microorganism are given in Figure
317 3. The difference between a point of the graph and the line of equivalence is a measure

318 of the accuracy of the corresponding estimation. The R^2, RMSE, accuracy (A_f), and bias
319 (B_f) factors from each prediction are also indicated in the figures. In general, the tertiary
320 models accurately predicted the UV-H inactivation of all microorganisms.
321 Tertiary models were used to compare the UV-H resistance of the investigated
322 microorganisms at different temperatures. Thus, Figure 4 shows the logarithm of the
323 treatment time and UV doses necessary to inactivate 5 Log_{10} cycles of *E. coli*,
324 *Salmonella* Typhimurium, *L. monocytogenes,* and *S. aureus* at temperatures between 25
325 ºC to 60.0 ºC in apple juice. UV doses were calculated from the existing relationship
326 between treatment time and UV dose of our equipment (data not shown). For
327 comparisons, thermal death time (TDT) curves to reach 5 Log_{10} reductions of each
328 microorganism in the same apple juice by heat treatments, obtained as detailed below,
329 have been included in the figure. As observed, the microbial inactivation of all species
330 increased with temperature, following a concave downward profile, so that the UV
331 lethality significantly improved at temperatures above 50 ºC. The most resistant
332 microorganism varied with the treatment temperature in a similar way that the thermo-
333 dependence of K_{max} of UV-H survival curves did (Figure 2 and Table 2). *L.*
334 *monocytogenes* was the most UV-H resistant microorganism at temperatures between
335 25 ºC to 44 ºC, achieving the 5 Log_{10} reductions with a UV dose ranging from 19.24
336 J/mL to 13.74 J/ml (for 12.63 min). *E. coli* became the target microorganism in the
337 temperature range from 44 ºC to 54 ºC, requiring a dose between 13.81 J/mL (12.71
338 min) and 5.20 J/mL (4.78 min), respectively, to meet the juice food safety goal. At
339 higher temperatures, *L. monocytogenes* was again the target microorganism, requiring a
340 UV dose from 5.20 J/mL (4.78 min) to 2.11 J/mL (1.93 min) between 54 ºC and 60 ºC.
341

342 **3.2. Heat resistance of *E. coli, Salmonella* Typhimurium, *L. monocytogenes*, and *S.***
343 ***aureus* in apple juice**
344 To evaluate the contribution of thermal effects on the lethality of combined UV-H
345 treatments, heat resistance characterization of *E. coli, Salmonella* Typhimurium, *L.*
346 *monocytogenes,* and *S. aureus* in apple juice was conducted. Inactivation curves did not
347 follow first-order kinetics, but shoulders were observed. Survival curves were fitted to
348 Geeraerd et al.'s model (primary model; Eq. 1), and heat resistance parameters (*Sl* and
349 K_{max}) are included in Table 3. The relationships between the treatment temperature and
350 the Log_{10} *Sl* and Log_{10} K_{max} for *E. coli, Salmonella* Typhimurium, *L. monocytogenes,*
351 and *S. aureus* are shown in Figure 5. As observed, *Sl* values decreased, and K_{max} values
352 increased log-linearly with temperature, following different patterns in each species.
353 The parameters (slope and intercept) of the linear regressions (secondary models) for
354 each microorganism are compiled in Table 4. As shown in Figure 5A, the shoulder
355 phase decline with temperature changes of *E. coli*, *Salmonella* Typhimurium, and *S.*
356 *aureus* was similar, whereas the shoulder length of *L. monocytogenes* decreased more
357 rapidly. Concerning the relationships of the inactivation rate, K_{max} values of *E. coli* and
358 *Salmonella* Typhimurium showed the same thermo-dependence ($p > 0.05$), but it was
359 rather higher than those of both Gram-positive microorganisms and primarily for *L.*
360 *monocytogenes* (Table 4). This means that the velocity of death of *L. monocytogenes*
361 was affected to a less extent by temperature changes than that of *E. coli* and *Salmonella*
362 Typhimurium.
363 Including the obtained secondary models for *Sl* and K_{max} in Geeraerd et al.´s equation
364 (primary model), tertiary models were obtained to predict the heat inactivation in apple
365 juice for each microorganism. The validation analyses of the obtained models showed
366 that there were good agreements between experimental and predicted data. R^2 values

ranged from 0.924 to 0.989; RMSE, from 0.187 to 0.353; A_f, from 1.251 to 1.476; and B_f, from 0.920 to 1.315. From tertiary models, the time required to achieve 5 Log_{10} reductions of each microorganism in apple juice by heat treatments at temperatures between 50 ºC to 62.5 ºC were calculated, and TDT curves were included in Figure 4. All TDT curves showed a log-linear profile from which was deduced *z* values (temperature increase for reducing 10-fold the treatment time) of 5.1 ºC, 5.1 ºC, 7.3 ºC, and 7.0 ºC for *E. coli, Salmonella* Typhimurium, *L. monocytogenes*, and *S. aureus*, respectively. These values are in the range of those that other authors obtained for fruit juices (Álvarez-Ordónez, Fernández, Bernardo, & López, 2009; Doyle & Mazzotta, 2000; Mazzotta, 2001). Accordingly, *E. coli* was the most heat-resistant bacterium when the temperature was increased to about 54 ºC, temperature at which the TDT curves of *E. coli* and *L. monocytogenes* intersected. Above this temperature *L. monocytogenes* became the most thermo-tolerant microorganism.

4. Discussion

In this investigation, the thermo-dependence of the UV inactivation of pathogenic bacteria of concern in apple juice was studied in order to establish the UV-H treatment conditions (process criteria) for obtaining a safe product. For this purpose, the effect of temperature on the UV lethality of UV tolerant strains of *Salmonella* Typhimurium (STCC 878), *L. monocytogenes* (STCC 5672), and *S. aureus* (STCC 4465) together with data previously obtained for *E. coli* (STCC 4201) was assessed. UV-H inactivation curves displayed shoulder phases, which are often observed in survival curves to UV-C light (Quintero-Ramos, Churey, Hartman, Barnard, & Worobo, 2004; Unluturk, Atilgan, Baysal, & Unluturk, 2010). According to the "multi-hit target theory," shoulders are related to DNA damage and repair phenomena (Jagger, 1967). DNA

392 repair systems can repair damage up to certain UV doses, resulting in shoulders. Once
393 the maximum DNA repair capability is surpassed, additional UV exposure is lethal for
394 microorganisms, and survivors exponentially decline (López-Malo & Palou, 2005).
395 When the maximum UV dose possible in one pass (3.92 J/mL) was applied, UV
396 treatments at room temperature (25 ºC) hardly decreased the microbial population of *E.*
397 *coli*, *Salmonella* Typhimurium, *L. monocytogenes,* and *S. aureus* (Figure 1A), which
398 can be attributed to the low UV transmittance of the apple juice (absorption coefficient
399 = 24.0 ± 2.5 cm^{-1}). The resistance variability observed among species was scarce due to
400 the low microbial inactivation achieved by UV light alone. Nevertheless, it can be
401 deduced that *L. monocytogenes* was the most UV-resistant microorganism and *S. aureus*
402 the most sensitive one, data that agree with those previously reported in laboratory
403 media (Gayán et al., 2012b). In general, it is believed that Gram-positive bacteria are
404 more UV-resistant than Gram-negatives are, which is attributed to the thicker
405 peptidoglycan cell wall of the formers (Beauchamp & Lacroix, 2012; Lu, Li, and Liu,
406 2011). The greater UV sensitivity of *S. aureus* compared with the two Gram-negative
407 bacteria tested demonstrates that this statement is not a general rule. In fact, other
408 authors have reported the higher susceptibility of *S. aureus* to UV technologies
409 compared with coliforms (Chang, et al., 1985; Hijnen, Beerendonk, & Medema, 2006).
410 The UV inactivation of all investigated microorganisms in apple juice considerably
411 improved when the treatment temperature was raised between 50.0 ºC and 60.0 ºC
412 (Figure 1). For instance, the UV inactivation of the most resistant microorganism, *L.*
413 *monocytogenes,* with a dose of 2.45 J/mL (2.23 min) increased 0.71, 1.09, 2.08, 4.82,
414 and more than 5.84 Log_{10} cycles at 50.0 ºC, 52.5 ºC, 55.0 ºC, 57.5, and 60.0 ºC,
415 respectively, compared with the same experiments carried out at room temperature.
416 These results indicate that combining UV light with mild heat increased the UV

417 inactivation of microorganisms of public health concern in apple juice, thereby
418 alleviating the problem of the low penetration depth of UV light into this product. This
419 fact opens the possibility of designing a feasible UV-H pasteurization process for juice.
420 However, to transfer UV-H technology to the food industry it is necessary for
421 identifying the target pathogen(s) and treatment conditions for achieving 5 Log_{10}
422 reductions as the U.S. FDA guidelines demand (U.S. FDA, 2001). The different
423 behavior of *E. coli*, *Salmonella* Typhimurium, *L. monocytogenes,* and *S. aureus* in
424 response to UV-H treatments in relation to the treatment temperature makes it difficult
425 to compare data and to establish the target microorganism for this technology in
426 different processing conditions. Therefore, it was essential to develop mathematical
427 models that enabled one to evaluate the effect of temperature on the UV lethality in
428 apple juice for each investigated microorganism.
429 Previously, we demonstrated that the improvement of UV-H inactivation was due to the
430 occurrence of a synergistic lethal effect and that the magnitude of such effect increased
431 when the treatment temperature was raised up to a threshold temperature (Gayán et al.,
432 2011; Gayán et al., 2012a). Above this temperature, thermal lethal effects began to
433 predominate on UV lethality, and UV-H synergism was reduced until disappearing so
434 that microbial death was exclusively due to heat. Thus, in the case of *E. coli* STCC
435 4201, the maximum UV-H synergism (38.1%) in the same apple juice used in this
436 investigation was observed at a treatment temperature of about 55 ºC, and above this
437 temperature, the synergism decreased until disappearing at 60.0 ºC, when UV-H and
438 heat survival curves overlapped (Gayán et al., 2012a). Therefore, to take advantage of
439 combined UV-H treatment, treatment temperature should be limited to temperatures
440 below the intersection of UV-H and heat lethality. This requires knowledge of heat
441 resistance of target microorganisms and its thermo-dependence.

442 Mathematical models used to predict the UV-H and heat inactivation of *E. coli*,
443 *Salmonella* Typhimurium, *L. monocytogenes,* and *S. aureus* in apple juice were
444 developed. These models were based on the log-linear regression plus shoulder equation
445 of Geeraerd et al. (2000), which allowed for the effect of temperature on the shoulder
446 length and the inactivation rate to be assessed independently. The models that were
447 developed were able to accurately predict the treatment time (and UV dose) needed to
448 achieve 5 Log_{10} reductions of the most resistant pathogens in apple juice by both UV-H
449 and heat treatments (Figure 4). As expected, the heat resistance of all microorganisms
450 increased log-linearly with temperature but showed two different tendencies: Gram-
451 negative species were more thermo-dependent than the two Gram-positive bacteria.
452 This behavior was related to the interaction between temperature and the inactivation
453 rate of heat treatments of each microorganism as described in Figure 5A and Table 4.
454 Hence, *E. coli* was the most resistant microorganism to heat at temperatures that were
455 lower than about 54 ºC, but above this temperature, *L. monocytogenes* became the
456 reference microorganism.
457 On contrary to heat treatments, the UV-H inactivation of all microorganisms increased,
458 with treatment temperature following a concave downward profile, evidencing that the
459 major advantages of the combined treatment occurred at temperatures above
460 approximately 50 ºC (Figure 4). However, the thermo-dependence of UV lethality
461 differed in each microorganism, and consequently, the target microorganism for UV-H
462 technology varied with the treatment temperature: *E. coli* was the most resistant
463 microorganism at temperatures between 50 ºC and 54 ºC, and *L. monocytogenes* was at
464 higher temperatures. This behavior was determined based on the thermo-dependence of
465 the inactivation rate of the UV-H lethality of each microorganism (Figure 2B).
466 Moreover, the inflexion temperature that determines the target microorganism

467 approximately corresponded to the intersection point of the TDT curves of *E. coli* and
468 *L. monocytogenes* (Figure 4).
469 In conclusion, when heat contributes to the UV-H inactivation improvement, the
470 velocity of death of UV-H treatments is related to the heat resistance of the
471 microorganism. Therefore, it is the heat resistance of pathogens of concern that
472 conditions the target microorganism of UV-H treatments. Thus, although *L.*
473 *monocytogenes* is the most resistant microorganism to UV light, *E. coli* proves to be the
474 target pathogen of UV-H treatments between 50 ºC and 54 ºC due to its higher heat
475 tolerance in comparison with *L. monocytogenes* at these temperatures, and despite the
476 fact that the UV resistance of *E. coli* is lower than that of *L. monocytogenes* and even
477 *Salmonella* Typhimurium.
478 Considering the most UV-H resistant microorganisms at a different range of
479 temperatures, *E. coli* and *L. monocytogenes* as indicated, models that have been
480 developed enable one to establish the process criteria (UV dose, time, and temperature)
481 required for 5 Log_{10} reductions of the four pathogens of reference in apple juice (Figure
482 4), that is to pasteurize apple juice. From a practical point of view, it can be deduced
483 that combining UV light with mild temperatures permits achieving the microbial
484 inactivation required for a safe product with lower UV doses and treatment times than
485 those needed for UV treatments alone. Thus, the UV dose required to achieve 5 Log_{10}
486 reductions of the pertinent pathogen (*L. monocytogenes*) by UV treatment at room
487 temperature would be reduced by 49.6% at 50.0 ºC, with *E. coli* being the target
488 microorganism. A more notorious improvement can be achieved, raising the treatment
489 temperature up to 55.0 ºC, 57.5 ºC, and 60.0 ºC at which the UV dose needed to
490 inactivate 5 Log_{10} cycles of *L. monocytogenes* were reduced by 76.4%, 83.7%, and
491 89.1%, respectively. On the other hand, the time required to achieve the food safety goal

by heat treatments would be decreased by 54.4%, 36.5%, and 25.8% at 55.0 ºC, 57.5 ºC, and 60.0 ºC, respectively, which evidence the occurrence of a synergistic lethal effect. Notice in Figure 4 that from 60–62 ºC UV-H, lethality coincides with heat inactivation, and therefore, the treatment temperature of the combined UV-H treatment should not surpass this threshold temperature.

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632 **Table 1**. Resistance parameters (*Sl* and K_{max}) obtained from the fit of UV-H survival

633 curves of *E. coli*, *Salmonella* Typhimurium, *S. aureus*, and *L. monocytogenes* at

634 different temperatures in apple juice to Geeraerd et al.'s model (Eq. 1).

Microorganism	Temperature (ºC)	*Sl* (min)	K_{max} (min^{-1})	R^2	RMSE
E. coli	25.0	1.15 (0.58)^{a}	0.88 (0.15)^{a}	0.979	0.036
	50.0	0.90 (0.43)^{a}	1.42 (0.45)^{a}	0.987	0.098
	52.5	0.72 (0.24)^{ab}	1.96 (0.09)^{a}	0.975	0.187
	55.0	0.24 (0.22)^{a}	3.55 (0.68)^{a}	0.988	0.201
	57.5	0.18 (0.03)^{a}	5.50 (0.73)^{a}	0.989	0.194
	60.0	0.00^{a}	10.47 (0.84)^{a}	0.974	0.374
Salmonella Typhimurium	25.0	1.06 (0.11)^{ab}	0.86 (0.03)^{a}	0.994	0.038
	50.0	0.97 (0.09)^{a}	2.03 (0.09)^{b}	0.985	0.116
	52.5	0.75 (0.1)^{a}	2.93 (0.02)^{b}	0.988	0.158
	55.0	0.59 (0.06)^{b}	4.67 (0.62)^{b}	0.985	0.297
	57.5	0.13 (0.12)^{ab}	8.06 (1.62)^{b}	0.990	0.374
L. monocytogenes	25.0	2.16 (0.44)^{c}	0.86 (0.04)^{a}	0.985	0.032
	50.0	1.22 (0.12)^{c}	1.65 (0.13)^{a}	0.992	0.064
	52.5	0.89 (0.07)^{c}	2.01 (0.21)^{a}	0.996	0.067
	55.0	0.67 (0.05)^{b}	3.55 (0.28)^{a}	0.985	0.255
	57.5	0.38 (0.08)^{c}	4.82 (0.31)^{a}	0.993	0.145
	60.0	0.14 (0.05)^{b}	6.29 (0.26)^{b}	0.990	0.084
S. aureus	25.0	0.92 (0.14)^{b}	1.43 (0.09)^{b}	0.986	0.078
	50.0	0.76 (0.08)^{b}	2.43 (0.19)^{c}	0.993	0.095
	52.5	0.61 (0.01)^{b}	3.23 (0.14)^{c}	0.976	0.284
	55.0	0.37 (0.03)^{a}	5.51 (0.60)^{b}	0.994	0.219
	57.5	0.00^{b}	6.71 (0.39)^{ab}	0.995	0.199

Values in parentheses represent the standard deviations of the means.

Letters a, b, and c indicate statistically significant differences ($p \leq 0.05$) among *Sl* and K_{max} values of UV-H survival curves of different microorganisms at the same treatment temperature.

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637 **Table 2**. Secondary model parameters estimated after fitting the evolution of *Sl* and K_{max} values obtained from UV-H survival curves of *E. coli*,

638 *Salmonella* Typhimurium, *L. monocytogenes*, and *S. aureus* (Table 1) with the treatment temperature to Albert and Mafart's (Eq. 3) and Mafart et

639 al.'s model (Eq. 4), respectively.

	Sl secondary model						K_{max} secondary model				
Miroorganism	**δ (min)**	**p**	**Sl_0 (min)**	**Sl_{res} (min)**	**R^2**	**RMSE**	**δ (min^{-1})**	**p**	**K_{max0} (min^{-1})**	**R^2**	**RMSE**
E. coli	54.42 (4.81)[a]	12.46 (3.72)[a]	1.23 (0.21)[a]	0 (0.27)[a]	0.925	0.181	52.17 (0.37)[a]	16.08 (0.90)[a]	-0.93 (0.12)[a]	0.998	0.183
Salmonella Typhimurium	56.44 (0.13)[a]	18.60 (3.11)[a]	1.05 (0.05)[a]	0 (0.05)[a]	0.994	0.050	49.84 (0.63)[b]	13.66 (1.13)[b]	-0.96 (0.19)[a]	0.997	0.205
S. aureus	55.86 (1.37)[a]	16.47 (0.21)[a]	0.91 (0.02)[b]	0 (0.02)[a]	0.998	0.021	48.59 (2.37)[b]	10.27 (1.46)[c]	-1.31 (0.54)[a]	0.971	0.270
L. monocytogenes	49.89 (0.54)[a]	5.19 (0.38)[b]	2.19 (0.04)[c]	0 (0.06)[a]	0.982	0.133	49.20 (1.56)[b]	8.82 (1.45)[c]	-0.74 (0.32)[a]	0.970	0.219

Values in parentheses represent the standard errors of the means.

Letters a, b, and c indicate statistically significant differences ($p \leq 0.05$) among values of different microorganisms.

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643 **Table 3**. Resistance parameters (*Sl* and K_{max}) obtained from the fit of heat inactivation
644 curves of *E. coli*, *Salmonella* Typhimurium, *S. aureus*, and *L. monocytogenes* at
645 different temperatures in apple juice to Geeraerd et al.'s model (Eq. 1).

Microorganism	Temperature (ºC)	*Sl* (min)	K_{max} (min^{-1})	R^2	RMSE
E. coli	56.0	2.34 (0.56)	2.34 (0.31)	0.996	0.133
	58.0	0.74 (0.09)	4.99 (0.15)	0.999	0.103
	60.0	0.44 (0.04)	15.42 (0.58)	0.993	0.144
	62.0	0.24 (0.03)	41.37 (4.42)	0.994	0.173
Salmonella Typhimurium	54.6	1.13 (0.05)	1.84 (0.07)	0.983	0.192
	56.1	1.10 (0.04)	4.27 (0.68)	0.990	0.193
	56.6	1.16 (0.06)	5.11 (0.80)	0.996	0.102
	58.1	1.20 (0.08)	9.90 (1.20)	0.980	0.472
L. monocytogenes	55.6	2.09 (0.04)	2.03 (0.30)	0.991	0.052
	57.1	1.14 (0.05)	2.65 (0.24)	0.999	0.075
	58.1	0.65 (0.02)	3.59 (0.01)	0.990	0.194
	60.6	0.10 (0.01)	4.97 (0.35)	0.986	0.235
S. aureus	53.1	1.19 (0.10)	1.45 (0,06)	0.987	0.060
	55.1	0.66 (0.17)	3.10 (0.51)	0.993	0.154
	56.1	0.42 (0.20)	3.76 (0.31)	0.992	0.165
	58.1	0.19 (0.02)	7.42 (0.18)	0.990	0.295

Values in parentheses represent the standard deviations of the means.

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653 **Table 4**. Secondary model parameters estimated from the log-linear regressions
654 between temperature and the *Sl* and K_{max} values obtained from heat survival curves of
655 *E. coli*, *Salmonella* Typhimurium, *L. monocytogenes*, and *S. aureus* in apple juice
656 (Table 3).

	Sl secondary model				K_{max} secondary model			
Miroorganism	**Slope (min^{-1})**	**Intercept (min)**	**R^2**	**RMSE**	**Slope (min^{-1})**	**Intercept (min)**	**R^2**	**RMSE**
E. coli	-0.157 (0.019)[a]	9.068 (1.101)[a]	0.970	0.194	0.212 (0.011)[a]	-11.54 (0.655)[a]	0.999	0.395
Salmonella Typhimurium	-0.157 (0.007)[a]	8.701 (0.399)[a]	0.999	0.205	0.208 (0.014)[a]	-11.05 (0.767)[a]	0.995	0.349
L. monocytogenes	-0.261 (0.013)[b]	14.87 (0.765)[b]	0.998	0.057	0.078 (0.010)[c]	-4.031 (0.560)[c]	0.996	0.152
S. aureus	-0.155 (0.012)[a]	8.294 (0.675)[a]	0.991	0.080	0.140 (0.009)[b]	-7.240 (0.479)[b]	0.996	0.151

Values in parentheses represent the standard errors of the means.

Letters a, b, and c indicate statistically significant differences ($p \leq 0.05$) among values of different microorganisms.

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668 **Figure captions**

669 **Figure 1**. Survival curves of *E. coli* (■), *Salmonella* Typhimurium (▲), *L.*
670 *monocytogenes* (●), and *S. aureus* (X) to UV treatment at different temperatures —25.0
671 (A), 50.0 (B), 52.5 (C), 55.0 (D), 57.5 (E), and 60.0 ºC (F)—in apple juice.
672 **Figure 2**. Relationships between temperature and the *Sl* (A) and K_{max} (B) parameters
673 obtained from the fit of UV-H survival curves of *E. coli* (■), *Salmonella* Typhimurium
674 (▲), *L. monocytogenes* (●), and *S. aureus* (X) in apple juice to Geeraerd et al.'s model
675 (Eq. 1) (Table 1). Curves obtained from the fits of the evolution of *Sl* and K_{max} with
676 temperature to Albert and Mafart's (Eq. 2) and Mafart et al.'s model (Eq. 3),
677 respectively, are also included—*E. coli* (—), *Salmonella* Typhimurium (·····), *L.*
678 *monocytogenes* (- - -), and *S. aureus* (- · -).
679 **Figure 3.** Correlation between observed and estimated data obtained with the tertiary
680 models for *E. coli* (A), *Salmonella* Typhimurium (B), *S. aureus* (C), and *L.*
681 *monocytogenes* (D) when treated by UV-H process. The R^2, RMSE, accuracy (A_f) and
682 bias (B_f) factors from each prediction are also indicated in the figures.
683 **Figure 4.** Log_{10} time and UV dose required to achieve 5 Log_{10} reductions by UV-H and
684 heat (H) treatments at different temperatures of *E. coli* (—), *Salmonella* Typhimurium (···
685), *L. monocytogenes* (- - -), and *S. aureus* (- · -) in apple juice
686 **Figure 5**. Relationships between treatment temperature and Log_{10} *Sl* (A) and K_{max} (B)
687 parameters obtained from the fit of heat survival curves of *E. coli* (■), *Salmonella*
688 Typhimurium (▲), *S. aureus* (X), and *L. monocytogenes* (●) in apple juice to the
689 Geeraerd et al.'s model (Eq. 1) (Table 3). Linear regressions are also included—*E. coli* (—
690), *Salmonella* Typhimurium (·····), *L. monocytogenes* (- - -), and *S. aureus* (- · -).

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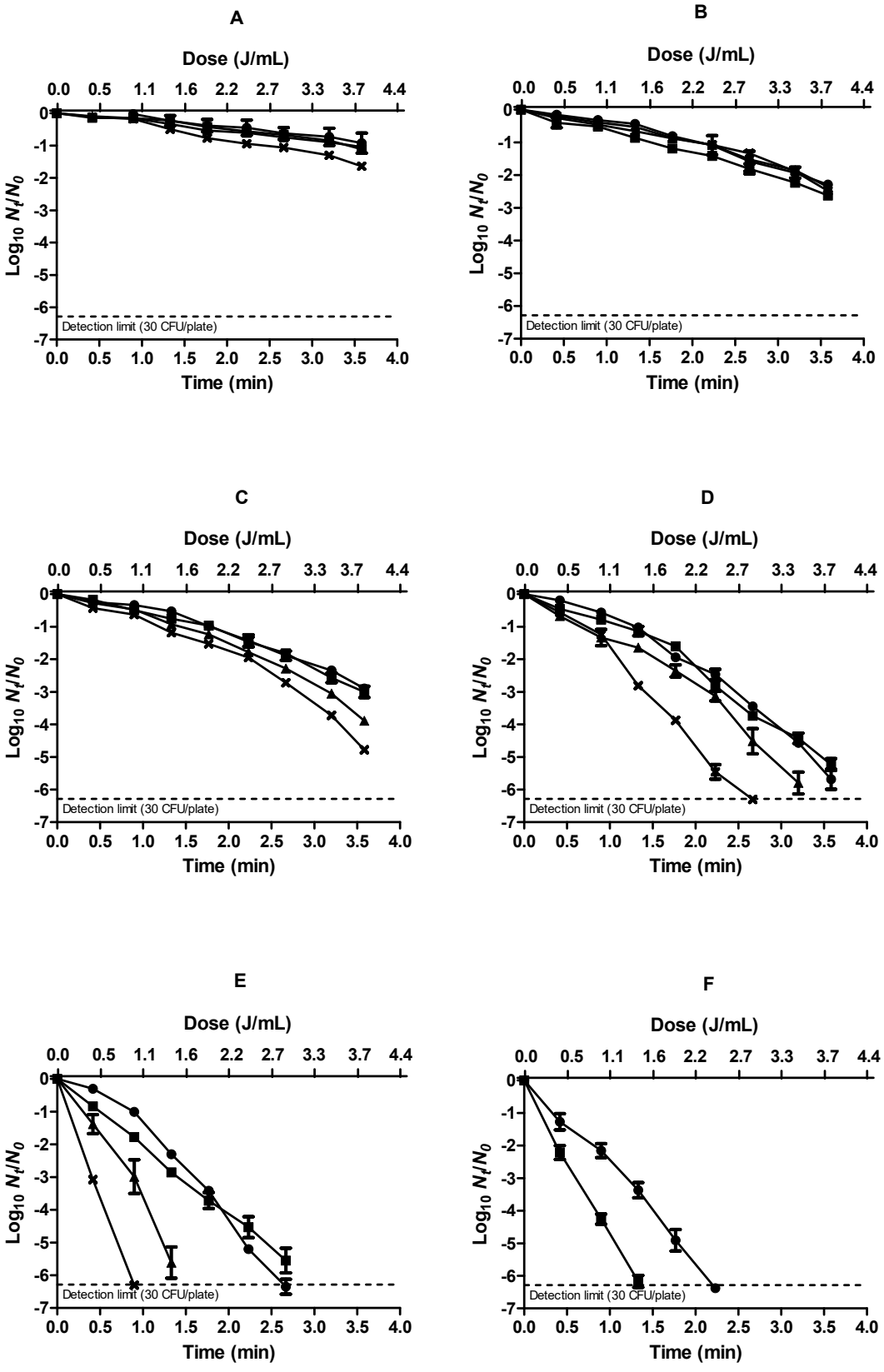
692 **Figure 1**



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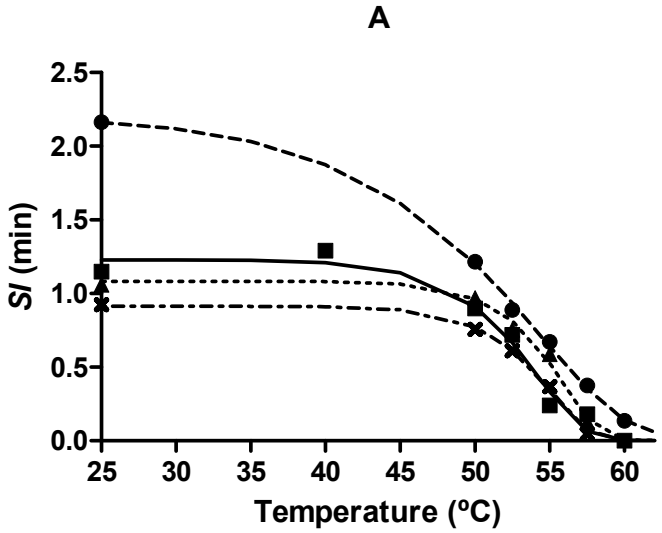
694 **Figure 2**



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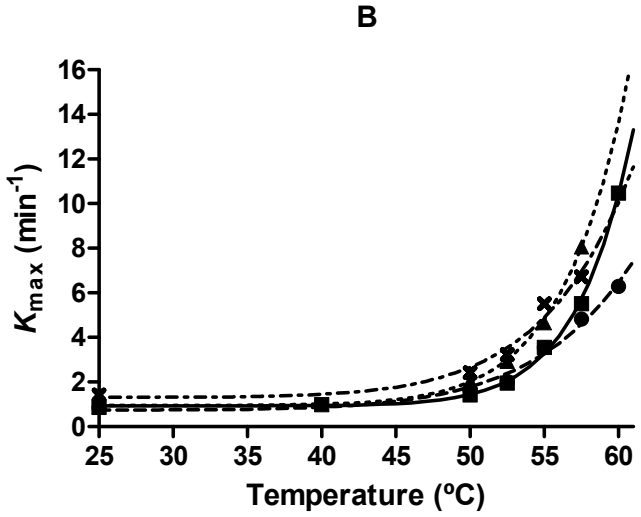

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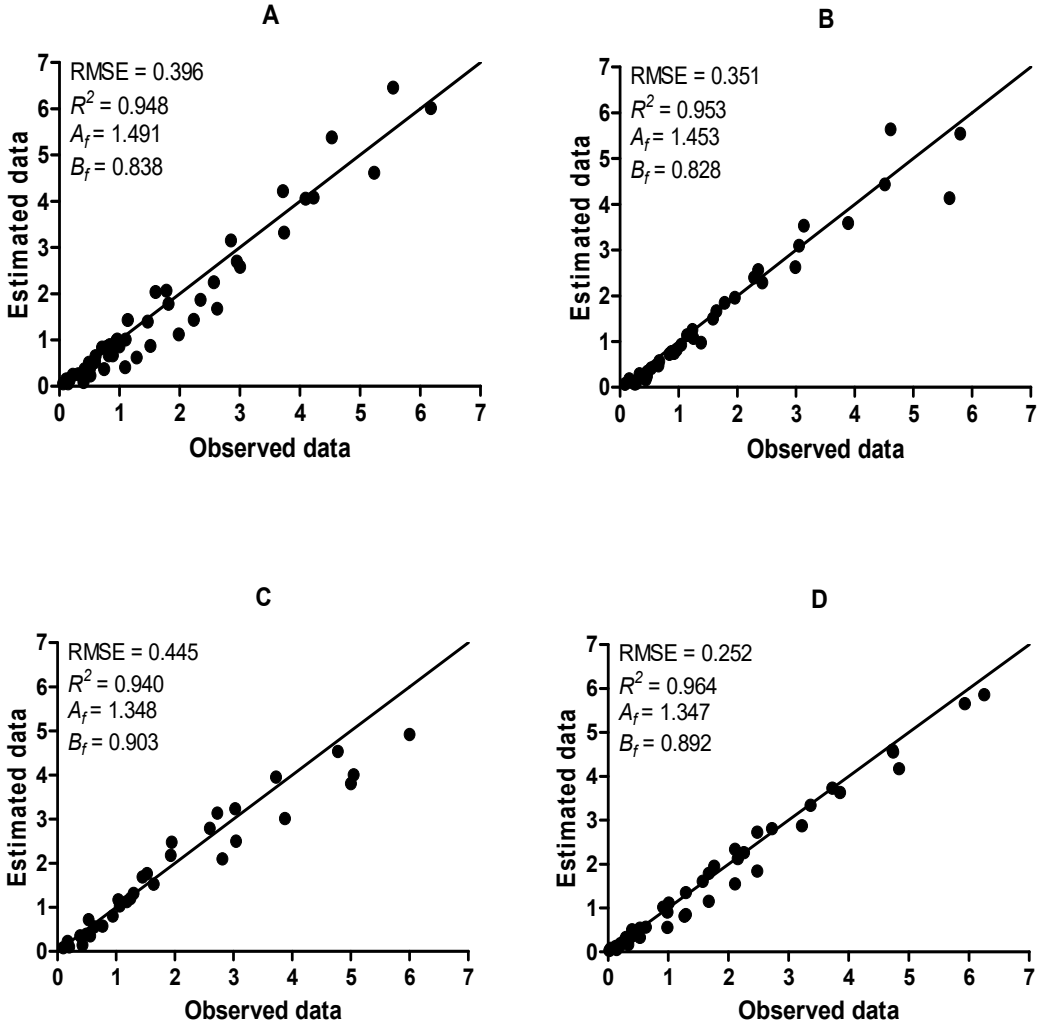
701 **Figure 3**



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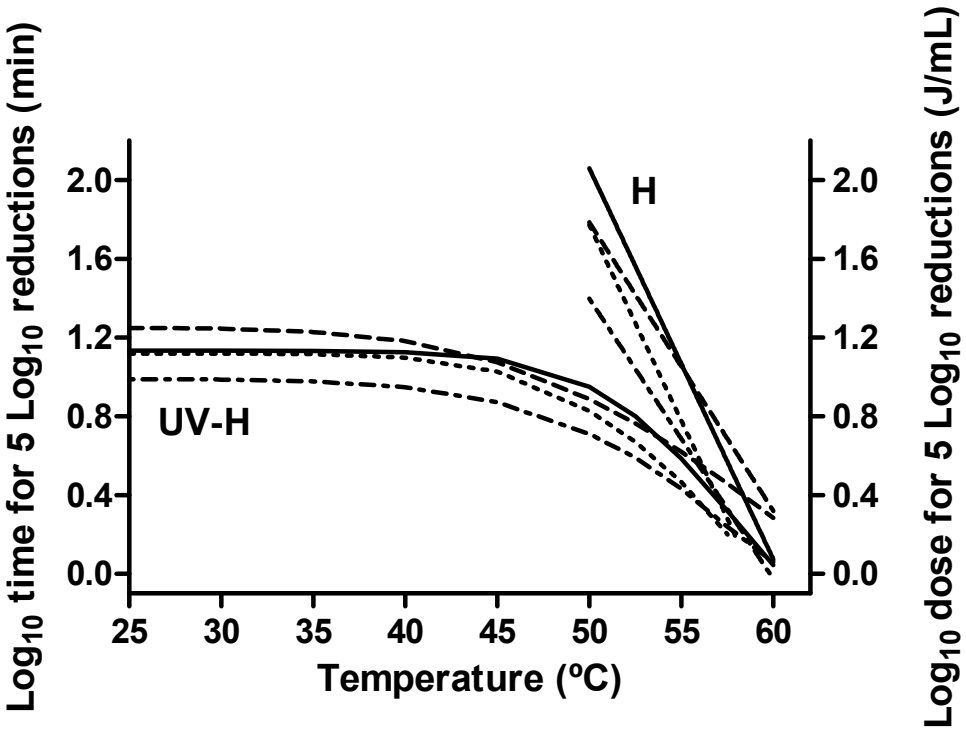
708 **Figure 4**



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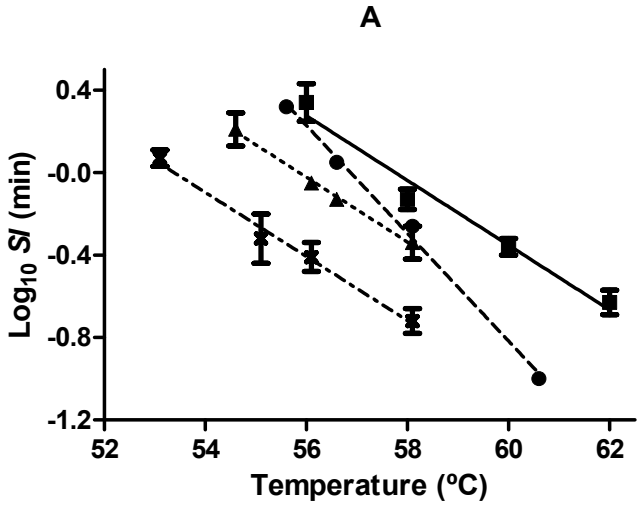
718 **Figure 5**



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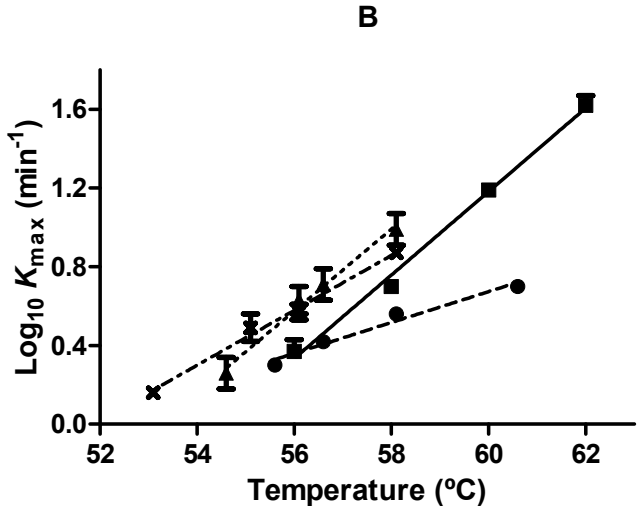

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