Environmental and biological factors influencing UV-C resistance of *Listeria monocytogenes.*

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ABSTRACT

The knowledge of microbial and processing factors that determine the resistance of *L. monocytogenes* to ultraviolet (UV) light is limited. The objective of this work was to investigate the effect of microbiological factors, treatment parameters, and treatment medium characteristics on its UV resistance. The dose to inactivate 99.99% (4*D*) of the initial population of five strains ranged from 21.84 J/mL (STCC 5672) to 14.66 J/mL (STCC 4031). Growth phase and sub-lethal stresses did not change the UV resistance of the most resistant strain. Also, *L. monocytogenes* EGD-e and its isogenic delete mutant *ΔsigB* showed the same UV resistance. Recovery in selective media did not change the number of survivors of *L. monocytogenes* STCC 5672, but the exposition to fluorescent daylight lamps increased the 4*D* value to 22.55 J/mL. The lethal effect of UV light synergistically increased with temperature between 50 and 60 ºC (UV-H treatment). Similarly, synergistic lethal effect was detected on *L. monocytogenes* inactivation by the combined process (27.10 J/mL at 55.0 ºC) in liquid foods, achieving 2.99±0.14 and 3.69±0.19 Log_{10} cycles in orange juice and vegetable soup, respectively, and more than 5 Log_{10} cycles reduction in apple juice and chicken soup. The pH and water activity of the treatment media did not influence the UV resistance at room temperature, whereas the inactivation rate (*Kmax*) exponentially decreased with the absorption coefficient.

Comentado [TdA1]: Se pasa en 22 palabras

1. Introduction

Heat is generally the most used preservation technique throughout the world for microbial inactivation since it can result in the death of both vegetative cells and bacterial spores. However, some detrimental effects on sensory and nutritional food characteristics often accompany the application of heat. Thus, emerging technologies such as high hydrostatic pressure, pulsed electric fields, and ultraviolet (UV) light arise as preservation techniques as substitutes for thermal treatments. Although in the food industry the main applications of UV technology are water disinfection and surface sterilization of packaging materials, this approach is currently gaining in popularity as a novel preservation technique for treating liquid foods due to its multiple advantages. Fruit and vegetable products have received considerable attention because UV treatments may produce safe but minimally processed foods with relatively low-cost production compared to other non-thermal preservation methods (Guerrero-Beltrán and Barbosa-Cánovas, 2004). However, UV-based pasteurization treatments are still under research and applications in the food industry are scarce. The transference of UV light processing to the industry requires a detailed knowledge of the following: the resistance of different microbial species of interest in food safety, its inactivation kinetics, external factors that influence UV resistance, and the effects of UV light on microbial response.

The efficacy of a preservation technology depends on a number of microbial-related factors that are independent of the preservation technique itself (López-Malo and Palau, 2005). The intrinsic UV resistance of each microorganism, partly related with the efficiency of DNA repair mechanisms, determines UV survival (Sastry, 2000). Another important microbial related factor influencing UV resistance to some extent is the physiological state of the cell, such as growth stage, environmental stress history, and

sub-lethal injuries. Stationary growth-phase cells of several bacterial species showed enhanced UV-C resistance compared to growing cells (Child et al., 2002; Bucheli-Witschel et al., 2010). Exposure of microbial pathogens to sub-lethal adverse environments (acid, heat, cold, and osmotic shock) lead to the development of adaptive stress responses, the pathogens becoming more resistant to later applications of further extreme food processing stresses, including non-thermal preservation technologies (McKinney, 2009; Bradley et al., 2012).

The application of UV light for treating liquid foods is limited due to its low penetration capability in the treatment medium. UV light transmittance is determined by the optical and physical properties of the treatment medium. This depends on the absorptivity of the liquid, which varies with the amount of color compounds and soluble and/or suspended solids (Koutchma et al., 2004). The penetration depth also depends on the turbidity of the media. Suspended solids increase the absorptivity but are also responsible for reflection and scattering phenomena (Liltved and Cripps, 1999; Koutchma et al., 2004). Additional critical factors are the homogeneity of the flow pattern and the geometric and conformation of UV equipment which determines the UV dose distribution inside the reactor (Koutchma and Parisi, 2004). Another important processing factor is the temperature of treatment. The combination of heat at middle temperatures with UV light has been reported to increase UV effectiveness to *E. coli* inactivation in laboratory media (Gayán et al., 2011), fruit juice (Ukuku and Geveke, 2010), and liquid egg whites (Geveke, 2008).

Listeria monocytogenes is a human pathogenic bacterium that can cause a serious food-borne infection. Listeriosis poses a real threat to children and pregnant women. Although the United States Food and Drug Administration (U.S. FDA, 2001) identified

as pertinent bacterial pathogens to juice safety *Escherichia coli* and *Salmonella enterica* because of their historical association with juice products, the Committee on Microbiological Criteria for Foods (NACMCF) also proposed *L. monocytogenes* to be a pertinent pathogen due to its ubiquity and its ability to grow in refrigerated and acidic conditions. UV light has proved to be effective in reducing *L. monocytogenes* contamination in the dairy industry (Matak et al., 2005), and meat, fish, and vegetable surfaces (Chun et al., 2010; Sommers et al., 2010). However, the knowledge of microbial and critical processing factors that determine the resistance of *L. monocytogenes* to UV light in liquid foods is limited. The objectives of this work were to investigate the effect of microbiological factors (strain, growth phase, exposition to sub-lethal stresses, and UV damage and repair capacity), treatment medium characteristics (pH, water activity, and absorption coefficient), and treatment parameters (dose and temperature) on its UV resistance in laboratory media and liquid foods (orange juice, apple juice, vegetable soup, and chicken soup).

2. Material and Methods

2.1. Bacterial culture and media

The strains of *L. monocytogenes* STCC 4301, 4302, 5366, 932, and 5672 were provided by the Spanish Type Culture Collection (STCC). *L. monocytogenes* EGD-e and its isogenic deletion mutant *ΔsigB* (chromosomal deletion of region 930.725 bp–931.393 bp) were used to study the role of sigma B factor on UV resistance. The *ΔsigB* mutant (Chatterjee, 2006) was kindly provided by Prof. Chakraborty (Institute for Medical Microbiology, Giessen, Germany). 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)

(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. 50 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. Analytical measurements

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 vs. path length correcting the dilution factor. Turbidity was measured using a HI 83749 nephelometer (Hanna Instrument, Szeged, Hungary). The pH was adjusted using a Basic 20 pH meter (Crison Instrument, Barcelona, Spain). Water activity was measured at room temperature with a specially designed instrument (Water Activity System, mod. CX-1, Decagon Devices, Pullman, Washington, USA). The mean illuminance (Klx) was measured with a radiometer FL A603 VL4 (Ahlborn, Holzkirchen, Germany).

2.3. UV treatments

UV treatments were carried out in the equipment previously described (Gayán et al., 2011). The whole system consisted of eight individual annular thin film flow-through reactors connected sequentially. Each reactor consisted of a low-pressure UV lamp (TUV 8WT5, Philips, U.S.A.) with 8 W of total power, emitting 85% of energy at a wavelength of 254 nm, fixed at the axis of an outer glass tube and enclosed by a quartz tube to prevent

direct contact of the lamp with the treatment medium. The equipment included a feed tank, a peristaltic pump (Ismatec, mod. ISM 10785, Glattbrugg, Switzerland), a heating/cooling coil exchanger, eight UV reactors connected in series, and eight 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 the control of the treatment medium temperature.

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.

McIlvaine citrate-phosphate buffers (Dawson, 1974) of different pH (3.0, 4.0, 5.0, 6.0, and 7.0), water activity (0.94, 0.96, 0.98, and >0.99), and absorption coefficients (from 6.12 to 22.77 cm^{-1}) were used as treatment media. Citrate-phosphate buffers of different water activities and absorption coefficients were prepared by adding different quantities of glycerol (Panreac, Barcelona, Spain) and tartrazine (Sigma-Aldrich, St. Louis, USA), respectively. Apple juice (Antonio Muñoz y Cia S.A., Spain), orange juice (DAFSA S.A., Spain), vegetable soup and chicken soup (Interal S.A., Spain) used as treatment medium was purchased from a local market in Zaragoza, Spain.

2.4. Adaptation to sub-lethal stresses

Previous to UV light exposure, stationary growth phase cells were exposed to different sub-lethal stresses (heat, acid, basic, and oxidative shocks) that were previously chosen as the conditions that exerted a highest increase in the homologous resistance of *L. monocytogenes* (Sagarzazu, 2010). For this purpose, 1 mL of cell suspension were resuspended in 9 mL of TSBYE either acidified with hydrochloric acid (Panreac, Barcelona, Spain) to a pH of 4.5; alkalinized with sodium hydroxide (Panreac) to a pH of 9.0; or prewarmed at a sub-lethal temperature of 48.0 °C. Cells were exposed to stress factors for 1 h, except for the basic shock for 2 h. For the oxidative shock cells were suspended in Tris-HCl buffer with hydrogen peroxide (Sigma, S. Louis, USA) added up to a concentration of 5 mM for 2 h. During acid, alkaline, and hydrogen peroxide adaptation, the temperature of the medium was kept at 25 °C.

2.5. Heat treatments

Heat treatments were carried out in a thermoresistometer TR-SC, as previously described by Condón et al. (1993). 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 (350 mL). After inoculation, 0.2 mL samples were collected at different heating times and immediately pour plated.

2.6. Incubation of treated samples and survival counting

The recovery medium was TSAYE. Where indicated, the maximum non-inhibitory concentrations (MNIC) of sodium chloride (Panreac) (TSAYE+SC) or 0.1% (w/v) of sodium piruvate (Panreac) (TSAYE+P) were added to estimate the percentage of sub-lethally injured cells (Mackey, 2000). The MNIC of sodium chloride used (5-6% w/v) was chosen in previous experiments with non-treated cells (data not shown). The

lack of tolerance to the presence of NaCl is attributed to damage to the functionality and/or integrity of the cytoplasmic membrane, whereas the sodium piruvate removes peroxide and improves recovery of oxidative stressed cells (Mackey, 2000). Samples recovered in the non-selective medium and the non-selective medium enriched with sodium pyruvate were incubated for 48 h at 35 ºC, and those recovered in the selective media for 72 h at 35 ºC. TSAYE+P plates were incubated under anaerobic conditions in a variable atmosphere incubator (MACS VA500, Don Whitley Scientific Limited, Shipley, United Kingdom), following manufacturer's instructions. Previous experiments demonstrated that longer incubation times did not change the profile of survival curves. 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).

For photoreactivation tests, 20 μL of different dilutions of each UV-treated samples were spread plated in TSAYE Petri dishes and incubated under daylight fluorescent lamps (13 W) as described Gayán et al. (2011). Plates were exposed under a mean illuminance of 11.15 Klx for a period of 60 min at room temperature. In each experiment, a sample of UV-irradiated suspension was kept in the dark at the same conditions. After photoreactivation, treatment plates were incubated 48 h at 35ºC.

2.7. Dosage calculation, curve fitting and statistical analysis

Applied dose may be expressed as incident UV intensity (J/cm^2) calculated with the irradiance in lamp surface and correcting UV depth penetration using the Lambert-Beer law (Quintero-Ramos et al., 2004). In our study, the exposure dose was expressed in terms of the actual amount of energy needed for the treatment; in other words, the energy consumption. The UV dose was calculated in J/mL, using the total power output

emitted by UV lights (8 W each UV lamp) and the experimentally calculated average residence time of our UV installation at 8.5 L/h (Gayán et al., 2011). This approach is more useful for industrial purposes because it facilitates comparing the energetic efficiency of these treatments with other technologies (Geveke, 2005).

Survival curves to UV treatments were obtained by plotting the logarithm of the survival fraction versus treatment doses, expressed in J/mL. 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. For comparison purposes, GInaFiT also provides the parameter 4*D*, defined as the treatment dose necessary to inactivate 99.99% of the microbial population.

Statistical analyses *t*-test (p=0.05), ANOVA tests (p=0.05), and 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 and 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

In this investigation, the effect of biological factors, physicochemical properties of the treatment medium, and processing parameters on the UV resistance of *L. monocytogenes* were evaluated. As biological factors, the effect of the strain, growth

phase and environmental stresses (heat, acid, basic, and oxidative shocks) before UV treatment were included. The presence of sub-lethal damaged cells in the survival population and the capacity to repair damages were also explored. As regard to environmental factors, the effect of the pH, water activity, and absorption coefficient of the treatment medium, as well as treatment temperature in laboratory media and liquid foods were studied on the most UV resistant strain of *L. monocytogenes*.

3.1. Biological factors influencing UV resistance of *L. monocytogenes*

3.1.1. UV resistance of *L. monocytogenes* strains

Figure 1 shows the profile of survival curves of five strains of *L. monocytogenes* treated by UV light in reference conditions: McIlvaine buffer pH 7.0 with 0.25 g/L of tartrazine added (absorption coefficient of 11.04 cm^{-1}) at 25 ºC. Although some authors reported that microbial death response to UV light followed a first-order kinetics (Franz et al., 2009; Oteiza et al., 2010), the typical death curve is often described as sigmoid with an initial lag phase, an exponential death phase, and a tailing phase towards the end of treatment (Sastry, 2000). In this work, survival curves showed shoulders that may be explained by the multi-hit kinetics theory, where the deaths of microorganisms are due to multiple UV hits on a single cell (Sastry, 2000). UV light acts by causing mutated bases that compromise cell functionality. However, bacteria have developed DNA repair mechanisms to restore DNA structure and functionality such as photoreactivation, nucleotide excision repair systems, and SOS repair systems (Häder and Sinha, 2005). This phenomenon is reflected in the shape of the inactivation curves. When DNA repair mechanisms are surpassed, minimal additional UV exposure would be lethal for microorganisms and survivor numbers rapidly decline (Sastry, 2000). Tailing phases, that have been related with different UV resistance of subpopulations, cellular aggregates,

and/or non-uniform dose distribution inside the reactor (Koutchma et al., 2004), were not found in this study.

To compare UV resistance, survival curves were fitted by the log linear regression plus shoulder proposed by Geeraerd et al. (2000). Although several models have been proposed to describe UV non-linear inactivation kinetics (Quintero-Ramos et al., 2004; Unluturk et al., 2010), there is not agreement about the most adequate model. Geeraerd et al.´s model was chosen because it allowed us to describe accurately and independently the shoulders (*Sl*) and the log linear rate of inactivation (*Kmax*), and therefore to correlate separately each phenomenon with the studied factors.

Table 1 shows the averages and standard deviations of the resistance parameters obtained after fitting survival curves of the five tested strains in the reference conditions, as well as the coefficient of determination (R^2) and the root mean square error (*RMSE*). Although some scientific studies have been conducted to assess the efficacy of UV for inactivation of *L. monocytogenes* by UV light, to the best of our knowledge there has not been reported data about the variability of UV light resistance of *L. monocytogenes* strains. UV susceptibility of the five tested strains varied widely. The dose to inactivate the 99.99% of the initial population ranged from 21.84 J/mL for the most UV resistant strain to 14.66 J/mL for the most sensitive one (Table 1). In other words, a treatment of 20.32 J/mL reached 3.82±0.02, 4.76±0.12, 4.98±0.04, 5.34±0.16 and more than 6 Log_{10} cycles of inactivation of *L. monocytogenes* STCC 5672, STCC 4032, STCC 5366, STCC 932, and STCC 4031, respectively (Figure 1).

It is difficult to compare the obtained UV resistance data of *L. monocytogenes* with those reported previously in the literature because conformation and geometry of UV equipment, flow pattern, and optical properties of the liquid play an important role in

UV germicidal efficacy (Müller et al., 2011). Our results can be compared with the UV resistance data of *E. coli* obtained in our installation in the same treatment medium. Thus, the 4*D* value of the most resistant strain (*L. monocytogenes* STCC 5672) was significantly higher than those most resistant strains of *E. coli* (16.6±0.48 J/mL) previously tested. It is well known that UV resistance of microorganisms varies from species to species, and in general gram-positive bacteria are more resistant to the effects of UV light than gram-negative bacteria (Guerrero-Beltrán and Barbosa-Cánova, 2004).Our results are in agreement with the observations of other authors that have demonstrated the higher resistance of *L. monocytogenes* compared to other tested bacteria in buffers (Gabriel and Nakano, 2009), milk (Lu et al., 2011), fruit juices (Guerrero-Beltrán and Barbosa-Cánovas, 2005; Gabriel and Nakano, 2009), and solid surfaces (Rowan, 1999). This fact may be attributed to the more efficient DNA repair systems of *L. monocytogenes* (Cheigh et al., 2012), the higher chromosome condensation, or cell wall structure of gram-positive bacteria (Beauchamp and Lacroix, 2011).

It is important to detect sub-lethal damage bacteria following exposure to UV light with regard to food safety, as sub-lethally injured cells under suitable conditions can recover and return to normal function. However, to date, little publications exist on the occurrence of injury cells inoculated in liquid food after exposure to UV light. In our study, the comparison of survivors recovered in the non-selective (TSAYE) and the selective media enriched with sodium chloride (TSAYE+SC) showed that UV light treatments did not damage the functionality and integrity of cell envelopes in all strains investigated (Table 1). Similarly, Pataro et al. (2011) found no appreciable differences in the proportion of sub-lethal damage in *L. innocua* by UV light pulses using selective growth media techniques. Nevertheless, the analysis of sub-lethal membrane damage by flow cytometry techniques showed that some stressed or injured sub-population of *L.*

innocua treated by UV light lost cytoplasmic membrane integrity while being metabolic active as so-called "viable but not culturable cells" (Schenk et al., 2011). Counts obtained in non-selective medium with 0.1% of sodium piruvate (TSAYE+P) indicated that there was no evidence of oxidative damage (Table 1). Photoreactivation may be used to detect and study DNA damage and to evaluate the capacity of cells to repair this damage by the photolyase enzyme (Bucheli-Witschel et al., 2010). Allowing the cells to perform light-dependent repair of the UV damages for 60 min, increased the recovery of survivors. The 4*D* value of the most resistant strain of *L. monocytogenes* increased but not significantly (p>0.05) from 21.84±0.77 to 22.55±1.35 J/mL when a photoreactivation step was included before incubation. This increase in UV resistance is due to a longer shoulder length, whereas the *Kmax* value barely changed. This would indicate that shoulders are related to DNA repair mechanisms.

3.1.2. UV inactivation of *L. monocytogenes* STCC 5672 at different growth phase.

The effect of cell age on the UV survival of *L. monocytogenes* in the reference treatment conditions was investigated. Table 1 includes the resistance parameters of strain STCC 5672 after 6 (early log phase), 8 (mid-log phase), 36 (early stationary phase) and 72 h (late stationary phase) of incubation. Depending on growth conditions, bacteria may change their cellular composition and their physiology. This is reflected in the different resistance of stationary phase cells of *L. monocytogenes* to non-thermal processes compared with growing cells (Mackey et al., 1995; Álvarez et al., 2002). Surprisingly, no significant differences (p>0.05) were found between exponential and stationary growth phase cells of *L. monocytogenes* STCC 5672 (Table 1).

In contrast, it has been reported that resistance to UV-C irradiation is dependent on the specific growth rate of *E. coli* (Bucheli-Witschel et al., 2010; Gayán et al., 2011)

and *Salmonella* (Child et al., 2002). This phenomenon was in part attributed to the transcription of the general stress sigma factor RpoS at the entrance of stationary growth phase in gram-negative bacteria. Gram-positive genera possess an alternative sigma factor, sigma B factor (σ^B), which is considered by many researchers as functionally homologous to the RpoS of gram-negative bacteria (Gertz et al., 2000). It is well known that some of the physiological and morphological changes in *L. monocytogenes* at the entrance on the stationary growth phase that may increase its resistance to most technologies are induced by the activation of σ^B (Becker, 1998).

Our results showed that UV resistance of *L. monocytogenes* is independent of the growth phase, suggesting that the expression of the alternative σ^B was not involved in the UV resistance of this species. To check the role of the σ^B factor on UV resistance, survival curves of *L. monocytogenes* EGD-e and its isogenic delete mutant *ΔsigB* were obtained (Table 2) at stationary and mid-log growth phase. No significant differences (p>0.05) were found between *4D* values of either the parental and mutant strains or stationary and exponential growth phases, confirming the independence of *L. monocytogenes* UV susceptibility to the sigma factor.

Our results did not show differences between stationary cultures of 24 and 72 h. These results are in agreement with those reported for *E. coli* at the same incubation times (Gayán et al., 2011). Likewise, Bucheli-Witschel et al. (2010) reported that *E. coli* susceptibility to UV-C irradiation was similar in populations cultivated under glucose-limiting conditions for more than six weeks. By contrast, Child et al. (2002) showed that the UV sensitivity of stationary-growth *Salmonella* after different periods of prolonged incubation varied randomly compared to early stationary cells. Similarly to early stationary phase cells, neither signs of cytoplasmic membrane injury nor oxidative damage was found after UV treatments in none of cell ages tested (Table 1).

3.1.3. UV resistance of *L. monocytogenes* STCC 5672 stressed cells

Exposure to sub-lethal environmental stresses may trigger adaptive responses in microbes that enhance their survival ability to the same stress applied in a later stage (homologous response) or to another unrelated processing intervention method (heterologous response) (van Schaik, 2005; Wesche et al., 2009). Although characterization of stress adaptation responses in microorganisms is important, only limited information is available about the development of resistance responses that could increase bacterial survival to non-thermal technologies, and particularly to UV light.

Thus, UV resistance in reference conditions of stationary growth phase cells of *L. monocytogenes* STCC 5672 were measured after being exposed to sub-lethal stresses (heat, acid, basic, and oxidative shocks). Microbial counts after stress exposition did not change compared to non-adapted cells (data not shown). As can be observed in Table 3, exposing *L. monocytogenes* cells to prior adverse conditions did not increase the UV resistance. Furthermore, 4*D* value after the shocks were slightly reduced compared to non-adapted controls, although only after basic shock were the differences statistically significant ($p<0.05$).

Little data are available on the UV resistance of *L. monocytogenes* stressed cells and results are contradictory. Bradley et al. (2012) also reported that the exposure of *L. monocytogenes* STCC 5672 cells to sub-lethal acid and heating conditions resulted in similar or increased susceptibility to pulsed UV light treatments. By contrast, McKinney et al. (2009) found that acid adaptation of *L. monocytogenes* provides cross-protection against UV exposure, while heat shocks made *L. monocytogenes* more vulnerable to UV radiation. Moreover, pre-illumination with infrared light increased UV survival, which has been attributed to a heat shock response (Lage et al., 2000).

Alternative σ^B factor regulon is regarded as the main regulator of general stress response in *L. monocytogenes* due to its role in the adaptation to multiple adverse conditions (van Schaik, 2005). In addition to the entry in the stationary growth phase, σ^B factor is activated under stress conditions (including salt, ethanol, organic acid, heat shock) (Becker et al., 1998), inducing the transcription of its regulon, which comprises genes that trigger a protective response (Somolinos et al., 2010). The absence of SigB made *L. monocytogenes* less resistant to stresses such as acid stress, heat treatments, osmotic stress, high hydrostatic pressure, antimicrobial compounds, disinfectants, and carbon starvation (Ait-Ouazzou et al., 2012). According to our results, the lack of environmental stress response is consistent with data obtained in growth phase cells, confirming again that the activation of stress promoters did not affect UV resistance.

3.2. Effect of environmental factors on the UV inactivation of *L. monocytogenes* STCC 5672.

3.2.1. pH, water activity and absorption coefficient

It is well known that the physicochemical characteristics of the treatment media may change the bactericidal efficacy of most food preservation technologies on *L. monocytogenes*. In general, the pH and water activity of the treatment medium are the most influential factors (Álvarez et al., 2002; Gómez et al., 2004). Table 4 includes resistant parameters of *L. monocytogenes* STCC 5672 treated by UV light in media of different pH and water activities. Both physicochemical properties appeared to have little effect on the UV sensitivity of *L. monocytogenes* between pH 3.0 to 7.0 and water activity between 0.94 to >0.99. The interaction between pH and water activity in extreme conditions did not show statistically significant differences (p>0.05). These results are in agreement with others previously published (Koutchma et al., 2004; Quintero-Ramos et al., 2004; Murakami et al., 2006).

Optical properties exert a major influence in UV effectiveness in liquid media due to absorption, reflection, scattering, and refraction phenomena (Koutchma et al., 2004; Oteiza et al., 2005; Murakami et al., 2006). However, the contribution of each individual factor has been scarcely investigated. We studied the effect of the absorbance of the treatment medium on the inactivation of *L. monocytogenes* STCC 5672 when the absorbance of the buffer was adjusted with tartrazine, while other physicochemical parameters (pH 7.0, aw> 0.99) and turbidity (5.92 NTU) were held constant. Table 4 shows the UV resistance parameters of *L. monocytogenes* STCC 5672 in media with absorption coefficients ranging from 6.12 to 22.77 cm^{-1}.

As observed, increasing absorption coefficient dramatically reduces UV lethality, increasing shoulder length (*Sl*) and 4*D* values, and decreasing the inactivation rate (*Kmax*). Plotting *Kmax* against the absorption coefficient demonstrated that there was an exponential relationship between the inactivation rate and the absorptivity (Figure 2). Other authors (Koutchma et al., 2004; Oteiza et al., 2005) have deduced a linear relationship between both parameters working in a narrow range of absorptivities. Overall our results are consistent with the Beer-Lambert-Bougerts Law which states that the amount of light that penetrates through a solution decreases exponentially with increases in the absorbance of the solution. The regression line that relates Log_{10} *Kmax* and the absorption coefficient ($Log_{10}\ Kmax = -0.0722\alpha + 0.652$, R^2=0.991) allowed us to conclude that the inactivation rate (*Kmax*) decreased ten times by increasing the absorption coefficient 13.9±0.7 cm^{-1}. This value does not significantly differ (p>0.05) from that obtained for *E. coli* (15.9 ±1.92 cm^{-1}) (Gayán et al, 2011). There are not other data in the bibliography with which our data can be compared.

3.2.2. Treatment temperature

As can be observed in Table 4, UV light scarcely reduced *L. monocytogenes* STCC 5672 population in treatment media of high absorption coefficients. Thus, applying the maximum possible dose in our installation with a single pass (27.10 J/mL), only 0.84±0.03 Log_{10} inactivation cycles were achieved in a medium of absorption coefficient of 22.77 cm^{-1}. Consequently, it can be deduced that treatment of liquid foods such as fruit juices whose absorption coefficient are close to these values (Koutchma et al., 2007) will be far from achieving the 5 Log_{10} reductions required by the U. S. FDA (2001). Some authors have observed that the combination of heat at sub-lethal temperatures with UV light enhances UV effectiveness of *E. coli* inactivation in laboratory media (Gayán et al., 2011), fruit juice (Ukuku and Geveke, 2010), and liquid egg whites (Geveke, 2008). However, there is not reported data about the combination of UV light and mild temperatures for the inactivation of *L. monocytogenes*.

Thus, the effect of treatment temperature on UV resistance of *L. monocytogenes* STCC 5672 was explored to evaluate prospective designs of a hurdle approach combining UV light and mild heat treatments (UV-H treatments). For this purpose, *L. monocytogenes* was UV treated at temperatures ranging from 50 to 60 ºC (Figure 3) in McIlvaine buffer pH 7.0 with an absorption coefficient of 22.77 cm^{-1}. As observed in Figure 3, UV resistance of *L. monocytogenes* hardly changed with temperature up to 50.0 ºC; above this temperature UV lethality improved, increasing with treatment temperature: a dose of 27.10 J/mL of UV light at 50.0, 52.5, 55.0, 57.5 and 60.0 ºC reduced the surviving population of *L. monocytogenes* cells 1.34±0.06, 2.26±0.23, 3.27±0.32, 4.41±0.55 and more than 6 Log_{10} cycles, respectively. Previously we reported that more than 5 Log_{10} cycles of inactivation of the most UV resistant strain of *E. coli* in the same treatment medium conditions was achieved at 55.0 ºC after applying 27.10 J/mL (Gayán et al., 2011). However, for *L. monocytogenes* inactivation, the temperature should be

raised up to 60 ºC to achieve the same goal. These differences might be explained by the greater heat and UV resistance of *L. monocytogenes* STCC 5672 compared to *E. coli* STCC 4201.

To determine the effect of temperature, heat-resistance characterization of *L. monocytogenes* STCC 5672 was performed in the same treatment medium conditions. *L. monocytogenes* inactivation by heat for the same time than UV treatments (3.58 min) at 50.0 ºC, 52.5 ºC, and 55.0 ºC (data not shown) as well as UV light at room temperature were negligible, but the corresponding lethal effect of the combined treatment was rather higher than the sum of the lethality of individual technologies (1.34±0.06, 2.26±0.23, 3.27±0.32 Log_{10} cycles, respectively). Therefore, a synergistic effect was deduced from the combination. At higher temperatures, lethality of the UV-H combined treatment was further increased, as was the heat inactivation rate. This phenomenon is reflected in Figure 4, in which the Log_{10} *Kmax* values of heat treatments and the combined UV-H treatment expressed in time units (min^{-1}) are plotted. An exponential relationship was deduced between the inactivation rate and temperature of heat treatment with a *z* value of 6.1±0.29 ºC, as has been observed by other authors (Pagán et al., 1998). By contrast, the inactivation rate to UV light hardly changed with temperature up to 50 ºC. Over this value, the *Kmax* value increased ten times by increasing the temperature 13.5±1.92 ºC, approximately. Therefore, the UV inactivation was less thermodependent than heat inactivation and the synergistic effect tended to disappear. This means that it is necessary to optimize treatment temperature to exploit the maximum synergistic effect of the combination of both technologies.

To verify if the lethal effect improvement of UV light in combination with mild temperatures is also provided in liquid food, combined UV-H treatments in orange juice (pH=3.57, absorption coefficient=91.10 cm^{-1}, turbidity=4460 NTU), apple juice

(pH=3.21, absorption coefficient=25.54 cm^{-1}, turbidity=3.34 NTU), vegetable soup (pH=5.81, absorption coefficient=29.56 cm^{-1}, turbidity=2340 NTU) and chicken soup (pH=5.20, absorption coefficient=23.63 cm^{-1}, turbidity=4310 NTU) were carried out. Figure 5 showed inactivation Log_{10} cycles of *L. monocytogenes* STCC 5672 in these products by the UV-H treatments for 27.10 J/mL or 3.58 min at 55.0 ºC, as well as the corresponding individual lethal factors. Thus, the combined treatment for this processing conditions achieved 2.99±0.14, 5.63±0.52, 3.69±0.19, and 5.03±0.32 Log_{10} cycles reduction of *L. monocytogenes* in orange juice, apple juice, vegetable soup and chicken soup, respectively. Again in all products was noticed a synergistic lethal effect due to the microbial reduction achieved with the combined treatment was higher than the sum of heat and UV inactivation (Figure 5). Inactivation Log_{10} cycles of synergistic effect observed at 3.58 min was 1.12±0.27, 2.72±0.52, 1.84±0.22, and 1.98±0.36 Log_{10} cycles for orange juice, apple juice, vegetable soup, and chicken soup, respectively. The inactivation reached with the combined treatment and magnitude of synergistic effect varied significantly between products that may be explained by their different physicoquemical characteristics (pH, absorption coefficient, and turbidity) that determine UV and heat resistance.

4. Conclusions

From our results we can conclude that the UV resistance of different strains of *L. monocytogenes* would differ widely. The 4*D* value of most UV resistant strain here studied (STCC 5672; 21.84 J/mL) was higher than previously obtained in the same experimental conditions for *E. coli* (Gayán et al., 2011; 16.60 J/mL).

Growth phase and sub-lethal shocks do not change the susceptibility of *L. monocytogenes* to UV light. These results and those obtained with strain EGD-e and its

isogenic delete mutant *ΔsigB* seemed to indicate, for the first time, that σ^B was not implicated in the UV resistance of *L. monocytogenes*.

Contrary to most non- thermal technologies, resistance of *L. monocytogenes* to UV light did not change with pH and water activity of the treatment media. However, the lethal effect of UV light exponentially decreased with the absorption coefficient.

The lethal effect of UV light synergistically increased with temperature between 50 and 60 ºC. Over this range of temperatures, the synergistic effect tended to disappear. Also a synergistic lethal effect was detected on *L. monocytogenes* inactivation by UV-H treatments at 55.0 ºC in liquid foods (orange juice, apple juice, vegetable soup, and chicken soup) that suggest the possibility to design a combined treatment to pasteurize these products at relatively low temperatures.

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Table 1. UV resistance parameters (*Sl*, *Kmax*, and 4*D*) obtained from the fitting of Geeraerd et al.´s model to the survival curves of five strains of *L. monocytogenes*, at different growth phase and recovered in different media. Letters a, b, c, and d indicate significant differences ($p \leq 0.05$) among mean values of each strain at stationary phase (24 h) recovered in TSAYE. Asterisks indicate significant differences ($p \leq 0.05$) among mean values of different recovery media comparing with counts in TSAYE for each *L. monocytogenes* strain.

Strain	*Growth phase*	*Recovery médium*	*Sl (J/mL)*	*Kmax (mL/J)*	*Dose for 4D reductions (J/mL)*	R^2	*RMSE*
L. monocytogenes STCC 5672	Stationary (24 h)	TSAYE	8.24 (0.14)[a]	0.71 (0.05)[a]	21.84 (0.77)[a]	0.983	0.331
		TSAYE+SC	7.58 (0.51)	0.78 (0.04)	19.88 (0.84)	0.993	0.232

		TSAYE+P	7.28 (0.97)	0.74 (0.07)	19.86 (0.59)	0.978	0.429
		TSAYE+visible light	9.42 (0.46)*	0.72 (0.06)	22.55 (1.35)	0.983	0.355
	Exponential (6 h)	TSAYE	7.59 (1.63)	0.84 (0.19)	20.07 (1.93)	0.986	0.270
		TSAYE+SC	8.91 (1.56)	0.87 (0.10)	18.05 (0.24)	0.974	0.486
		TSAYE+P	8.63 (0.56)	0.98 (0.20)	18.15 (0.54)	0.981	0.344
	Exponential (8 h)	TSAYE	8.00 (0.84)	0.94 (0.13)	21.04 (0.94)	0.993	0.199
		TSAYE+SC	8.55 (1.17)	0.98 (0.04)	20.69 (0.57)	0.946	0.168
		TSAYE+P	8.97 (1.24)	0.87 (0.09)	21.00 (0.33)	0.982	0.285
	Stationary (72 h)	TSAYE	8.31 (1.12)	0.87 (0.07)	20.24 (1.20)	0.993	0.208
		TSAYE+SC	9.13 (0.82)	0.89 (0.08)	19.65 (0.53)	0.991	0.526
		TSAYE+P	8.47 (1.23)	0.82 (0.12)	19.90 (0.36)	0.990	0.365
L. monocytogenes STCC 4031	Stationary (24 h)	TSAYE	4.53 (0.11)[b]	0.92 (0.02)[b]	14.66 (0.10)[b]	0.988	0.355
		TSAYE+SC	3.59 (0.87)	0.86 (0.03)	14.80 (0.28)	0.996	0.203
		TSAYE+P	3.71 (0.44)*	0.96 (0.05)	15.75 (0.23)	0.980	0.455
L. monocytogenes STCC 4032	Stationary (24 h)	TSAYE	6.50 (0.49)[c]	0.75 (0.07)[a]	18.97 (0.75)[c]	0.995	0.207
		TSAYE+SC	6.32 (1.21)	0.73 (0.15)	18.28 (0.96)	0.977	0.486
		TSAYE+P	6.64 (0.55)	0.71 (0.02)	19.54 (0.43)	0.998	0.136
L. monocytogenes STCC 5366	Stationary (24 h)	TSAYE	5.92 (0.17)[d]	0.72 (0.01)[a]	18.86 (0.17)[c]	0,993	0.264
		TSAYE+SC	5.34 (0.87)	0.71 (0.03)	18.37 (0.32)	0.996	0.210
		TSAYE+P	6.51 (0.73)	0.75 (0.03)	18.96 (0.26)	0.996	0.190
L. monocytogenes STCC 932	Stationary (24 h)	TSAYE	5.71 (0.84)[c]	1.36 (0.16)[a]	17.98 (0.25)[c]	0.984	0.144
		TSAYE+SC	5.76 (1.36)	1.10 (0.14)	18.37 (0.47)	0.996	0.210
		TSAYE+P	6.14 (1.62)	1.07 (0.15)	16.84 (0.74)	0.971	0.409

Table 2. UV resistance parameters (*Sl*, *Kmax*, and *4D*) obtained from the fitting of Geeraerd et al.´s model to the survival curves of *L. monocytogenes* EGD-e and its isogenic *ΔsigB* at stationary and mid-log growth phase.

Strain	***Growth phase***	***Sl (J/mL)***	***Kmax (mL/J)***	***Dose for 4D reductions (J/mL)***	***R^2***	***RMSE***
Parental strain	Stationary (36 h)	5.55 (0.69)	0.88 (0.08)	16.59 (0.52)	0.992	0.277
Parental strain	Exponential (7 h)	4.60 (0.36)	0.79 (0.15)	16.45 (0.36)	0.995	0.201
Mutant strain	Stationary (36 h)	5.18 (1.06)	0.85 (0.12)	16.32 (0.83)	0.987	0.367
Mutant strain	Exponential (7 h)	4.47 (0.62)	0.80 (0.07)	16.21 (0.59)	0.988	0.307

Table 3. UV resistance parameters (*Sl*, *Kmax*, and 4*D*) obtained from the fitting of Geeraerd et al.´s model to the survival curves of *L. monocytogenes* STCC 5672 after the exposition to heat, acid, basic and oxidative shocks. Asterisk indicates significant differences ($p \leq 0.05$) among mean values of different stressed cells and non-adapted control cells.

Stress	***Sl (J/mL)***	***Kmax (mL/J)***	***Dose for 4D reductions (J/mL)***	***R^2***	***RMSE***
Control	8.24 (0.14)	0.71 (0.05)	21.84 (0.77)	0.983	0.331
Heat shock	8.34 (1.20)	0.74 (0.07)	19.45 (1.27)	0,985	0.335
Acid shock	7.32 (1.02)	0.78 (0.06)	18.97 (1.15)	0.983	0.397
Basic shock	7.11 (0.43)*	0.83 (0.07)	18.31 (0.93)*	0.998	0.136
Oxidative shock	8.27 (0.48)	0,78 (0,10)	18.94 (1.26)	0.975	0.440

Table 4. UV resistance parameters (*Sl*, *Kmax*, and *4D*) obtained from the fitting of Geeraerd et al.´s model to the survival curves of *L. monocytogenes* STCC 5672 in different media.

pH	*aw*	*Absorption coefficient (cm^{-1})*	*Sl (J/mL)*	*Kmax (mL/J)*	*Dose for 4D reductions (J/mL)*	R^2	*RMSE*
3.0	0.99	11.04	8.64 (0.73)	0.71 (0.2)	19.98 (0.62)	0.980	0.121
4.0	0.99	11.04	7.65 (0.53)	0.71 (0.07)	20.12 (0.13)	0.990	0.247
5.0	0.99	11.04	8.18 (0.37)	0.79 (0.02)	20.00 (0.41)	0.984	0.381
6.0	0.99	11.04	8.21 (0.29)	0.70 (0.09)	21.58 (0.62)	0.989	0.277
7.0	0.99	11.04	8.24 (0.14)	0.71 (0.05)	21.84 (0.77)	0.983	0.331
7.0	0.98	11.04	8.12 (0.77)	0.74 (0.04)	20.61 (0.62)	0.979	0.433
7.0	0.96	11.04	7.82 (0.32)	0.71 (0.08)	21.00 (0.21)	0.991	0.223
7.0	0.94	11.04	7.49 (0.99)	0.65 (0.09)	20.51 (0.95)	0.995	0.172

3.0	0.94	11.04	8.10 (0.11)	0.71 (0.03)	20.01 (0.66)	0.996	0.169
7.0	0.99	6.12	2.82 (0.48)	1.65 (0.34)	10.81 (0.68)	0.993	0.315
7.0	0.99	8.89	6.01 (0.35)	1.04 (0.01)	13.55 (0.31)	0.990	0.372
7.0	0.99	12.94	8.98 (1.50)	0.56 (0.05)	24.94 (0.54)	0.992	0.172
7.0	0.99	14.61	9.12 (0.54)	0.34 (0.03)	-	0.985	0.147
7.0	0.99	17.02	9.49 (1.22)	0.24 (0.02)	-	0.994	0.071
7.0	0.99	18,91	9.06 (0.89)	0.22 (0.01)	-	0.996	0.046
7.0	0.99	19,89	10.10 (1.74)	0.17 (0.02)	-	0.989	0.058
7.0	0.99	22.77	11.24 (3.50)	0.10 (0.02)	-	0.985	0.240

Figure captions

Figure 1. Survival curves of *L. monocytogenes* STCC 5672 (●), 5366 (▲), 4032 (■), 932 (○) and STCC 4031 (Δ) in McIlvaine buffer of pH 7.0 and absorption coefficient of 11.04 cm^{-1}.

Figure 2. Relationship between the absorption coefficient and Log_{10} *Kmax* obtained from the fitting of survival curves of *L. monocytogenes* STCC 5672 in McIlvaine buffer of pH 7.0 with different concentration of tartrazine.

Figure 3. Survival curves of *L. monocytogenes* STCC 5672 treated by UV light at 25.0 (●), 50.0 (■), 52.5 (▲), 55.0 (○), 57.5 (□), and 60.0 ºC (X) in McIlvaine buffer of pH 7.0 and absorption coefficient of 22.77 cm^{-1}.

Figure 4. Relationship between Log_{10} *Kmax* and temperature from the fitting of survival curves of *L. monocytogenes* STCC 5672 treated by the combined UV-H treatment (●) and heat alone (○) in McIlvaine buffer of pH 7.0 and absorption coefficient of 22.77 cm^{-1} at different temperatures.

Figure 5. Inactivation of *L. monocytogenes* STCC 5672 by UV light (UV, black) (27.10 J/ml or 3.58 min at room temperature), heat treatment (H, white) (3.58 min at 55.0 ºC), and combined treatment of both technologies simultaneously (UV-H, grey) (27.10 J/mL or 3.58 min) in different food matrices: orange juice (OJ), apple juice (AJ), vegetable soup (VS), and chicken soup (CS).

Figure 1

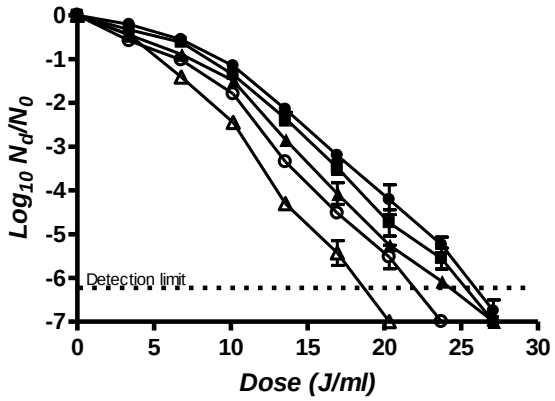

0
-1
-2
-3
-4
-5
-6
-7
Log10 Nd/N0
Detection limit
0
5
10
15
20
25
30
Dose (J/ml)

Figure 2

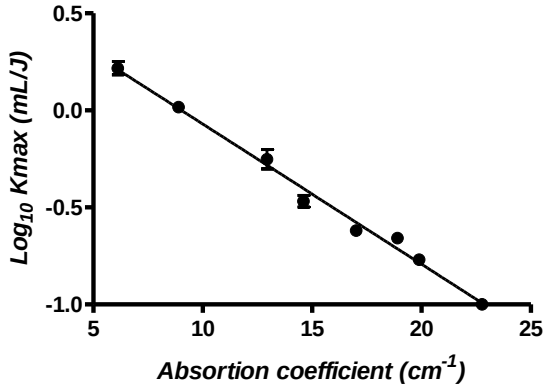

0.5
0.0
-0.5
-1.0
5
10
15
20
25
Log10 Kmax (mL/J)
Absortion coefficient (cm-1)

Figure 3

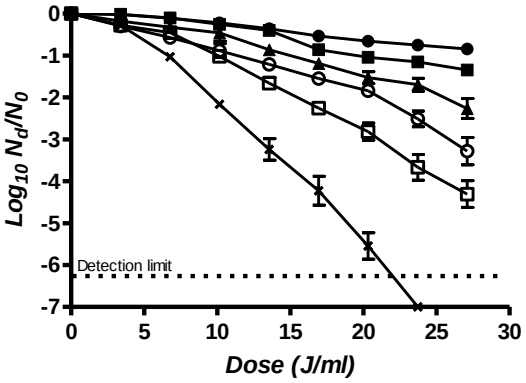

0
-1
-2
-3
-4
-5
-6
-7
Log10 Nd/N0
Detection limit
0
5
10
15
20
25
30
Dose (J/ml)

Figure 4

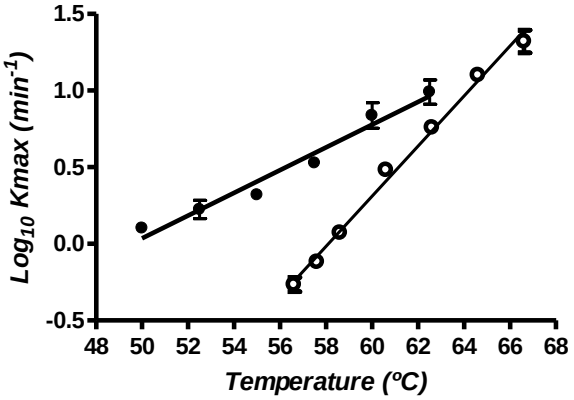
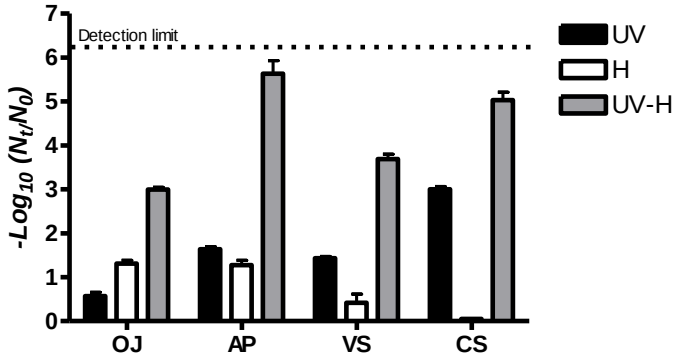

1.5
1.0
0.5
0.0
-0.5
48
50
52
54
56
58
60
62
64
66
68
Log10 Kmax (min-1)
Temperature (ºC)

Figura 5


Detection limit
UV
H
UV-H
-Log10 (Nt/N0)
7
6
5
4
3
2
1
0
OJ
AP
VS
CS