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Citric acid as alternative to sodium hypochlorite for washing and disinfection of experimentally-infected spinach leaves

G. Finten*, M.V. Agüero [1], R.J. Jagus [1]

Universidad de Buenos Aires (UBA), Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Instituto de Tecnologías y Ciencias de la Ingeniería (INTECIN), Facultad de Ingeniería, Departamento de Ingeniería Química, Laboratorio de Microbiología Industrial: Tecnología de alimentos, Av. Int. Guiraldes 2620, C1428EGA, Ciudad Autónoma de Buenos Aires, Argentina

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ABSTRACT

This research aims to investigate citric acid (CA) 0.5% as alternative to sodium hypochlorite (SH) 200 ppm for washing and disinfecting spinach leaves (*Spinacia oleracea* L.). The initial disinfection achieved in leaves spot-inoculated with *Escherichia coli* and *Listeria innocua*, pathogen surrogates, was investigated along with the effects of time and temperature conditions before processing on the performance of CA and SH. Next, the effectiveness of CA and SH was evaluated throughout refrigerated storage (6.5 °C, 9 days) at a low and high contamination load, 5–6 and 8–9 log CFU.g^{-1}, respectively. And lastly, sensory impact was assessed through a trained panel and instrumental color. Results indicated that there were not significant differences between the initial disinfection achieved by CA and SH. Storing infected spinach under refrigeration, between harvest and processing, played a key role not only in reducing their deterioration but also in assuring their safety by maintaining CA and SH effectiveness against the inoculated surrogates. Citric acid performance was better in controlling surrogates' regrowth along refrigerated storage. And there were not significant differences between CA and SH treated samples with respect to their sensory quality. Therefore, CA could constitute an alternative washing and disinfection method for spinach leaves.

1. Introduction

Leafy greens are constantly demanding feasible technologies and optimized preservation methods to minimize safety risks in fresh and fresh-cut products. Minimally processed vegetables such as bagged leafy greens have increased greatly in the last years (Matthews, 2014), and in association with this pattern numerous foodborne outbreaks occurred worldwide. In 2006 contaminated fresh spinach with *Escherichia coli* O157:H7 was linked to a multi-state outbreak in North America, where the number of cases reached 206 (Lynch, Tauxe, & Hedberg, 2009). Furthermore, the lack of Good Agricultural Practices (GAP's) and improper post-harvest handling represent a safety risk on their consumption (Matthews, 2014).

Alternative washing and disinfection methods for leafy greens have been extensively studied, but most of the time they are not easy to apply or not effective enough to replace the existing ones (Matthews, 2014). Sodium hypochlorite solutions (chlorinated water), the most widespread disinfection agent, is criticized because it has limited efficacy against pathogens and it is believed that its use generates carcinogenic compounds that might reach consumers (Gómez-López, Lannoo, Gil, & Allende, 2014). Therefore, several alternative approaches to increase the microbial safety of fresh produce arose in the last years. Among them, organic acids have demonstrated their bactericidal activity through multiple mechanisms: pH reduction, disruption of cell membrane transport system and permeability, accumulation of ions, and a reduction in the internal cellular pH which affects their homeostasis (Neal et al., 2012). However, their effectiveness varies greatly depending on the type of organic acid, application method, microorganism tested, produce, and processing parameters. Several authors (Akbas & Ölmez, 2007; Almasoud, Hettiarachchy, Rayaprolu, Horax, & Eswaranandam, 2015; Bermúdez-Aguirre and Barbosa-Cánovas, 2013; Dikici, Koluman, & Calicioglu, 2015; Choi et al., 2012;

Abbreviations: CA, Citric acid; SH, Sodium hypochlorite; FT, Fresh tissue; C, Control.

* Corresponding author.
E-mail address: fintengabriel@hotmail.com (G. Finten).

[1] These authors contributed equally in the research presented in this manuscript.

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Ganesh et al., 2010, Ganesh, Hettiarachchy, Griffis, Martin, & Ricke, 2012; Ho, Rodde, Tang, & Phan, 2011; Huang, Ye, & Chen, 2012; Neal et al., 2012; Ölmez & Temur, 2010; Park et al., 2011) have studied the efficacy of organic acids (acetic, ascorbic, citric, lactic, malic, propionic, and tartaric acids) for washing and disinfecting leafy greens; nevertheless, only Almasoud et al., 2015 and Ölmez & Temur, 2010 considered biofilm formation and/or time before processing which mimics the lapse between microbial contaminations occur and the washing and disinfecting step is carried out. The lapse in-between contamination and processing along with temperature conditions during that period are factors that have been barely studied and might reduce the effectiveness of organic acids as leafy greens' sanitizers.

In our previous study (Finten, Finten, Agüero, & Jagus, 2015), it was demonstrated that a dip washing with citric acid (CA) 0.5% was effective against native microbiota present in spinach leaves without affecting their sensory quality, and it could constitute an alternative sanitizer to the traditional disinfection agent against foodborne pathogens. Therefore, the aim of the present research was to broaden the study of spinach leaves' washing and disinfection with citric acid 0.5%. Firstly, the initial disinfection achieved in spot-inoculated *Escherichia coli* and *Listeria innocua*, pathogen surrogates, was investigated along with the effects of time and temperature conditions before processing on the performance of sodium hypochlorite (SH) and CA. Next, the effectiveness of CA and SH was evaluated throughout 9 days of refrigerated storage (6.5 ± 1 °C) at a low and high contamination load, 5–6 and 8–9 log CFU.g^{-1}, respectively. And lastly, sensory impact of the selected disinfection treatment on spinach leaves was assessed through a trained panel and instrumental color.

2. Materials and methods

In the present study three experiments were carried out sequentially. The first and the second were microbial studies where the effectiveness of citric acid against *E. coli* and *Listeria monocytogenes*' pathogen surrogates inoculated on spinach disks was compared to that achieved by the traditional disinfection agent, sodium hypochlorite. Finally, the third experiment was a sensory study, carried out in order to establish whether or not citric acid could constitute a feasible alternative in the washing and disinfection step of spinach leaves minimally processed.

2.1. Microbial studies

2.1.1. Raw material, sample preparation, and initial disinfection

Spinach leaves (*Spinacia oleracea* L.) were directly obtained from local markets in Buenos Aires city, Argentina. Leaves without visual defects were sampled with a circular cutting edge (d = 60 mm) and weighed, each disc constituted an experimental unit (1.06 ± 0.03 g). With the purpose of reducing native microbiota each disc was disinfected with sodium hypochlorite 200 ppm at 25 °C. The procedure was the following: experimental units were placed in falcon tubes (50 mL) with a weight:volume of solution (w:v) ratio of 1:40, then gently shaken for 2.5 min, and finally air dried in tissue paper and placed in sterile Petri dishes.

2.1.2. Bacterial strains and culture conditions

Two strains of *Escherichia coli* (ATCC® CRM-8739™ and ATCC® PTA-3526™) and one of *Listeria innocua* (ATCC® 33090™) were grown in 100 mL of Tryptic Soy broth supplemented with 0.6% yeast extract (TSYE, Biokar Diagnostics, France), in a temperature-controlled shaker at 28 °C overnight. An aliquot (2 mL) of this preculture was transferred to a fresh TSYE broth and incubated under agitation until it reached the desired cells concentration (roughly 10^7 or 10^{10} CFU mL^{-1}). Cells concentration was determined by optical density using an UV-VIS spectrophotometer UV-1800 (Shimadzu, Japan) at 630 and 540 nm, respectively for *E. coli* and *L. innocua*; then microbial load was confirmed by plate count.

2.1.3. Spot inoculation

In the first experiment, experimental units were inoculated separately on the adaxial side with 100 μL of 10^{10} CFU mL^{-1} of each strain. Inoculum was spread on the surface of the disc by randomly depositing 20 droplets. Incubation for 3 h at 22 °C was performed in order to allow bacterial attachment. For the second experiment, the concentration of each strain was 10^7 and 10^{10} CFU mL^{-1} to reach a final count of 5–6 or 8–9 log cycles CFU per gram of fresh tissue (FT), respectively.

2.1.4. Washing/disinfection solutions

Citric acid 0.5% at 20–25 °C was prepared with anhydrous citric acid and distilled water; solutions presented a pH equal to 2.3. Sodium hypochlorite solution 200 ppm at 20–25 °C was prepared by adding commercial bleach into distilled water, its pH was adjusted to 6.5–7.0 with a solution of hydrochloric acid. Chlorine Test Papers and Free Chlorine High Range Test Strips (LaMotte®, USA) were used to check total chlorine and free chlorine, respectively. Solutions' pH was measured using a digital pH meter (Thermo Scientific Orion 3 Star, USA). Conditions tested are results of unpublished preliminary studies where processing parameters time and temperature of the washing/disinfecting solution were optimized.

2.1.5. Procedure for washing/disinfecting produce

For the first experiment, the effects of time and temperature conditions before processing on the effectiveness of citric acid (CA) and sodium hypochlorite (SH) to reduce pathogen surrogates were compared; inoculated experimental units were incubated for 24 and 48 h at 5 and 25 °C. Treatments were carried out as follows: inoculated leaves were aseptically placed in falcon tubes with a w:v ratio of 1:40, dip-washing (with CA and SH) was performed for 2.5 min with gentle shaking, and finally air dried on tissue paper. Inoculated samples that did not receive disinfection treatments were kept and analyzed as controls (C).

In the second experiment, units inoculated at two contamination loads were dip-washed by following the same procedure described previously. Both *E. coli* strains presented similar resistance to CA and SH, hence it was decided to continue the research with only one of them. Counts of *E. coli* ATCC 8739 and *L. innocua* were evaluated immediately after treatments and throughout refrigerated storage (6.5 ± 1 °C) for 9 days.

2.1.6. Microbial analysis

Each disk was aseptically homogenized for 2 min with 20 mL of sterile 0.1% peptone water (Biokar Diagnostics, France) using a vortex shaker. Next, serial dilutions were carried out using sterile 0.1% peptone water. *Escherichia coli* and *L. innocua* counts were performed using MacConkey Agar and Oxford Agar added with Oxford Selective Supplement (Biokar Diagnostics, France), respectively. Petri dishes were incubated at 37 °C for 24 h. Detection limit of the method was 2.3 log CFU.g^{-1} of FT.

2.2. Sensory study

2.2.1. Raw material, sample preparation, and treatments procedure

Raw material (*Spinacia oleracea* L.) for sensory analyses was prepared as follows: spinach leaves obtained from local markets in Buenos Aires city were transported to the laboratory in refrigerated

Table 1
Microbial loads of pathogen surrogates inoculated on spinach leaves after disinfection with citric acid as affected by contact time and temperature before processing.

Contact time [h]	Treatment[x]	Microbial load [log CFU.g^{-1}][y]					
		E. coli ATCC 8739		*E. coli* ATCC 3526		*L. innocua* ATCC 33090	
		5 °C	25 °C	5 °C	25 °C	5 °C	25 °C
3[z]	C	9.0 ± 0.0^{a}		8.2 ± 0.1^{a}		8.3 ± 0.1^{a}	
	SH	6.2 ± 0.7^{b}		5.8 ± 0.0^{b}		5.2 ± 0.3^{b}	
	CA	6.7 ± 0.0^{b}		5.1 ± 0.4^{b}		5.8 ± 0.4^{b}	
24	C	8.8 ± 0.1^{a}	9.7 ± 0.0^{a}	9.2 ± 0.1^{a}	8.4 ± 0.3^{a}	9.0 ± 0.2^{a}	8.9 ± 0.3^{a}
	SH	5.0 ± 0.9^{b}	7.5 ± 0.2^{b}	6.2 ± 0.2^{b}	5.9 ± 0.4^{b}	5.6 ± 0.6^{b}	5.6 ± 0.5^{b}
	CA	6.5 ± 0.6^{a,b}	7.8 ± 0.1^{b}	6.6 ± 0.3^{b}	7.9 ± 0.1^{c}	5.7 ± 0.3^{b}	7.1 ± 0.4^{a,b}
48	C	8.9 ± 0.1^{a}	9.7 ± 0.1^{a}	8.2 ± 0.2^{a}	9.3 ± 0.2^{a}	8.9 ± 0.1^{a}	8.7 ± 0.0^{a}
	SH	6.1 ± 0.0^{b}	7.3 ± 0.2^{b}	6.0 ± 0.7^{b}	6.4 ± 0.4^{b}	5.3 ± 0.2^{b}	6.2 ± 0.1^{b}
	CA	6.1 ± 0.2^{b}	7.9 ± 0.7^{b}	5.9 ± 0.6^{b}	8.0 ± 0.3^{c}	5.8 ± 0.2^{b}	7.3 ± 0.3^{c}

[x]: Control, sodium hypochlorite (200 ppm, pH: 6.5–7.0), and citric acid (0.5%) samples are represented by C, SH and CA, respectively.
[y]: Data are presented as the means ± standard errors (n = 3). Different lowercase letters in the same column for each microorganism at each incubation time indicate significant differences (Bonferroni's p-adjusted values, $p < 0.05$).
[z]: Initial incubation for all samples was at 22 °C.

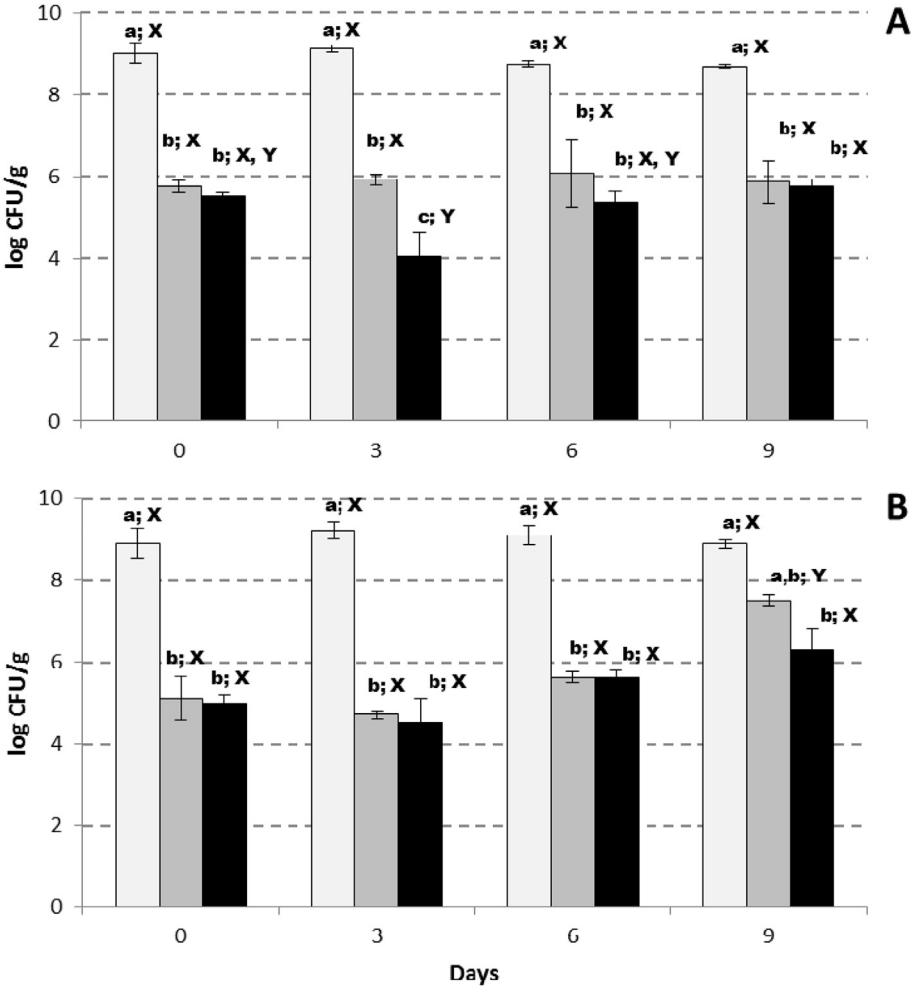

Fig. 1. Effects of treatments on inoculated pathogen surrogates at a high load (≈9 log CFU.g^{-1}) on spinach leaves throughout storage at 6.5 °C. A: *Escherichia coli* ATCC 8739, B: *Listeria innocua* ATCC 33090. Control, sodium hypochlorite (200 ppm, pH: 6.5–7.0), and citric acid (0.5%) samples are represented by light-grey, dark-grey, and black bars, respectively. Data are presented as the means ± standard errors expressed as vertical segments (n = 3). Lowercase [a, b] and uppercase letters [X, Y] indicate significant differences ($p < 0.05$) under factors treatment and time, respectively.

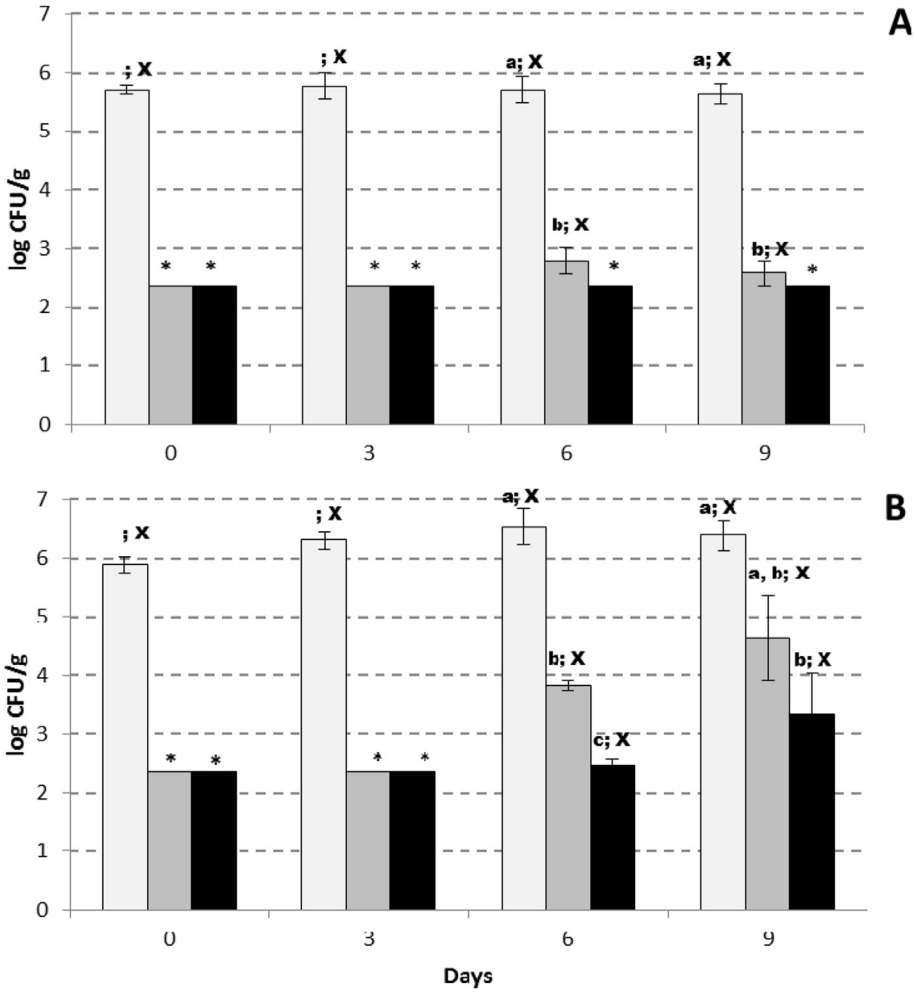

Fig. 2. Effects of treatments on inoculated pathogen surrogates at a lower load (≈6 log CFU.g^{-1}) on spinach leaves throughout storage at 6.5 °C. A: *Escherichia coli* ATCC 8739, B: *Listeria innocua* ATCC 33090. Control, sodium hypochlorite (200 ppm, pH: 6.5–7.0), and citric acid (0.5%) samples are represented by light-grey, dark-grey, and black bars, respectively. Data are presented as the means ± standard errors expressed as vertical segments (n = 3). Lowercase $^{a, b}$ and uppercase letters $^{X, Y}$ indicate significant differences ($p < 0.05$) for bars under factors treatment and time, respectively. Microbial counts below detection limit are indicated by an asterisk (*).

containers, cut to discard the roots and the lower portions of stems, and then selected by uniformity in size and color, and lacking imperfections. Selected leaves were dip-washed with CA and SH (solutions prepared as described in *2.1.4*) 1:20 (w:v) for 2.5 min, centrifuged for 30 s in a domestic salad spinner, and packed in 10 g units (experimental unit) using polyolefin PD960 (Cryovac®, Duncan, USA). The packaging material presented an O_2 and CO_2 transmission rate of 6000–8000 and 19000-22000 cm^3 m^{-2}.24 h^{-1}, respectively; and a water vapor transmission rate of 5–6 g cm^{-2}.24 h^{-1}. Samples were stored under refrigeration (6.5 ± 1 °C) for 14 days.

2.2.2. Sensory analysis

Sensory analysis was carried out by a trained panel composed of 4 members aged 30–60, who evaluated fresh-odor, off-odor, color, texture and overall liking of samples along storage using a 5-point hedonic scale; with 3 as the acceptability threshold for each attribute, and 5 as the rating which indicates the best quality. Codified experimental units (10 g) consisted of 4–8 spinach leaves. Samples were analyzed on days 0 (after treatments), 5, 11, and 14, but on day 14 they were not tested. Panelists were requested to open each experimental unit, sniff for detecting odors, evaluate color, texture by pressing leaves with their hands and tasting them, and give ratings and commentaries for each attribute. Water and crackers were used for palate cleansing in between the samples. For color evaluation each panelist had an "indicative color image", which is presented as supplementary data (Fig. S2).

2.2.3. Measurement of instrumental color

Instrumental color was measured using a colorimeter (Minolta CM508b, Japan) calibrated with a white standard. The illuminating/viewing geometry was D65/10° and the color space used was the CIELab system. Leaves color was measured on the upper side of 3 individual leaves per package, and four experimental units for each replicate. For evaluating color changes after treatments, chroma (C*), hue angle (H°) and color difference (ΔE) were calculated with the equations (1) (Koukounaras, Siomos, & Sfakiotakis, 2009), (2) (Koukounaras et al., 2009), and (3) (Chisari, Todaro, Barbagallo, & Spagna, 2010):

$$C^* = [(a^*)^2+(b^*)^2]^{0.5} \quad (1)$$

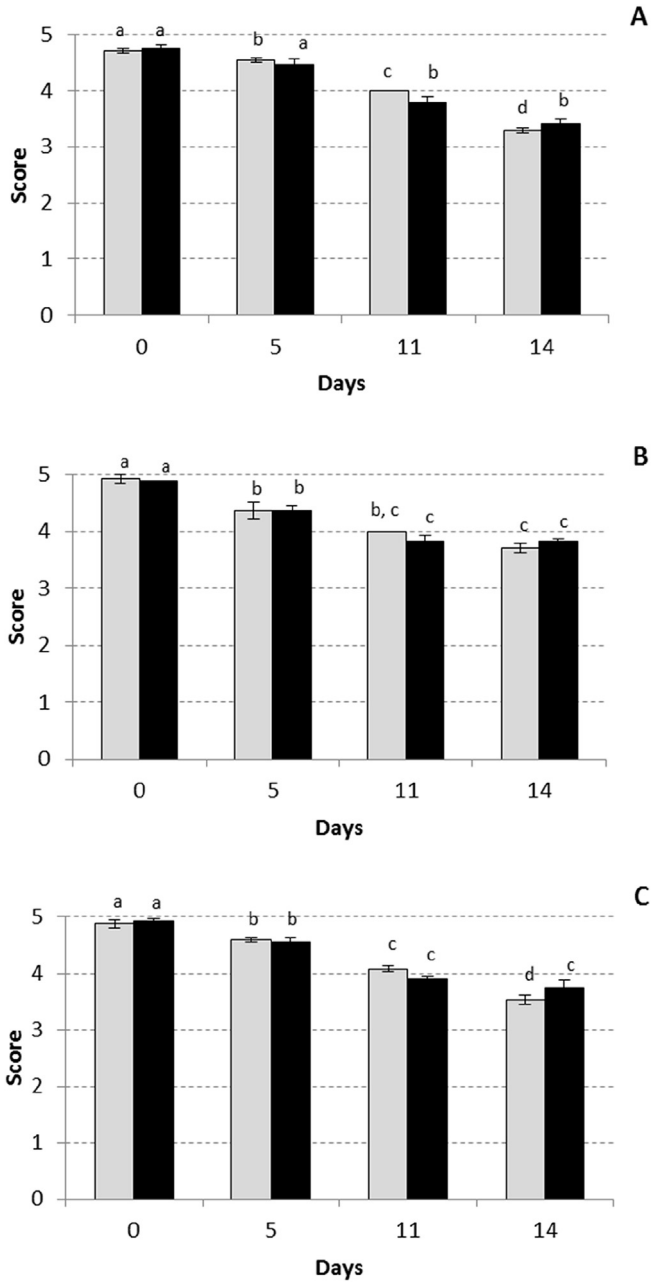

Fig. 3. Sensory analysis of citric acid disinfected spinach leaves during storage at 6.5 °C. Samples treated with SH (sodium hypochlorite 200 ppm, pH = 6.5–7.0) and CA (citric acid 0.5%) are represented by grey and black bars, respectively. Attributes evaluated: Color (A), Texture (B), and Overall Liking (C). Data are presented as the means ± standard errors expressed as vertical segments (n = 3). Letters $^{a, b, c, d}$ indicate significant differences between days for each treatment. (Bonferroni's p-adjusted values, $p < 0.05$).

$$H^\circ = 180+(\tan\text{-}1(b^*/a^*))\cdot(180/\pi) \quad (2)$$

$$\Delta E = [(L_c^*\text{-}L^*)^2+(a_c^*\text{-}a^*)^2+(b_c^*\text{-}b^*)^2]^{0.5} \quad (3)$$

where L_s^*, a_c^* and b_c^* are the parameters L^* (lightness), coordinate a^*, and coordinate b^* for the sample treated with sodium hypochlorite, respectively.

2.3. Statistical analysis

Data were analyzed using R 3.2.2 version (R Core Team, 2015). For all experiments, General Linear Model procedure was used for analysis of variance (ANOVA). Differences between levels of factors were assessed by pairwise comparisons using t tests with pooled standard deviation and adjusted p-values by Bonferroni's methodology (pairwise.t.test( ) R function). Additionally, two-sample *t*-test and Welch two-sample *t*-test, the latter when variances were not equal by means of the F test, were performed when required. The fulfillment of the normal distribution and the homoscedasticity of the data were tested by means of the Shapiro-Wilk's and the Bartlett's tests, respectively. Adjusted models and surface plots presented as supplementary data (Table S1 and Fig. S1) were performed with the R package "rsm" (Lenth, 2009); insignificant terms were deleted by backward elimination. Furthermore, models were checked by analyzing distribution of residuals and residuals vs. fitted values. Pearson's product-moment correlation was performed to assess correlation between instrumental color and color judged by the sensory panel. Treatments were carried out by triplicate and the significance level for all the statistical procedures was 5%.

3. Results and discussion

3.1. Effectiveness against pathogen surrogates

3.1.1. First experiment: effects of time and temperature conditions before processing on the effectiveness of citric acid and sodium hypochlorite

Microbial counts of experimentally-infected samples, control and disinfected spinach, are presented along with the effects of contact time and temperature conditions before processing on CA and SH performances (Table 1). Adjusted models and surface plots for the responses of each strain can be found as supplementary data; Table S1 and Fig. S1, respectively.

Statistical analyses indicated that results presented in Table 1 did not show significant differences in the initial disinfection achieved by CA and SH (incubation for 3 h); reductions between 2.3 and 3.1 log cycles were found. Furthermore, both strains of *E. coli* and *L. innocua* presented similar sensitivity. Then, it was found that when time and temperature increased the effectiveness of CA and SH was reduced; but SH performed significantly better against *E. coli* ATCC 3526 and *L. innocua*. The reduction in CA and SH effectiveness could be attributed to biofilm formation by inoculated microorganisms. Biofilms present a protective nature that makes microorganisms embedded therein resistant to harsh environments and disinfection treatments; thus introducing another challenge in assuring microbial safety of fresh and fresh-cut produces (Ölmez & Temur, 2010). Supporting this hypothesis, in Table 1 it can be seen that control samples inoculated with *L. innocua* presented a similar increase at both temperatures (less than 1 log cycle) before being processed, but sanitizers effectiveness was only reduced at the highest and more favorable temperature for microbial attachment and perhaps biofilm formation as well. Biofilm formation represents a key concern in the fresh market, and consequently this aspect must be studied further.

On the other hand, when temperature was kept at 5 °C, time in-between contamination and processing did not affect neither CA nor SH effectiveness (Table 1). It is well known that storage temperature plays a key role in reducing spinach leaves' deterioration and assuring their safety, but it could also maintain sanitizers' effectiveness against postharvest contamination by foodborne pathogens; possibly by preventing or lowering biofilm formation/bacterial attachment. Regarding biofilm formation, Ölmez and Temur (2010) studied the efficacy of different sanitizing treatments on lettuce. These authors artificially inoculated lettuce samples by dipping them in solutions with *E. coli* and

Table 2
Instrumental colour in samples treated with SH (sodium hypochlorite 200 ppm, pH = 6.5–7.0) and CA (citric acid 0.5%).

Treatments[x]	Parameters	Days			
		0	5	11	14
SH	(-a*)	11.70 ± 0.2[a]	11.60 ± 0.2[a]	11.44 ± 0.2[a]	10.94 ± 0.3[a]
	b*	30.35 ± 1.1[a]	30.89 ± 0.7[a, b]	34.19 ± 1.3[a, b]	34.95 ± 0.0[b]
	L*	39.13 ± 0.9[a]	40.62 ± 0.2[a, b]	42.79 ± 0.6[b, c]	43.98 ± 0.7[c]
	C*	32.5 ± 1.0[a]	33.0 ± 0.7[a]	36.1 ± 1.3[a]	36.6 ± 0.1[a]
	H°	111.1 ± 0.5[a]	110.6 ± 0.1[a]	108.5 ± 0.4[b]	107.4 ± 0.5[b]
CA	(-a*)	11.53 ± 0.1[a]	11.60 ± 0.2[a]	10.95 ± 0.0[a,b]	10.72 ± 0.2[b]
	b*	29.45 ± 0.6[a]	30.19 ± 1.2[a]	32.72 ± 1.2[a]	33.28 ± 0.2[a]
	L*	38.69 ± 0.5[a]	40.08 ± 0.7[a, b]	42.02 ± 0.7[b, c]	43.30 ± 0.3[c]
	C*	31.6 ± 0.6[a]	32.3 ± 1.2[a]	34.5 ± 1.1[a]	35.0 ± 0.2[a]
	H°	111.4 ± 0.3[a]	111.0 ± 0.4[a]	108.5 ± 0.6[b]	107.9 ± 0.3[b]
	ΔE*	1.4 ± 0.5[a]	1.8 ± 0.8[a]	2.4 ± 0.7[a]	1.9 ± 0.2[a]

[x]: Data are presented as the means ± standard errors (n = 3).
[a, b, c, d]: Different letters in the same row indicate significant differences (Bonferroni's p-adjusted values, $p < 0.05$).

L. monocytogenes, and incubated the samples at 10 °C for 6, 24 and 48 h. They found that none of the sanitizing treatments, among them citric acid 0.25% plus ascorbic acid 0.50%, could reduce or detach bacterial cells within biofilms. Likewise, Almasoud et al. (2015) studied the efficacy of electrostatic spraying of malic and lactic acids, alone and in combination, to reduce biofilms formed by *E. coli* 0157:H7 on spinach leaves. Nevertheless, they found a 4 log cycle reduction in *E. coli* incubated for 24 h at 37 °C by a combination treatment of both acids, each at a concentration of 2%. Furthermore, they confirmed their results using a confocal microscope. Unfortunately they did not carry out sensory analyses to confirm whether the treatment is feasible or not.

3.1.2. Second experiment: comparison of the effectiveness of citric acid with sodium hypochlorite along refrigerated storage at two contamination loads

In Fig. 1 and Fig. 2 inoculated spinach samples were evaluated after disinfection treatments, with CA and SH, along refrigerated storage (6.5 ± 1 °C) for 9 days. At the highest contamination load (Fig. 1), reductions achieved by both disinfection agents were greater than 2 log cycles, and there were not significant differences among them. *Escherichia coli*'s growth was not reassumed nor further reduced during storage (Fig. 1A). With regard to *L. innocua*, their counts were slightly increased in the last sampling point (Fig. 1B), especially in samples treated with SH. These results can be explained by the psychrothrophic nature of *L. innocua*.

On the other hand, at the lowest and more realistic contamination load (Fig. 2), reductions registered after treatments were similar to those at the highest load (Fig. 1). However, when samples were inoculated with low loads of surrogates disinfection treatments reduced their counts below the detection limit (2.3 log CFU.g^{-1} of FT). Therefore reductions could have been greater (Fig. 2). The initial disinfection achieved in *E. coli* inoculated samples and treated with SH and CA persisted 3 and 9 days, respectively (Fig. 2A). Meanwhile, in Fig. 2B it can be seen that samples inoculated with *L. innocua* reassumed their growth after day 3. Once again, likely because of the ability of the genus *Listeria* spp. to growth under refrigeration, being more evident in samples treated with SH.

There is scarce information of the effects of organic acid disinfection treatments for leafy greens on controlling possible regrowth of inoculated surrogates under refrigerated storage. Huang et al. (2012) evaluated the evolution of baby spinach inoculated with *E. coli* O157:H7 under refrigerated storage. They found that lactic acid enhanced the antimicrobial efficacy of allyl isothiocyanate employed in the washing and disinfection step during refrigerated storage. Ganesh et al. (2010, 2012), who treated spinach leaves with electrostatic spraying of organic acids alone and in combination with grape seed extract, found that the bactericidal effect of organic acids persisted and further reduced microbial counts of *Salmonella typhimurium* and *E. coli* O157:H7 along refrigerated storage, respectively.

3.2. Impact on sensory quality

Results of sensory quality are presented in Fig. 3 and Table 2. Data indicated that there were not significant interactions between factors treatment and time. Additionally, the effect of treatment was not significant either. Hence sensory attributes and instrumental color did not differ after citric acid and sodium hypochlorite treatments, demonstrating that the traditional treatment can be replaced from a sensory point of view. On the other hand, factor time affected the quality indices evaluated as it was expected due to leaves senescence. Sensory attributes' ratings presented in Fig. 3 decreased along refrigerated storage, but even in the last sampling point scores were above the acceptability threshold. Results are consistent with the study carried out by Piagentini, Güemes, and Pirovani (2002), who evaluated sensory quality of spinach leaves treated with citric acid under other processing conditions and packed with another packaging material. Furthermore, it is noteworthy that neither fresh-odor nor off-odor were detected in samples during the whole refrigerated storage period, hence those data are not presented in Fig. 3.

Purchase decision of fresh vegetables, including minimally processed leafy greens, is strongly affected by color as it impacts directly on consumer visual perception (Agüero, Bevilacqua, & Roura, 2012), thus it is important to evaluate it through an instrumental method. As Negueruela (2012) indicated, visual perception of related colors should be analyzed bearing in mind L* (lightness), C* (chroma), and H° (hue). The increase in L* and the reduction in H° detected in disinfected samples over refrigerated storage can be interpreted as leaves turning from green to a lighter green (Table 2), most likely because of leaves senescence. Chroma (C*) increased over refrigerated storage, possibly due to water loss and pigments concentrations; however, significant differences were only detected at 7 and 15% significance level in SH and CA samples, respectively (Table 2). An increase in C* can be interpreted as an increment in color intensity, which was not perceived by the sensory panel (Fig. 3). In addition, color differences expressed by ΔE remained below 5.0 (Table 2), indicating no remarkable differences

according to the threshold established by Melgosa, Pérez, Yebra, Huertas, and Hita (2001). Finally, instrumental color evaluated through H° significantly correlated with color judge by the trained panel (r = 0.88). When comparing results with other studies, similarly, Bermúdez-Aguirre and Barbosa-Cánovas (2013), Choi et al. (2012), and Park et al. (2011) did not find significant differences in instrumental color in lettuce (and spinach in the second study) treated with organic acids. Ho et al. (2011), who treated a lettuce and spinach mix with peroxyacetic and lactic acid in combination, did not find a negative influence on product quality when compared with chlorinated water; they also reported that decay was significantly reduced.

It is noteworthy that even though overall sensory quality was acceptable till day 14, in a previous unpublished experiment where spinach leaves were treated and stored following the same procedures, microbial quality was only assured till day 13. The latter was established taking into account the following criteria: for mesophilic bacteria, a maximum count of 7.7 log CFU.g^{-1}, taken from French regulations (Corbo, Nobile, & Sinigaglia, 2006; 2004), and for yeasts and molds Fleet (1992) suggested a maximum level of 5 log CFU.g^{-1}.

4. Conclusion

Storing spinach under refrigeration between harvest and processing played a key role not only in reducing their deterioration but also in assuring their safety by maintaining citric acid and sodium hypochlorite effectiveness against *E. coli* and *L. innocua*. Overall, citric acid performed better than sodium hypochlorite in controlling the inoculated surrogates' regrowth in spinach samples along refrigerated storage (6.5 ± 1 °C; 9 days). Furthermore, there were not significant differences between CA and SH treated samples with respect to their sensory quality. Therefore, a single-step washing with citric acid 0.5% (2.5 min, at 25 °C) represents an effective, feasible, and compatible with organic produce disinfection treatment for spinach leaves; and could constitute an alternative to their traditional washing and disinfection method.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.lwt.2017.04.047.

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