Title: Antimicrobial activity of suspensions and nanoemulsions of citral in combination with heat or pulsed electric fields

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Running head: Nanoemulsions of citral in combined treatments

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SIGNIFICANCE AND IMPACT OF THE STUDY

The exploration of different delivery systems of antimicrobial compounds such as citral in aqueous food products aids in the establishment of successful combined treatments for food preservation. While at room temperature, citral in form of a nanoemulsion shows a higher antimicrobial activity; its combination with heat would imply a partial loss of the outstanding synergistic lethal effect achieved when added in suspension form. Therefore, the most suitable procedure to magnify the synergism between heat and citral when processing juices would merely require an intense homogenization step prior to the combined treatment.

ABSTRACT

The application of essential oils in form of nanoemulsions has been proposed as a method to improve their solubility in aqueous solutions, and hence their antimicrobial activity. The objective of this study was to evaluate the antimicrobial activity of citral, applied directly or in combined treatments with heat or pulsed electric fields (PEF), as a function of the inoculation procedure assayed: (i) a simple, vigorous shaking method by vortex agitation (suspension of citral; s-citral) or (ii) the previous preparation of nanoemulsions by the emulsion phase inversion (EPI) method (nanoemulsion of citral; n-citral). N-citral was more effective in either inhibiting or inactivating *Escherichia coli* O157:H7 Sakai than s-citral. However, when combined with heat, a greater synergistic effect was observed with s-citral rather than with n-citral, either in lab media (pH 7.0 and 4.0) or apple juice. For instance, while almost 5 log_{10} cell cycles were inactivated in apple juice after 15 min at 53°C in the presence of 0.1 µl ml^{-1} of s-citral, the use of n-citral required 30 min. The use of nanoemulsions did not modify the slight synergism observed when citral and mild PEF were combined (150 µs, 30 kV cm^{-1}).
INTRODUCTION

The design of successful food preservation processes relies on the establishment of those treatment conditions which guarantee the innocuity of the product with minimal detriment of nutritional and sensory parameters. A way of achieving such effective treatment conditions is through the combination of antimicrobial agents and physical methods, following the hurdle theory proposed by Leistner and Gorris (1995). Over the last few decades, essential oils (EOs) extracted from aromatic and medicinal plants or citrus fruits, as well as their individual constituents, have been tested as antimicrobial agents in combination with heat or pulsed electric field pulses (PEF). In many cases, remarkable synergistic effects in the lethality of these combinations allowed to decrease treatment temperatures and/or antimicrobial doses, or to potentiate the inactivation achieved by PEF (Corbo et al. 2009; Espina et al. 2010, 2012; de Souza et al. 2016).

Among EO constituents, citral has displayed a broad-spectrum antimicrobial activity and has been shown to be one of the most effective antimicrobials applied either directly or in combined treatments. For instance, very low doses of citral (0.018-0.2 µl ml⁻¹) in combination with heat were capable of inactivating five log₁₀ cell cycles of *Escherichia coli* O157:H7, showing promising results for the preservation of apple juice (Espina et al. 2010).

Nevertheless, many authors observe that the hydrophobicity of EOs may hamper their homogenous dispersion in aqueous food products (Maswall and Dar 2013; Piorkowski and McClements 2014). As an effective approach to improve the dispersion of EOs into food products and minimize the phase separation, the formation of food-grade emulsions using low-energy preparation methods is a field of great interest (Komaiko and McClements 2016). Procedures such as the emulsion phase inversion (EPI) method
generate metastable oil-in-water nanoemulsions, are simple to implement, and no expensive equipment is required. While the effect of emulsification on the antimicrobial activity of EOs has been studied in depth (Donsi et al. 2011; Maswall and Dar 2013; Moghimi et al. 2016; Zhang et al. 2017), few studies have evaluated their influence when applied in combination with other treatments such as heat or PEF. In this regard, to the best of our knowledge, there are no documented studies on the behavior of EPI nanoemulsions of citral under heat or PEF treatments. The first objective of this study was to evaluate the antimicrobial activity of citral against *Escherichia coli* O157:H7 Sakai, applied directly or in combined treatments with heat or PEF, as a function of the citral preparation procedure used: (i) a simple vigorous shaking method by vortex agitation (suspension of citral; s-citral), and (ii) the previous preparation of nanoemulsions by the EPI method (nanoemulsion of citral; n-citral). As a second objective, the combination of heat with s- and n-citral to inactivate *Escherichia coli* O157:H7 Sakai was assayed in apple juice.

**RESULTS AND DISCUSSION**

**Droplet size and stability of nanoemulsions**

Nanoemulsions of citral were prepared by the EPI method and characterized during a period of storage under refrigeration. As shown in Table 1, droplet size remained below 200 nm during the 4 months of storage. On the other hand, there were no significant differences ($p>0.05$) among survival curves of *E. coli* O157:H7 Sakai obtained in the presence of 0.6 µl ml$^{-1}$ at pH 4.0, either from different nanoemulsion preparations and different storage times, which indicates that the EPI method assayed allows the
obtention of reproducible and stable nanoemulsions of citral. Figure 1A shows the mean values and the standard deviation of nine survival curves corresponding to different emulsions and storage times. To the best of our knowledge, there are no documented studies on the production and characterization of nanoemulsions of citral using the EPI method. The stability of nanoemulsions of D-limonene obtained with the same methodology has already been shown by Zhang et al. (2014) and Mate et al. (2016) for 6 months.

**Effect of citral as a suspension or nanoemulsion on antimicrobial activity**

The use of citral in the form of a nanoemulsion decreased the MIC from 0.8 (s-citral) to 0.7 (n-citral) µl ml⁻¹ (p<0.05) against *E. coli* O157:H7 Sakai. This result reveals that n-citral can inhibit microbial growth more efficiently than s-citral. Nevertheless, this increase in the antibacterial efficacy of citral after its emulsification was much lower than that observed for other antimicrobials in previous works (Komaiko and McClements 2016). For example, Moghimi et al. (2016) demonstrated a 4-fold reduction of the MIC value with a nanoemulsion of sage oil.

Moreover, as shown in Figure 1, n-citral was also more effective than s-citral in the inactivation of *E. coli* O157:H7 Sakai in both pH 4.0 and 7.0 treatment media. While the kinetics of inactivation obtained with s-citral showed a prolonged shoulder followed by a rapid decrease, those obtained with n-citral were approximately linear. The greater antimicrobial activity of EOs in the form of nanoemulsions has been associated with their increased polarity, thanks to the coating of the surfactants that reduces surface tension of the oil droplets (Piorkowski and McClements, 2014). Thus, the emulsification of hydrophobic substances might reduce their immiscibility in aqueous solutions, making them readily dispersible in the treatment media. In this regard,
Moghimi et al. (2016) proved that conversion of sage oil into a nanoemulsion improved its antibacterial activity by enhancing its ability to promote the destruction of bacterial cell membranes. Therefore, the preparation of a nanoemulsion of citral seems to be the best option in treatments applied at room temperature. In addition, the nanoemulsion would also provide the chemical stability required for prolonged inhibitory or bactericidal treatments (Maswall and Dar 2013).

On the other hand, the comparison of survival curves shown in Figures 1A and 1B confirms the exceptional higher resistance of *E. coli* O157:H7 Sakai in acid than in neutral pH, already described by Somolinos et al. (2010), and shows that this phenomenon also occurs when citral is applied as a nanoemulsion.

**Effect of citral as a suspension or nanoemulsion on microbial inactivation in combined treatments**

The synergism observed when combining heat or PEF with EOs has been directly related to the detection of injured cells in the cytoplasmic and outer membranes of Gram-negative bacteria after the application of physical technologies as a single agent (Mackey 2000; Somolinos et al. 2009; Arroyo et al. 2010; Espina et al. 2012). In those studies, heat and PEF treatments were applied, and survivors were recovered in non-selective and selective media (Figure 2). Results obtained in the non-selective medium show that heat and PEF treatments acting as single agents inactivated less than 1 log10 cell cycle. Based on the differences in the log10 cycles of inactivation achieved when comparing the non-selective with selective media, heat treatments at pH 4.0 (Figure 2A) injured more survivors in the outer than in the cytoplasmic membrane (*p*<0.05), whereas PEF treatments (Figure 2C) did the opposite. Under these treatment conditions,
at least 90% of survivors were injured and susceptible to a citral attack during the combined treatments.

Regarding the combination of heat and citral, a remarkable synergism was observed: almost $5 \log_{10}$ cells cycles of inactivation were achieved at both pH as a function of the citral addition procedure (s- or n-citral) and the antimicrobial concentration. In contrast with the results shown when citral was acting as a single agent (Figure 1), the addition of s-citral was to some extent more effective than n-citral either at pH 7.0 or at pH 4.0, showing the greatest difference in the presence of 0.1 $\mu l$ ml$^{-1}$ at pH 7.0 ($p<0.05$) (Figure 2B). Thus, maintaining the synergism when using n-citral would require higher concentrations of the hydrophobic compound to achieve the desired level of microbial inactivation. It should be noted that the high levels of inactivation achieved with the combined treatment might correspond to the tail of the survival curves. As a consequence, greater differences in the antimicrobial efficacy between s- and n-citral might be expected at lower concentrations or shorter treatment times of the combined processes. Further experiments should be performed to explain this unexpected inversion of the compared activity of s-citral and n-citral when increasing the treatment temperature up to 53°C. Possible hypotheses to consider include the increased solubility of s-citral under mild thermal treatments, providing the optimum dispersion in the treatment medium, and a greater availability of s-citral to interact with microbial cells in the short time span of the treatment in comparison with n-citral, which might be partially retained by the surfactant.

Nevertheless, these results differ from those obtained with the EPI method, using propylene glycol instead of ethanol as co-surfactant, to obtain nanoemulsions of D-limonene for the inactivation of *Listeria monocytogenes* (Mate et al. 2016). Therefore, it should be considered that the result of using nanoemulsions in combined treatments...
with heat is likely to vary as a function of the type or complexity of the antimicrobial molecules and/or the microorganism investigated.

According to Arroyo et al. (2010), the detection of sublethal injury, specifically on the outer membrane of Gram-negative bacteria after PEF, is the key when identifying treatment conditions under which PEF may act synergistically with citral. Thus, the scarce presence of sublethally injured cells on the outer membrane of *E. coli* 0157:H7 Sakai after PEF (Figure 2C and D) might justify the limited synergism observed with s-citral. To the best of our knowledge, the bacterial inactivation of PEF treatments with EPI nanoemulsions has never been previously tested. In the present study, no significant differences were found between the efficacy of the combined treatments using PEF and s- or n-citral, and no worsening or improvement of the overall lethality of the combined treatment was observed when emulsifying citral prior to its incorporation. Further experiments combining s-citral or n-citral with PEF applied at higher temperatures could be conducted to further explore the effect of emulsification on each one of these physical preservation treatments.

**Effect of citral in the form of suspension or nanoemulsion on microbial inactivation by combined treatments applied to apple juice**

In order to validate in a food model the results obtained with citral in form of suspension or nanoemulsion in lab media, apple juice was contaminated with *E. coli* O157:H7 Sakai and treated with a combined treatment of mild heat and citral (s- and n-citral) (Figure 3). Again, the combined treatment with s- or n-citral was more effective than the use of mild heat as a single agent, showing a remarkable synergistic effect. In addition, the main conclusion obtained in lab media was confirmed in apple juice: s-citral was more effective than n-citral when applied at mild temperature at any treatment
time. For instance, while almost $5 \log_{10}$ cell cycles were inactivated in apple juice after 15 min at 53°C in the presence of 0.1 µl ml$^{-1}$ of s-citral, the use of n-citral required doubling the treatment time. Thus, the vigorous agitation in vortex method for suspending citral, which might simulate the action of the actual industrial homogenizers employed as a previous stage in the pasteurization process of liquid foods, seems to be sufficient to disperse the oil correctly and favor its antimicrobial action in apple juice, providing enough stability at least during the short duration of the combined treatment. In this regard, the treatment time required to comply with FDA regulation (FDA 2001), which recommended that juices should be hygienized reaching $5 \log_{10}$ reductions (99.999%) of pathogens of concern such as *E. coli* O157:H7, would be approx. 2 times shorter when using s- than n-citral. Nevertheless, it should be highlighted that citral in the form of nanoemulsion also attained the $5 \log_{10}$ reductions of the pathogen. Thus, if its use represented any advantage – for instance, for the purpose of limiting the modification of the flavor in food due to high EO concentrations in comparison with non-encapsulated ones – then it would be interesting to reconsider its use in the development of combined treatments for food preservation. Further studies are required in order to evaluate the influence of this and other encapsulation methods on the efficacy of combined processes with heat, PEF, or other successful emerging technologies, such as high hydrostatic pressure, and other EOs and EO constituents.

MATERIALS AND METHODS

**Bacterial strain and cultures**

*E. coli* O157:H7 Sakai *stx* 1A$^{-}$/stx 2A$^{-}$ was kindly provided by Kyu-Tae Chang (The National Primate Research Center, KRIBB, Ochang, South Korea). This strain was isolated from an outbreak involving white radish sprout (Michino *et al.* 1999). During
this investigation, the cultures were maintained and kept frozen at −80 °C in cryovials. Broth subcultures were prepared by inoculating one single colony from a plate into a test tube containing 5 mL of sterile tryptic soy broth (Biolife, Milan, Italy) with 0.6% yeast extract added (Biolife) (TSBYE). After inoculation, the tubes were incubated overnight at 37 °C. Along with these subcultures, 250-ml Erlenmeyer flasks containing 50 mL of TSBYE were inoculated to a final concentration of $10^4$ cells ml$^{-1}$. These flasks were incubated under agitation (130 rpm; Selecta, mod. Rotabit, Barcelona, Spain) at the appropriate temperature until the stationary growth phase was reached (24 ± 2 h).

**Antimicrobial and direct addition procedure**

Citral was obtained from Sigma Aldrich Chemie (Steinheim, Germany). Following the procedure described by Friedman *et al.* (2002), a vigorous shaking method was used to prepare citral suspensions (s-citral) in the treatment media: citrate-phosphate buffer (McIlvaine’s buffer) at pH 7.0 and pH 4.0 (Dawson *et al.* 1974) and apple juice.

**Preparation of nanoemulsions**

The preparation of nanoemulsions of citral (n-citral) was based on the catastrophic phase inversion method (Zhang *et al.* 2014, 2017), also known as the emulsion phase inversion (EPI) method. The aqueous phase was prepared by mixing 1.5 ml of ethanol (Sigma) with 40.5 ml of sterile distilled water. The oily phase was prepared by mixing 3 ml of Tween 80 (Panreac, Barcelona, Spain) with 5 ml of citral. Nanoemulsions were prepared from a mixture of oily phase by slowly adding aqueous phase with gentle magnetic agitation. The addition rate of aqueous phase was kept constant at approximately 1.0 ml min$^{-1}$. A water-in-oil (W/O) emulsion with a high oil-to-water ratio was formed, and then increasing amounts of water were added to the system with
continuous stirring. The amount of water added to a W/O emulsion was progressively increased, until a phase inversion occurred and an oil-in-water (O/W) emulsion was formed. Final concentration of citral in the nanoemulsion was 587 mM.

**Droplet size and stability of nanoemulsions**

The emulsion droplet size and size distribution (polydispersity index-PDI) was determined using a particle size analyzer (Brookhaven, 90 Plus, New York, USA). Droplet size was analyzed using dynamic light scattering (DLS) technique. Prior to all the experiments, the nanoemulsion formulations were diluted with water to eliminate the multiple scattering effects. Emulsion droplet size was estimated by an average of three measurements and is presented as the mean diameter of volume distribution. Droplet size was evaluated after fresh preparation, and then after 1 month of storage under refrigeration. The reproducibility of the protocol for preparing nanoemulsions and their stability during 30 days was also evaluated by comparing the survival curves of *E. coli* O157:H7 Sakai in the presence of 0.6 µl ml⁻¹ of n-citral at pH 4.0, as described below.

**Evaluation of the antimicrobial activity of citral**

Citral (s-citral and n-citral) was evaluated to determine the minimum inhibitory concentration (MIC) and to obtain survival curves against *E. coli* O157:H7 Sakai. Regarding the MIC, tubes containing 5 mL of TSBYE and different concentrations of citral (0.5–1 µl ml⁻¹) were inoculated to a final concentration of 10⁵ cells ml⁻¹. Negative control (without microorganisms), positive control (without citral), and diluent control (the amount of ethanol corresponding to the maximum n-citral concentration assayed -1 µl ml⁻¹) were also prepared. After 24 h of incubation at the appropriate temperature in a
shaking thermostatic incubator (Bunsen, mod. BTG, Madrid, Spain), survivors were enumerated, as described below. The MIC was the lowest concentration of citral at which bacteria failed to grow, showing counts equal to the initial concentration.

Moreover, the antimicrobial properties of s-citral and n-citral were evaluated by determining survival curves in treatment media of different pH. Cells from stationary-phase cultures were added at final concentrations of $10^7$ cells ml$^{-1}$ to buffers with citral (0.6 μl ml$^{-1}$ at pH 4.0 and 0.4 μl ml$^{-1}$ at pH 7.0). Buffer pH was not modified as a consequence of adding antimicrobial compounds. Antimicrobial compound treatments were carried out at room temperature (23 ± 2 °C). Samples were taken at preset intervals and survivors were enumerated, as described below. Previous experiments showed that untreated cells of \textit{E. coli} O157:H7 Sakai at concentrations of $10^7$ cells ml$^{-1}$ were insensitive to incubation at pH 7.0 or 4.0 for 1 h at room temperature (data not shown).

\textbf{Evaluation of microbial inactivation by heat and heat combined with citral}

For the preparation of heat-treated samples, microorganisms were resuspended at a concentration of $10^7$ cells ml$^{-1}$ in treatment media thermostated at 53 ± 0.2°C (FX Incubator, A.F. Ingeniería S. L., Valencia, Spain). Buffer of pH 7.0 and 4.0, as well as the same treatment media with s-citral or n-citral to a final concentration of 0.1 and 0.2 μl ml$^{-1}$ were used. Antimicrobials were added once the treatment media were thermostated, and prior to microbial inoculation. The actual temperature was controlled with a thermocouple wire introduced in a 0.9 mL buffer test tube inside the incubator. After 15 min at 53 ºC, samples were taken, immediately placed on ice, and survivors and sublethally-injured cells were evaluated, as explained below. Following the same methodology, heat treatments were also carried out in apple juice (Don Simón, Murcia, España), as well as in the presence of s-citral and n-citral (0.1 μl
Samples were collected at pre-set intervals and survivors were evaluated to obtain survival curves.

**Evaluation of microbial inactivation by PEF and PEF combined with citral**

PEF treatments were carried out using ScandiNova equipment (Modulator PG, ScandiNova, Uppsala, Sweden), described by Saldaña *et al.* (2010). Before treatments, micro-organisms were centrifuged at 6000 x g for 5 min and resuspended at a concentration of 10^7 cells ml^{-1} in citrate–phosphate buffer of pH 7.0 and 4.0 (electrical conductivity was adjusted to 1 mS/cm), as well as in the same treatment media with s-citral or n-citral to a final concentration of 0.2 μl ml^{-1}. Then, 0.5 ml of the microbial suspensions was placed into the treatment chamber with a sterile syringe. Exponential waveform pulses at an electrical field strength of 30 kV cm^{-1} and a pulse repetition rate of 1 Hz were used in this study. The specific energy input of each pulse 2.7 kJ kg^{-1}. Cell suspensions were treated for 50 pulses (pulse width 3 μs). Experiments started at room temperature (23 ± 2 ºC). In all experiments, the temperature of the samples after the application of 50 pulses was lower than 35 ºC. After treatment, samples were taken, and survivors and sublethally-injured cells were evaluated, as explained below.

**Counts of viable cells**

After treatments, samples were adequately diluted in 0.1% w/v peptone water (Biolife). Subsequently, 0.1 ml samples were pour-plated onto Tryptic Soy Agar (Biolife) with 0.6% Yeast Extract added (Biolife) (TSAYE). Plates were incubated for 24 h at 37ºC. Previous experiments showed that longer incubation times did not influence the surviving cell counts. After incubation, colonies were counted with an improved image.
analyzer automatic counter (Protos; Analytical Measuring Systems, Cambridge, United Kingdom), as previously described (Condón et al. 1996).

Detection of sublethal injury

In order to determine bacterial cell injury, treated samples were also plated onto TSAYE with 4% sodium chloride (Panreac) added (TSAYE-SC) and onto TSAYE with 0.25% bile salts (Oxoid, Hampshire, United Kingdom) added (TSAYE-BS) in order to evaluate cytoplasmic membrane damage and outer membrane damage, respectively (Mackey, 2000). These levels of sodium chloride and bile salts were previously determined as the maximum non-inhibitory concentrations for native cells (data not shown). Samples recovered in selective media were incubated for 48 h. Previous experiments showed that longer incubation times did not influence survival counts.

The proportion of sublethally injured cells was estimated by the difference in the number of log_{10} cycles of colony forming units (CFU) obtained after plating treated cells in the nonselective (TSAYE) and selective (TSAYE-SC) media.

Data analyses

The error bars in the figures indicate the mean ± standard deviations from the data obtained from at least three independent experiments. All analyses were performed with GraphPad PRISM® software (GraphPad Software, Inc., San Diego, CA). Unpaired t-Student and one-way ANOVA tests were performed to test statistically significant differences among two or more groups, respectively (p = 0.05).

ACKNOWLEDGEMENTS
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CONFLICT OF INTEREST

No conflict of interest declared.

REFERENCES


Table 1. Droplet size and polydispersity index (PDI) of nanoemulsions of citral stored under refrigeration. Data represent the mean ± standard error of the mean of at least three independent experiments.

<table>
<thead>
<tr>
<th>Storage time (months)</th>
<th>Droplet size (nm)</th>
<th>PDI</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>161 ± 5</td>
<td>0.096 ± 0.012</td>
</tr>
<tr>
<td>1</td>
<td>160 ± 2</td>
<td>0.159 ± 0.011</td>
</tr>
<tr>
<td>4</td>
<td>191 ± 0</td>
<td>0.291 ± 0.005</td>
</tr>
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</table>
FIGURE LEGENDS

Figure 1. Survival curves of *Escherichia coli* O157:H7 Sakai (initial concentration: 3 x 10^7 CFU/mL) after exposure to s-citral (●) or n-citral (○) in buffer of pH 4.0 (A) (0.6 μl ml^-1) and 7.0 (B) (0.4 μl ml^-1) at room temperature and recovered in TSAYE. Data represent the mean ± standard error of the mean (error bars) of at least three independent experiments. The dotted line represents the detection limit.

Figure 2. Cycles of inactivation of *Escherichia coli* O157:H7 Sakai (initial concentration: 3 x 10^7 CFU/mL) after a heat treatment at 53 ºC for 15 min (A,B) and after a PEA treatment at 30 kV cm^-1 for 150 μs (50 pulses of 3 μs) (C, D) in buffers of pH 4.0 (A, C) and 7.0 (B, D), and recovered in TSAYE (grey bars; NS), TSAYE-SC (grey bars; SC), TSAYE-BS (grey bars; BS), or recovered in TSAYE after combined treatment with heat and s-citral (black bar) or n-citral (white bar) (0.1 and 0.2 μl ml^-1). Data represent the mean ± standard error of the mean (error bars) of at least three independent experiments. Statistical differences (Student’s t-test, p<0.05) among combined treatments is represented by different superscript letters. The dotted line represents the detection limit.

Figure 3. Survival curves of *Escherichia coli* O157:H7 Sakai (initial concentration: 10^7 CFU/mL) to a heat treatment at 53 ºC in apple juice (▲), and a combined treatment of heat and s-citral (●) or n-citral (○) (0.1 μl ml^-1) and recovered in TSAYE. Data represent the mean ± standard error of the mean (error bars) of at least three independent experiments. The dotted line represents the detection limit.
Figure 1

A

B

Log$_{10} \frac{N_t}{N_0}$ vs. Time (min)

Log$_{10} \frac{N_t}{N_0}$ vs. Time (min)
Figure 2

A

B

C

D
Figure 3

![Graph showing Log Nt/N0 vs. Time (min)]