International Journal of Food Microbiology 331 (2020) 108786

Contents lists available at ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

Chitosan nanoemulsions of cold-pressed orange essential oil to preserve fruit juices

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ARTICLE INFO

Keywords:
Escherichia coli O157:H7, *Citrus sinensis* essential oil
Chitosan
Nanoemulsion
Heat
Combined process
Synergism
Sensory analyses
Fruit beverages

ABSTRACT

Sweet orange essential oil is obtained from the peels of *Citrus sinensis* (CSEO) by cold pressing, and used as a valuable product by the food industry. Nanoencapsulation is known as a valid strategy to improve chemical stability, organoleptic properties, and delivery of EO-based products. In the present study we encapsulated CSEO using chitosan nanoemulsions (cn) as nanocarrier, and evaluated its antimicrobial activity in combination with mild heat, as well as its sensorial acceptability in orange and apple juices. CSEO composition was analyzed by GC–MS, and 19 components were identified, with limonene as the predominant constituent (95.1%). cn-CSEO was prepared under low shear conditions and characterized according to droplet size (< 60 nm) and polydispersity index (< 0.260 nm). Nanoemulsions were stable for at least 3 months at 4 ± 2 °C. cn-CSEO were compared with suspensions of CSEO (s-CSEO) (0.2 μL of CSEO/mL) in terms of antibacterial activity in combination with mild heat (52 °C) against *Escherichia coli* O157:H7 Sakai. cn-CSEO displayed a greater bactericidal activity than s-CSEO at pH 7.0 and pH 4.0. The validation in fruit juices showed an improved bactericidal effect of cn-CSEO in comparison with s-CSEO when combined with mild heat in apple juice, but not in orange juice. In both juices, the combination of CSEO and mild heat exerted synergistic lethal effects, reducing the treatment time to cause the inactivation of up to 5 Log_{10} cycles of *E. coli* O157:H7 Sakai cells. Finally, the sensory characteristics of both juices were acceptable either when using s-CSEO or CSEO nanoemulsified with chitosan. Therefore, as a promising carrier for lipophilic substances, the encapsulation of EOs with chitosan nanoemulsions might represent an advantageous alternative when combined with mild heat to preserve fruit juices.

1. Introduction

The protection of inner tissues of crustaceans and insects is highly dependent on the presence of chitin in their exoskeletons. Chitin is a linear (1 → 4) β-linked homopolymer of the aminosugar *N*-acetyl-d-glucosamine with mechanic and permeability barrier functions (Merzendorfer and Zimoch, 2003). The industrial deacetylation of chitin produces chitosan, which has received great scientific and industrial attention because of its diverse biological activities, and biocompatibility (Yuan et al., 2016). Moreover, due to its low toxicity, chitosan has been listed as a GRAS product (Generally Recognized As Safe) in the U.S., and it is recognized as food additive in other countries (Japan, Italy, Finland, etc.) (Matica et al., 2017). Thanks to its emulsifying properties, chitosan can be used as a coating material to encapsulate bioactive compounds while avoiding their oxidation or degradation. Thus, chitosan has been explored as a drug delivery system in pharmaceutical applications (Morin-Crini et al., 2019). In the food industry, encapsulated systems based on chitosan could be used to protect sensitive ingredients from environmental conditions, to improve water solubility of lipophilic compounds, and to mask possible undesirable flavoring properties of active ingredients (Rocha et al., 2017).

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https://doi.org/10.1016/j.ijfoodmicro.2020.108786
Received 25 March 2020; Received in revised form 5 June 2020; Accepted 3 July 2020
Available online 06 July 2020

Essential oils (EOs) are great candidates for incorporation into chitosan-based capsules with the aim of preserving beverages for several reasons: a) their sensitivity to oxygen, light, and heat during food processing and storage, b) their high volatility and low solubility in aqueous phase, and c) their possible undesired taste and odor, as a function of EO and food composition, and especially at high concentrations (Mahato et al., 2019). The encapsulation of EOs normally improves their distribution in food, while minimizing possible unpleasant organoleptic qualities in fruit juices (Donsì et al., 2011; Viacava et al., 2018). The most widely used edible polymers in nanoemulsion-based EO delivery systems for food preservation include starch, alginate, gellan gum, chitosan, zein, gellatin, and cyclodextrin (Froiio et al., 2019). However, under certain conditions, certain emulsifiers could reduce the antimicrobial activity of encapsulated EOs as compared to free EOs (Salvia-Trujillo et al., 2014).

Citrus EOs have been shown to exert a powerful antimicrobial effect against juice-related bacteria (de Souza et al., 2016). The application of citrus EOs during heat treatments of fruit juices displayed a synergistic lethal effect against pathogen and spoilage bacteria (de Souza Pedrosa et al., 2019; Espina et al., 2014, 2012). This combination allowed the reduction of intensity of effective heat treatment and of the final concentration of EOs for the hygienization of fruit juices.

Sweet orange essential oil (CSEO) is obtained from the peels of *Citrus sinensis* (L.) Osbeck by cold pressing, and is one of the most widely used EOs on an industrial level, with over 100 tons produced worldwide each year (Lubbe and Verpoorte, 2011). This product finds applications in different fields such as food, cosmetics, pharmaceutics, and agrochemicals (Bica et al., 2011; Charara et al., 1992; Isman, 2017; McClements, 2013).

The addition of 0.2 μL/mL of CSEO in orange juice reduced 2.5 fold the duration of heat treatment for inactivation of 5 Log_{10} cycles of *Escherichia coli* O157:H7, and maintained the degree of sensory acceptance of the resulting juice (Espina et al., 2014). A combination of this process with nanoemulsions of citrus EOs might reduce heat treatment intensity even further.

The antimicrobial properties of encapsulated EOs for purposes of food preservation have been extensively demonstrated in diverse fresh foods, such as fruits, sausages, cheese, and chicken fillet, mainly applied during food storage [see the review by Ju et al., 2019]. However, there is still a lack of knowledge regarding the effect of food processing conditions, e.g. heat treatments, on the antimicrobial effects of encapsulated EOs. In this regard, Amiri et al. (2020) noted that the thermal resistance of EOs increased when they were loaded into chitosan particles.

We therefore carried out this research with the following goals: (i) to evaluate the chemical composition of CSEO; ii) to obtain and characterize chitosan nanoemulsions of CSEO (cn-CSEO); iii) to assess the antimicrobial efficacy of cn-CSEO as a single hurdle or in combination with mild heat in laboratory media at pH 7.0 and 4.0; iv) to assess the antimicrobial efficacy of cn-CSEO in combination with mild heat for the inactivation of 5 Log_{10} cycles of *Escherichia coli* O157:H7 in orange juice and apple juices; and (v) to evaluate the acceptability of orange and apple juices with added cn-CSEO by means of sensory analysis.

2. Material and methods

2.1. Citrus sinensis essential oil (CSEO)

Sweet orange (*Citrus sinensis*) essential oil (CSEO) used in this investigation was kindly provided by Indulleida S.A. (Lérida, Spain). This commercial EO was prepared using a mixture of different orange varieties ('Washington Navel', 'Navelate', 'Navelina', 'Salustiana', 'Valencia Late') by cold press system extraction. The peels of fresh fruits were cold-pressed, the EO was separated from the crude extract by centrifugation, and stored in the dark in sealed glass vials at 4 °C until use.

Following the method described by Friedman et al. (2002), a vigorous shaking procedure was applied to prepare CSEO suspensions (s-CSEO) either in citrate-phosphate buffer at pH 7.0 (23.38 g/L Na_2HPO_4 + 3.70 g/L citric acid) and 4.0 (10.94 g/L Na_2HPO_4 + 12.9 g/L citric acid), or in squeezed orange and apple juices (0.2 μL of CSEO/mL), prepared as described below.

2.2. Chemical analysis of Citrus sinensis essential oil (CSEO)

CSEO was diluted 1:100 in n-hexane (Carlo Erba, Milan, Italy) then injected (1 μL, split ratio: 1:50) into a GC–MS system consisting in an Agilent 6898N gas chromatograph equipped with an autosampler and fitted with a 5973 N mass spectrometer. The stationary phase was composed of an HP-5MS capillary column (5% phenylmethylpolysiloxane, 30 m length × 0.25 mm i.d., 0.1 μm film thickness, Agilent, Folsom, CA), while the mobile phase was helium (99.999%) at 1 mL/min. Oven temperature was programmed from 60 °C to 220 °C at 4 °C/min, then raised to 280 °C at 11 °C/min. The mass spectra were acquired in electron impact mode (EI, 70 eV) in the range 29–400 *m/z*. Qualitative and quantitative analysis was performed by using the MSD ChemStation software (Agilent, Version G1701DA D.01.00) (Maggi et al., 2010). A mixture of n-alkanes (C8-C24) was purchased from Supelco (Bellefonte, CA) and used to calculate the linear retention indices (RIs). As components, α-Pinene, sabinene, β-pinene, myrcene, α-phellandrene, n-octanal, δ-3-carene, limonene, terpinolene, linalool, citronellal, α-terpineol, neral, geranial, and (*E*)-caryophyllene were identified by comparing retention times (RTs), RIs, and mass spectra (MS) of chromatographed peaks with those of authentic standards purchased from Sigma-Aldrich (Milan, Italy). n-Nonanal, n-decanal, α-copaene and γ-murolene were identified by interactive combination of RI and MS of peaks with those recorded in ADAMS, NIST 17, and FFNSC3 libraries. Relative peak area percentages were obtained by peak area normalization without applying correction factors. Values are the mean of three independent injections (three different preparations of CSEO solution).

2.3. Nanoemulsion preparation, droplet characterization and stability

Nanoemulsions of CSEO (cn-CSEO) were prepared by catastrophic phase inversion method (also known as the emulsion phase inversion, or EPI method; see Zhang et al., 2017) according to Pagán et al. (2018), with adaptations. Aqueous and oil phase solutions were produced. Chitosan (medium molecular weight [190,000–310,000 Da, deacetylation degree 75–85%, Sigma-Aldrich] solution [0.5% (w/v)]) was prepared by agitating chitosan in an aqueous acetic acid solution (1%, v/v [Panreac]) at 40 °C overnight.

The aqueous phase was prepared by mixing 1.5 mL of ethanol (Sigma-Aldrich) with 35.5 mL of sterile distilled water and 5 mL of chitosan solution. The oily phase was prepared by mixing 3 mL of Tween 80 (Panreac, Barcelona, Spain) with 5 mL of CSEO. Nanoemulsions were prepared from a mixture of the oily phase by slowly adding the aqueous phase with gentle magnetic agitation. The addition rate of aqueous phase was kept constant at approximately 1.0 mL/min. A water-in-oil (W/O) emulsion with a high oil-to-water ratio was formed, after which increasing amounts of water were added to the system by 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 CSEO in the nanoemulsion was 10%, determined by calculation.

The emulsion droplet size and size distribution (polydispersity index, PDI) was determined using a particle size analyzer (Brookhaven, 90 Plus, New York, NY). Droplet size was analyzed using dynamic light scattering (DLS). Prior to all experiments, the nanoemulsion formulations were diluted with water to eliminate 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 just after preparation, and then after 1, 2, and 3 months of storage under refrigeration (4 ± 2 °C). The reproducibility of the protocol for preparing nanoemulsions, as well as their stability for 3 months, were likewise evaluated by comparing the survival curves of *E. coli* O157:H7 Sakai obtained after a heat treatment (at 52 °C for 30 min) in the presence of cn-CSEO at pH 4.0, as described below.

2.4. Fruit juices

Oranges ('Valencia') and apples ('Golden') were purchased at a local supermarket (Zaragoza, Spain) in the commercial maturation stage, and selected for similar shape and uniform color, with absence of mechanical damages and no visible signs of infection. The fruits were surface-disinfected by immersion in a sodium hypochlorite solution (0.15 μL/mL, pH 7.2 adjusted using 1 M NaOH) for 5 min, then washed with sterile distilled water, and dried for 30 min in a biosafety cabinet. Oranges were subsequently squeezed (mod. WDF-OJ150; Mizumo S.L., Elche, Spain), and apples were aseptically peeled, cut into small pieces, and crushed using a food processor (Robot-Coupe, Blixer 6 V.V., Burgundy, France). Strained orange and apple juices were sealed and stored at −18 °C in 20 mL plastic tubes. The final pH of the orange and apple juices was 3.8 ± 0.1 and 4.1 ± 0.1, respectively.

For sensory analysis, the fruits were purchased in the same week of their evaluation, transformed into juice following the procedure described above, and stored at 0–4 °C until sensory evaluation within the following 6 h.

2.5. Micro-organisms and growth conditions

E. coli O157:H7 Sakai Δstx1A/Δstx2A- (Kim et al., 2010) was kindly provided by Prof. Kyu-Tae Chang, a strain isolated from an outbreak associated with white radish sprout (Michino et al., 1999), and genetically modified thereafter in order to remove Shiga toxin genes. Mutant strain was obtained following the one-step PCR mutagenesis method (Datsenko and Wanner, 2000). In addition to PCR verification, VTEC-RPLA® and Vero cell cytotoxicity assays were performed to confirm the deletion of stx1A and stx2A (Kim et al., 2010). Culture preparation and growth conditions were the same as those reported by Luis-Villaroya et al. (2015).

2.6. Cell inactivation assessment by s-CSEO or cn-CSEO

The antimicrobial activity of s-CSEO and cn-CSEO was evaluated by ascertaining the degree of bacterial inactivation in citrate-phosphate buffer (7.0 and 4.0), as well as in orange and apple juices. In order to match previously published data (Espina et al., 2011; Luis-Villaroya et al., 2015; Pagán et al., 2018), we added cells from stationary-phase cultures at final concentrations of 3×10^7 CFU/mL to the treatment media, with and without s-CSEO and cn-CSEO (CSEO final concentration: 0.2 μL/mL). Buffer pH was not altered by the addition of antimicrobial compounds. We applied antimicrobial compound treatments for 30 min at room temperature (20 ± 2 °C). Samples were taken at preset intervals, and survivors were counted as described below.

2.7. Cell inactivation assessment by heat treatment, and by combined treatments (heat and s-CSEO or cn-CSEO)

Heat treatments and combined treatments were carried out in an incubator (FX Incubator, mod. ZE/FX, Zeulab, Zaragoza, Spain). To monitor heating temperature, a thermocouple was used (Ahlborn, mod. Almemo 2450, Holzkirchen, Germany). Treatment temperature was chosen on the basis of preliminary results (data not shown). As treatment media, we used 1 mL of citrate-phosphate buffer at pH 7.0 and 4.0, orange and apple juices, as well as the same media with s-CSEO or cn-CSEO added (CSEO final concentration: 0.2 μL/mL). Once the treatment temperature was reached, the microbial suspension was added to a final concentration of 3×10^7 CFU/mL. Treatment temperature was kept constant at 52 ± 0.2 °C, and at preset intervals, samples were taken and survivors were counted.

2.8. Counts of viable cells

Samples were diluted after treatment in 0.1% *w/v* peptone water (Oxoid). Subsequently, 0.1-mL samples were pour-plated onto a recovery medium (TSAYE). Plates were incubated at 37 °C for 24 h. An image analyzer automatic counter (Protos; Analytical Measuring Systems, Cambridge, United Kingdom) was used in order to count the colony-forming units (CFUs.) Inactivation was expressed in terms of the extent of reduction in Log_{10} counts (CFU) after each type of treatment, and the detection limit was -5.0 Log_{10}.

2.9. Statistical analysis for microbial experiments

In order to evaluate the efficacy of lethal treatments, results were obtained from at least three independent experiments carried out on separate working days with different microbial cultures. Results were represented as the mean ± standard deviation using the PRISM® program (GraphPad Software, Inc., San Diego, USA). Data were analyzed and submitted to comparison of averages via ANOVA followed by a *post-hoc* Tukey's test and *t*-tests with GraphPad PRISM®. Differences were considered significant if $p < 0.05$.

2.10. Sensory analyses procedure

The sensory portion of the study was performed in the sensory laboratory at the Pilot Plant of Food Science and Technology (University of Zaragoza). A total of 65 untrained panelists were recruited from the staff and students of the Veterinary Faculty at the University of Zaragoza, Spain. The panelists were distributed in 9 private booths in different shifts to minimize distractions and possible interactions during sensory analysis, under white fluorescent light. Prior to sensory analysis, panelists were provided with instructions on how to proceed during the test.

Natural orange and apple juices, prepared as described above, were poured into 1.5 L glass bottles, after which the necessary quantities of s-CSEO and cn-CSEO were added to reach a concentration of 0.2 μL/mL CSEO, followed by vigorous shaking to ensure an even distribution. Natural orange and apple juices were also prepared without adding CSEO and used as a control. The samples and the control were kept under refrigeration (4 ± 2 °C); 30 min before each sensory test shift, a portion of the prepared juices was placed at room temperature. The samples of each juice (with s-CSEO and cn-CSEO) and the control were presented to the panelists at the same time in counterbalanced order; yogurt was offered as a palate cleanser. For each sample, 20 mL of juice was offered in a transparent 10 cL coded glass cup at room temperature. The same procedure was followed for the testing of the apple juice samples.

Panelists were asked to compare the samples of each juice (with s-CSEO and cn-CSEO) and the control, and to determine the hedonic acceptance of all the samples by ranking them in a 1–9 scale (from 'dislike extremely' to 'like extremely') for 4 sensory parameters: flavor, color, odor and overall acceptability.

2.11. Statistical analysis for sensory test data

The results of the sensory analysis were automatically collected and subsequently processed for statistical analysis. The GraphPad PRISM® program was employed to represent the results, to study the distribution of the samples, and to evaluate statistically significant differences.

Table 1
Chemical composition of the essential oil of *Citrus sinensis*.

No	Component[a]	RI[b]	RI adams[c]	Peak area%[d]	ID[e]
1	α-Pinene	925	932	0.7 ± 0.2	Std
2	Sabinene	964	969	0.7 ± 0.2	Std
3	β-Pinene	967	974	0.1 ± 0.0	Std
4	Myrcene	987	988	2.0 ± 0.4	Std
5	α-Phellandrene	1001	1002	tr[f]	Std
6	*n*-Octanal	1003	998	0.3 ± 0.1	Std
7	δ-3-Carene	1006	1008	0.2 ± 0.1	Std
8	Limonene	1025	1024	95.1 ± 1.0	Std
9	Terpinolene	1083	1086	tr	Std
10	Linalool	1099	1095	0.2 ± 0.0	Std
11	*n*-Nonanal	1104	1100	0.1 ± 0.0	RI,MS
12	Citronellal	1152	1148	tr	Std
13	α-Terpineol	1187	1186	tr	Std
14	*n*-Decanal	1205	1201	0.3 ± 0.1	RI,MS
15	Neral	1240	1235	tr	Std
16	Geranial	1270	1264	0.1 ± 0.0	Std
17	α-Copaene	1366	1374	tr	RI,MS
18	(*E*)-Caryophyllene	1411	1417	tr	Std
19	γ-Muurolene	1478	1478	0.1 ± 0.0	RI,MS
	Total identified (%)				99.9
	Chemical classes (%)				
	Monoterpene hydrocarbons				98.8
	Oxygenated monoterpenes				0.4
	Sesquiterpene hydrocarbons				0.1
	Others				0.6

[a] The order of compounds is consistent with elution from a HP-5MS column (30 m × 0.25 mm i.d., 0.1 μm f.t.).
[b] RI, temperature-programmed retention index calculated using a mixture of *n*-alkanes (C_8–C_{24}).
[c] Literature retention index taken from ADAMS library.
[d] Relative percentage values are mean of three replicates ± standard deviation.
[e] ID, identification method: Std, comparison of retention time, retention index and mass spectrum with those of analytical standard (Sigma-Aldrich, Milan, Italy); RI, coherence of the calculated RI with respect to those reported in ADAMS library; MS, marching with spectra stored in ADAMS, NIST 17 and FFNSC2 libraries.
[f] tr, traces, % < 0.1.

Histograms of distribution were prepared, and a D'Agostino & Pearson normality test ($p < 0.05$) was conducted to determine the normality of distribution of each parameter for all samples. Sensory results were represented by box and whisker plots indicating the mean and the 2.5, 25, 50, 75, and 97.5 percentiles. Data were analyzed and submitted to comparison of averages using analysis of variance (ANOVA) followed by *post-hoc* Tukey test, considering differences as significant if $p < .05$.

3. Results and discussion

3.1. Chemical composition of Citrus sinensis essential oil (CSEO)

The chemical composition of CSEO is reported in Table 1, in which a total of 19 identified components are listed according to their elution from a HP-5MS capillary column. The oil was almost entirely made up of monoterpene hydrocarbons (98.8%), whereas oxygenated monoterpenes (0.4%), sesquiterpene hydrocarbons (0.1%), and aliphatic aldehydes (0.6%) were scarce. The CSEO composition was dominated by limonene, which accounted for 95.1% of the total composition. Among the minor components, only myrcene (2.0%) exceeded 1%. α-Pinene (0.7%), sabinene (0.7%), β-pinene (0.1%), and δ-3-carene (0.2%) were the other compounds representative of monoterpene hydrocarbons; linalool (0.2%) and geranial (0.1%) for oxygenated monoterpenes; α-copaene (traces), (*E*)-caryophyllene (traces) and γ-muurolene (0.1%) for sesquiterpene hyrocarbons; n-octanal (0.3%), n-nonanal (0.1%) and n-decanal (0.3%) for aliphatic aldehydes.

Table 2
Droplet size and polydispersity index (PDI) of nanoemulsions of *Citrus sinensis* essential oil storage under refrigeration. Data represent the mean ± standard error of the mean of at least three independent experiments.

Storage (months)	Droplet size (nm)	PDI
0	55.5 ± 7.9[a]	0.251 ± 0.021[a]
0.5	54.9 ± 5.7[a]	0.231 ± 0.013[a]
1.0	59.2 ± 4.6[a]	0.242 ± 0.022[a]
1.5	55.6 ± 5.3[a]	0.240 ± 0.022[a]
2	55.0 ± 3.5[a]	0.236 ± 0.014[a]
3	58.2 ± 4.5[a]	0.262 ± 0.018[a]

Different superscript letters in the same column show a significant difference ($p < 0.05$).

The obtained data are in agreement with studies available in the literature that characterized CSEO produced by different suppliers (Aboudaou et al., 2019; Bica et al., 2011; Espina et al., 2011; Gonçalves et al., 2018; Oboh et al., 2017; Uprety and Rakshit, 2017). In this regard, Geraci et al. (2017) studied 12 cultivars of CSEO and observed that the main component in all cultivars was limonene, at a percentage ranging from 73.9 to 97%.

3.2. Characterization and stability of chitosan nanoemulsion of CSEO (cn-CSEO)

The most common method to obtain chitosan nanoparticles is by ionic gelation with tripolyphosphate (TPP) (Feyzioglu and Tornuk, 2016; Ghaderi-Ghahfarokhi et al., 2017); however, with the aim of simplifying the production method, we fabricated cn-CSEO with an oil-in-water emulsion technique, thereby avoiding the use of TPP and other conditioning stages.

DLS technique was applied to evaluate the hydrodynamic diameter and polydispersity index (PDI) of nanoemulsions (Table 2). PDI is a measure of the size distribution of particles in cn-CSEO. The PDI values obtained here, lower than 0.3, indicate that the droplets have a narrow size distribution, which is related to the stability of the nanoemulsions (Dickinson, 2003). In this regard, as shown in Table 2, size distribution remained stable over 3 months, as well as the hydrodynamic diameter of the nanoparticles (55.5–59.2 nm), without any significant differences during storage time at 4 ± 2 °C ($p > 0.05$). This method is thus advantageous, since it provides stable solutions for at least 3 months under refrigeration.

The particle size obtained with this methodology was very small in comparison to other studies that also used chitosan as nanocarrier, and which achieved a droplet size of around 100 (Woranuch and Yoksan, 2013), 300 (Hasani et al., 2018; Hasheminejad et al., 2019) and 500 nm (Keawchaoon and Yoksan, 2011). This difference may be due to the method we followed to prepare the emulsions, the degree of acetylation of chitosan, or the low concentration of chitosan in the nanoparticle-forming solution, since a greater relative amount of this polymer in the dispersion may lead to greater particle sizes (Guinebretière et al., 2002; Sreekumar et al., 2018).

3.3. Synergistic effect of heat combined with s-CSEO or cn-CSEO in laboratory media

The main problem in using EOs to preserve food is that their flavor, is so strong that the doses required to achieve sufficient antimicrobial efficacy affect the sensory properties of food (Burt, 2004; Hyldgaard et al., 2012; Mani-López et al., 2017). However, when the use of EOs in combination with other technologies (mild heat, high hydrostatic pressure, etc.) results in a synergistic effect (Berdejo et al., 2019; de Carvalho et al., 2018; Guevara et al., 2015; Mate et al., 2016; Pagán et al., 2018), antimicrobial doses can be reduced until they lie below the sensory rejection limit (de Souza et al., 2016; Espina et al., 2012).

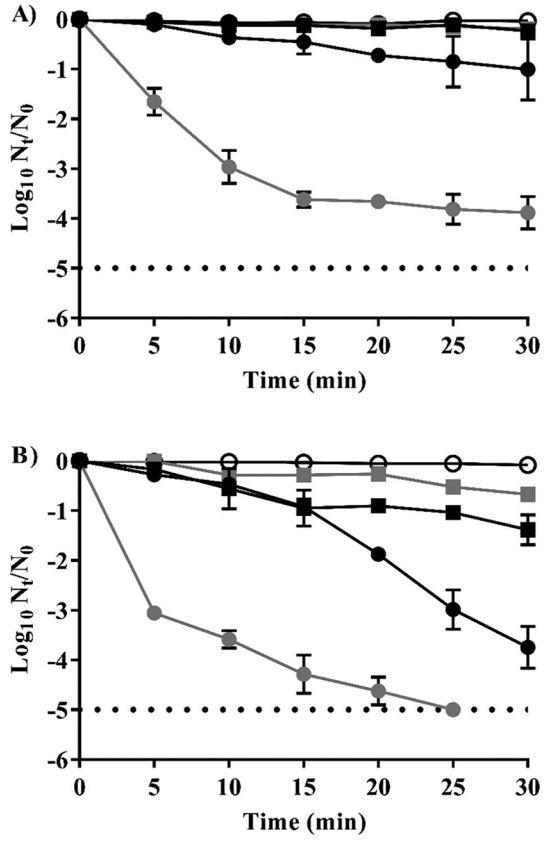

Fig. 1. Survival curves of *Escherichia coli* O157:H7 Sakai (initial concentration: 3 × 107 CFU/mL) after heat treatment at 52 °C (○), *Citrus sinensis* essential oil (CSEO) treatment in form of a suspension (■) or chitosan nanoemulsion (■) (0.2 μL/mL of CSEO) and combined treatment with heat and CSEO in form of a suspension (●) or chitosan nanoemulsion (●) (0.2 μL/mL of CSEO), for 30 min in phosphate-citrate buffers of pH 7.0 (A) and 4.0 (B). Data represent the mean ± standard deviation (error bars) of at least three independent experiments. The dotted line represents the detection limit (−5.0 Log_{10}).

The second major problem of EOs is their chemical instability due to oxidation, high reactivity, and hydrophobicity, which impairs homogeneous distribution (Mahato et al., 2019). Chitosan, which, like EOs, is a natural product, has been proposed as a biocompatible carrier for the preparation of EO nanoemulsions in order to overcome those disadvantages. Nevertheless, it is unknown whether the use of chitosan would hamper the synergism between heat and EOs described above, or whether its presence would negatively affect the sensory characteristics of food.

As a previous step to assess the synergism between mild heat and CSEO in form of a suspension (s-CSEO) or of a chitosan nanoemulsion (cn-CSEO), we tested the antimicrobial efficacy of heat treatment (52 °C for 30 min) of both forms of CSEO (0.2 μL/mL) applied individually against *E. coli* O157:H7 Sakai. This EO concentration (0.2 μL/mL) was established for comparative purposes based on previous results (Ait-Ouazzou et al., 2011, 2013; Espina et al., 2011) and because of its successful sensorial acceptance in fruit juices (Espina et al., 2014). Thus, Fig. 1 shows the inactivation curves obtained by the heat treatment and by the EO treatments (s-CSEO or cn-CSEO) at 20 °C acting separately at pH 7.0 (Fig. 1A) or pH 4.0 (Fig. 1B). As shown in the figure, after a 30-min treatment the sum of the lethality caused by the heat treatment and by the EO treatments effect scarcely inactivated 0.2 Log_{10} cycles at pH 7.0, both with s- or cn-CSEO, and up to 1 Log_{10} cycles when s-CSEO was applied at pH 4.0. At pH 4.0, cn-CSEO displayed a lower ($p < 0.05$) antimicrobial activity as compared to s-CSEO. Monoterpenes from CSEO might interact with chitosan molecules, e.g. by hydrogen bonds, thereby controlling the release of CSEO during treatment time (Yuan et al., 2016). Consequently, a lower release of CSEO from cn-CSEO when short treatments are applied might contribute to this result. This could be associated with the lower bioavailability of EOs to interact against the bacterial population when short treatments are applied (Merino et al., 2019). In the case of prolonged bacteriostatic activity, however, other authors have shown an increase of the antibacterial efficacy of different EOs after their emulsification (Komaiko and McClements, 2016; Moghimi et al., 2016).

Fig. 1 shows the survival curves of *E. coli* O157:H7 Sakai after a combined treatment at 52 °C for 30 min in the presence of s-CSEO or cn-CSEO (0.2 μL/mL of CSEO) in buffers of pH 7.0 and 4.0. As can be observed, the inactivation of *E. coli* O157:H7 Sakai by the combined treatment always occurred more rapidly than the additive effect. An outstanding synergism was observed between heat and cn-CSEO, rather than with s-CSEO. At pH 7.0 (Fig. 1A), the heat treatment in the presence of s-CSEO scarcely increased the degree of inactivation up to 1 Log_{10} cell cycle after 30 min; however, a reduction of 4 Log_{10} cycles was achieved with cn-CSEO after 15 min, followed by a prolonged tail for at least 15 min. At pH 4.0 (Fig. 1B), the combined treatment was more effective than at pH 7.0, achieving almost 4 and 5 Log_{10} cycles of inactivation after 30 min in the presence of s-CSEO and 20 min in the presence of cn-CSEO, respectively. The results obtained at the two pH levels suggest that the preparation of nanoemulsions with chitosan is the most efficient method to enhance synergism between heat and CSEO. In fact, these results showed that the antimicrobial efficacy of CSEO in the combined treatment increased with the manner of preparation of the EO (cn-CSEO vs s-CSEO), with treatment temperature (52 °C vs room temperature), and with the acidification of the treatment medium pH (pH 4.0 vs pH 7.0). The smaller droplet size achieved with chitosan (> 60 nm) in comparison with other nanoemulsions of D-limonene (Garre et al., 2020; Mehanna, 2020) or *Thymbra capitata* EO (Merino et al., 2019) (> 100 nm) prepared with lecithin or zein, respectively, might be responsible for the remarkable antimicrobial synergistic effect exerted by cn-CSEO in this study.

The greater antimicrobial activity of EOs in the form of nanoemulsions has been explained by their increased polarity, thanks to the coating of the surfactants that reduces the oil droplets' surface tension (Piorkowski and McClements, 2014). Thus, the emulsification of hydrophobic substances might reduce their immiscibility in aqueous solutions, making them more readily dispersible in the treatment media. In this regard, Moghimi et al. (2016) proved that conversion of sage EO into a nanoemulsion improved its antibacterial activity by enhancing its ability to promote the destruction of bacterial cell membranes. Moreover, the synergism observed when combining heat with EOs has been directly associated with 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 (Arroyo et al., 2010; Espina et al., 2012; Somolinos et al., 2010). The greater antimicrobial effect of cn-CSEO as compared with s-CSEO at 52 °C might be explained by several factors: a) chitosan coating prevents the degradation of CSEO at mild temperatures (Amiri et al., 2020); and/or b) temperature rise destabilizes weak interactions between chitosan and CSEO, thereby increasing the release of CSEO (Yuan et al., 2016); and/or c) the interaction between CSEO nanoemulsions and bacterial envelopes improves thanks to membrane fluidification, and higher solubility of the emulsifier caused by temperature (Shao et al., 2018).

Fig. 2 shows the mean values and the standard deviation of four survival curves of *E. coli* O157:H7 Sakai, corresponding with different nanoemulsions and storage times in combination with heat treatments. The similarity of the obtained survival curves ($p < 0.05$) corroborates the stability of the antimicrobial activity of cn-CSEO for a period of at least three months. The stability for at least 3 months of nanoemulsions of D-limonene, which is the main component of CSEO, obtained with other nanoemulsifiers using similar methodologies, has already been shown by Mate et al. (2016), Zhang et al. (2017), and Mehanna (2020).

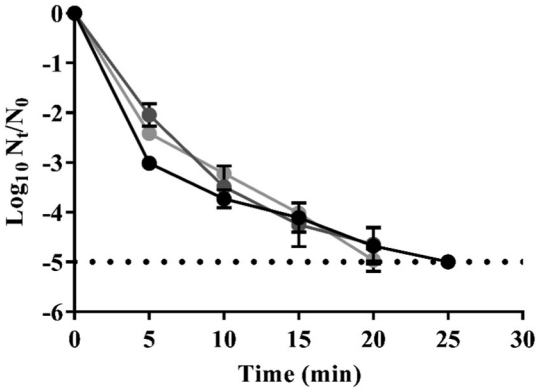

Fig. 2. Survival curves of *Escherichia coli* O157:H7 Sakai (initial concentration: 3×10^7 CFU/mL) after a heat treatment at 52 °C in combination with 0.2 μL/mL chitosan nanoemulsion of *Citrus sinensis* essential oil (cn-CSEO) for 30 min in phosphate-citrate buffer of pH 4.0. Survival curves correspond to three different cn-CSEO: freshly prepared (●), after 1 month (●), and after 3 months (●) of storage at 4 ± 2 °C. Data represent the mean ± standard deviation (error bars) of at least three independent experiments. The dotted line represents the detection limit (−5.0 Log_{10}).

However, to the best of our knowledge, no documented studies have been previously carried out on the stability of the antimicrobial activity of chitosan nanoemulsions during a storage period, or on the stability of their antimicrobial activity when applied in combination with mild heat.

3.4. Synergistic effect of heat and s-CSEO and cn-CSEO in orange and apple juices

Orange and apple juices were selected to validate in a food model the results obtained with CSEO in the form of suspension or nanoemulsion in lab media. The selection of fruit juices was based on the best performance of the nanoemulsions of chitosan at acid pHs and, on their sensory compatibility with CSEO flavor (Espina et al., 2014). Orange (pH 3.8) and apple (pH 4.1) juices were contaminated with *E. coli* O157:H7 Sakai and treated with a simultaneous combination of mild heat and CSEO (s- and n-CSEO) (Fig. 3). As shown by Fig. 3, the simultaneous application of mild heat and s-CSEO or cn-CSEO was more effective than the separate application of the hurdles, showing remarkable synergistic effects in both fruit juices. Nevertheless, the results were different as a function of the fruit juice assayed: while the use of s-CSEO or cn-CSEO in combination with mild heat described a similar survival curve ($p < 0.05$) in orange juice (Fig. 3A), causing the inactivation of 5 Log_{10} cycles of *E. coli* O157:H7 Sakai cells after 25 min, cn-CSEO was much more effective in apple juice (Fig. 3B) than s-CSEO ($p < 0.05$). The lower synergism when combining heat and cn-CSEO in orange juice than when doing so in apple juice might be related to the higher pectin concentration in orange juice. Amine groups of chitosan present a pKa value ~6.5, meaning that in acid beverages, such as fruit juices, chitosan nanoemulsions are positively charged, thereby enabling their solubility as a function of pH (Abdelmalek et al., 2017; Szymańska and Winnicka, 2015). Positively charged chitosan has been found to be effective as a clearing agent to precipitate negatively charged pectins (Chatterjee et al., 2004). Electrostatic interaction between chitosan and pectins might limit the antimicrobial activity of cn-CSEO in orange juice. In apple juice, cn-CSEO caused an extra 1.5 Log_{10} cycles of inactivation after 30 min at 52 °C, almost reaching 5 Log_{10} cycles of *E. coli* O157:H7 Sakai, which is a requirement established by FDA regulation for the hygienization of fruit juices (FDA, 2001). Therefore, the preparation of nanoemulsions of CSEO with chitosan might represent an advantageous alternative for the preservation of fruit juices when combined with mild heat since, as has been shown in apple juice, it would allow for the destruction of the pathogenic microorganisms at reduced treatment intensity while minimizing the loss of nutritional and sensory characteristics of the fresh fruit juices.

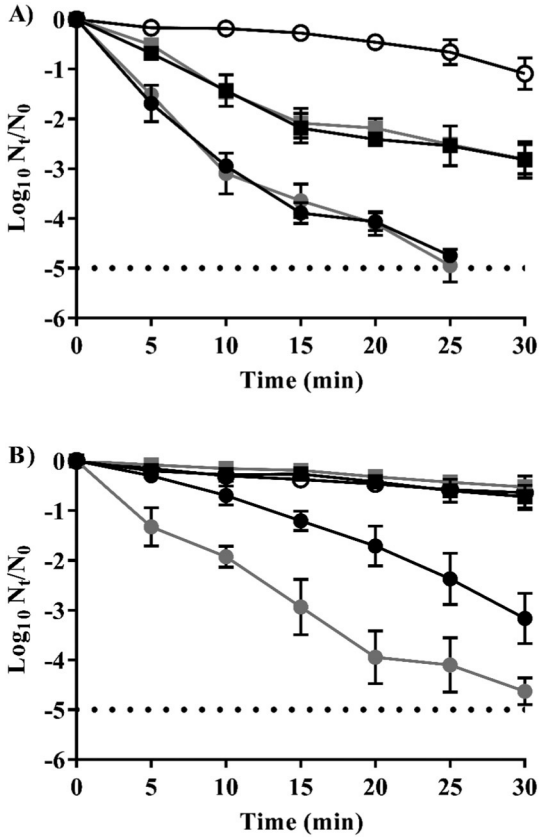

Fig. 3. Survival curves of *Escherichia coli* O157:H7 Sakai (initial concentration: 3×10^7 CFU/mL) after heat treatment at 52 °C (○), *Citrus sinensis* essential oil (CSEO) treatment in form of a suspension (■) or chitosan nanoemulsion (■) (0.2 μL/mL of CSEO) and combined treatment with heat at 52 °C and *Citrus sinensis* essential oil (CSEO) in form of a suspension (●) or chitosan nanoemulsion (●) (0.2 μL/mL of CSEO) for 30 min in natural orange (A) and apple (B) juices. Data represent the mean ± standard deviation (error bars) of at least three independent experiments. The dotted line represents the detection limit (−5.0 Log_{10}).

3.5. Determination of the hedonic acceptability of orange and apple juices with s-CSEO and cn-CSEO added

Once the antimicrobial activity of CSEO applied in suspension (s-CSEO) and nanoemulsion (cn-CSEO) had been assayed, a sensory analysis was conducted with an untrained panel to determine the acceptability of s-CSEO and cn-CSEO in apple and orange juices.

Figs. 1S and 2S show the dispersions of the hedonic score data for the 4 sensory parameters tested in orange (S1) and apple (S2) juices with and without 0.2 μL/mL of s-CSEO or cn-CSEO added: overall acceptability, flavor, color, and odor. The D'Agostino & Pearson normality test was carried out on each sensory parameter for both juices in order to study the dispersion of the sensory score test and to know which statistical analysis should be applied. All the hedonic data for both juices revealed a Gaussian distribution of the samples ($p < 0.05$), thus analysis of variance (ANOVA) followed by a *post-hoc* Tukey test was performed to evaluate significant differences ($p < 0.05$) among the averages of the samples (control, s-CSEO and cn-CSEO).

Fig. 4 depicts the box and whisper plots corresponding to the hedonic data for the overall acceptability, flavor, color, and odor of orange juice. As observed in Fig. 4, the hedonic data for orange juice show

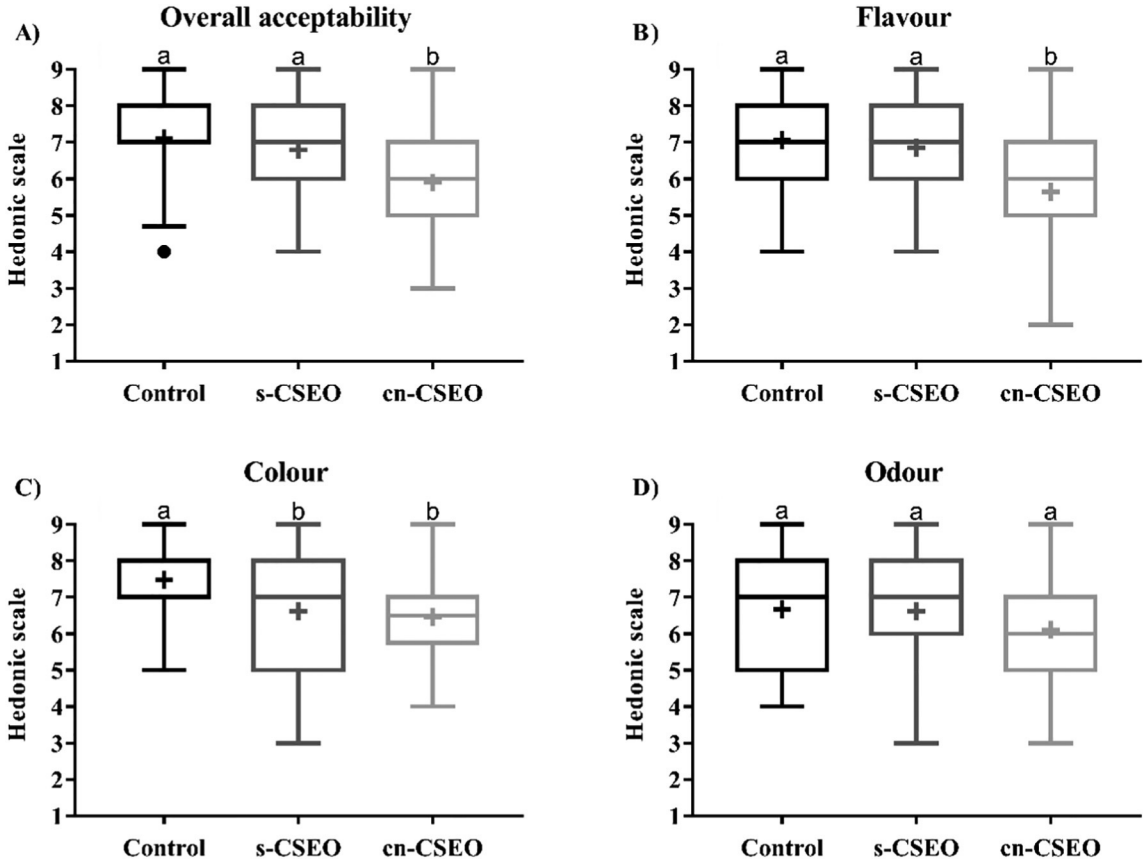

Fig. 4. Box and whisker plots displaying the hedonic data values of natural orange juice (■; control) and with 0.2 μL/mL of *Citrus sinensis* essential oil (CSEO) in form of a suspension (■) or chitosan nanoemulsion (■) added for overall acceptability (A), flavor (B), color (C) and odor (D). The cross represents the hedonic mean, the single points represent the extreme values, the whiskers correspond to 2.5 and 97.5 percentiles, the box correspond to the 25 and 75 percentiles and the median is represented by the central bar in the box. The hedonic values range from 1 to 9. Different letters over the bars represent statistically significant differences ($p < 0.05$) among the means of each group as determined by one-way ANOVA followed by Tukey's multiple pairwise comparison post hoc test.

that the panelists gave a mean score higher than 6 for all the tested parameters. The concentration of 0.2 μL/mL of CSEO in suspension (s-CSEO) as well as in nanoemulsion (cn-CSEO) would thus be accepted by the consumer in orange juice. However, the samples with added CSEO showed lower ratings than control for all parameters. In the case of s-CSEO, although the average values of the 4 parameters were slightly lower than control, the only significant observed differences ($p < 0.05$) were associated with color. On the other hand, cn-CSEO-added samples were sensorially rated lower than control in terms of acceptability, taste and color ($p < 0.05$), although mean scores were always above 6; overall acceptability changed from 7.0 to 6.0 when cn-CSEO was added to orange juice. Comparing the addition of CSEO in the form of suspension or nanoemulsion, overall acceptability and flavor were rated higher in orange juice with s-CSEO than with cn-CSEO ($p < 0.05$). Taking into account that the efficacy of the combined application of heat and s-CSEO or cn-CSEO was exactly the same (Fig. 3A) ($p < 0.05$) under the treatment conditions tested, the results of the sensory test would point toward s-CSEO as the best option for designing a combined treatment with mild heat to preserve orange juice.

Fig. 5 shows the box and whisper plots corresponding to the hedonic data collected for the overall acceptability, flavor, color and odor of apple juice. As shown in Fig. 5, apple juice also achieved a mean score higher than 5.0 (acceptable) for both control and for juice with added cn-CSEO, but not for juice with added s-CSEO in the parameters of color (4.9) and odor (4.9). As in the orange juice, the best hedonic scores for the 4 tested parameters were obtained in the apple control juice but, in this case, the only significant differences observed ($p < 0.05$) were in the parameters of flavor and odor. While the average odor score of control apple juice was 5.8, the odor of apple juice with s-CSEO and cn-CSEO was rated at an average of 4.9 and 5.2, respectively. In contrast with the hedonic data of orange juice, apple juice with cn-CSEO achieved a better flavor rating than apple juice with s-CSEO; the 50-percentile was 6.0 for cn-CSEO juice in comparison to 5.5 for s-CSEO juice. As a result, the use of cn-CSEO would be the best option when combining mild heat and CSEO to preserve apple juice not only because of the better sensory results obtained in comparison with the application of s-CSEO, but also because of its higher antimicrobial efficacy in combination with heat as applied to apple juice (Fig. 3B) ($p < 0.05$).

It is known that most EOs have strong odors and flavors that prevent their direct use as a sole method of food preservation because of the high concentrations required for this purpose (Burt, 2004; Hyldgaard et al., 2012; Mani-López et al., 2017). Thus, in order to reduce EO doses and, consequently, their sensory impact, several studies seek to apply these natural antimicrobials in combination with other technologies (Berdejo et al., 2019; de Carvalho et al., 2018). Furthermore, the use of low concentrations of EOs in combination with other technologies could be a promising method for juice preservation, since consumers accept effective doses in these foods (de Souza et al., 2016). In a previous study, Espina et al. (2014) reported that 0.2 μL/mL of CSEO added to orange juice was accepted by the panelists, and there were no significant differences ($p > 0.05$) in the acceptability of control in comparison to s-CSEO juice. Another recent study demonstrated that concentrations ≤ 0.25 μL/mL of *Citrus limon* (L.) Osbeck EO or ≤ 0.50 μL/mL of *Citrus reticulata* Blanco EO were also accepted by consumers in orange and apple juices (de Souza Pedrosa et al., 2019).

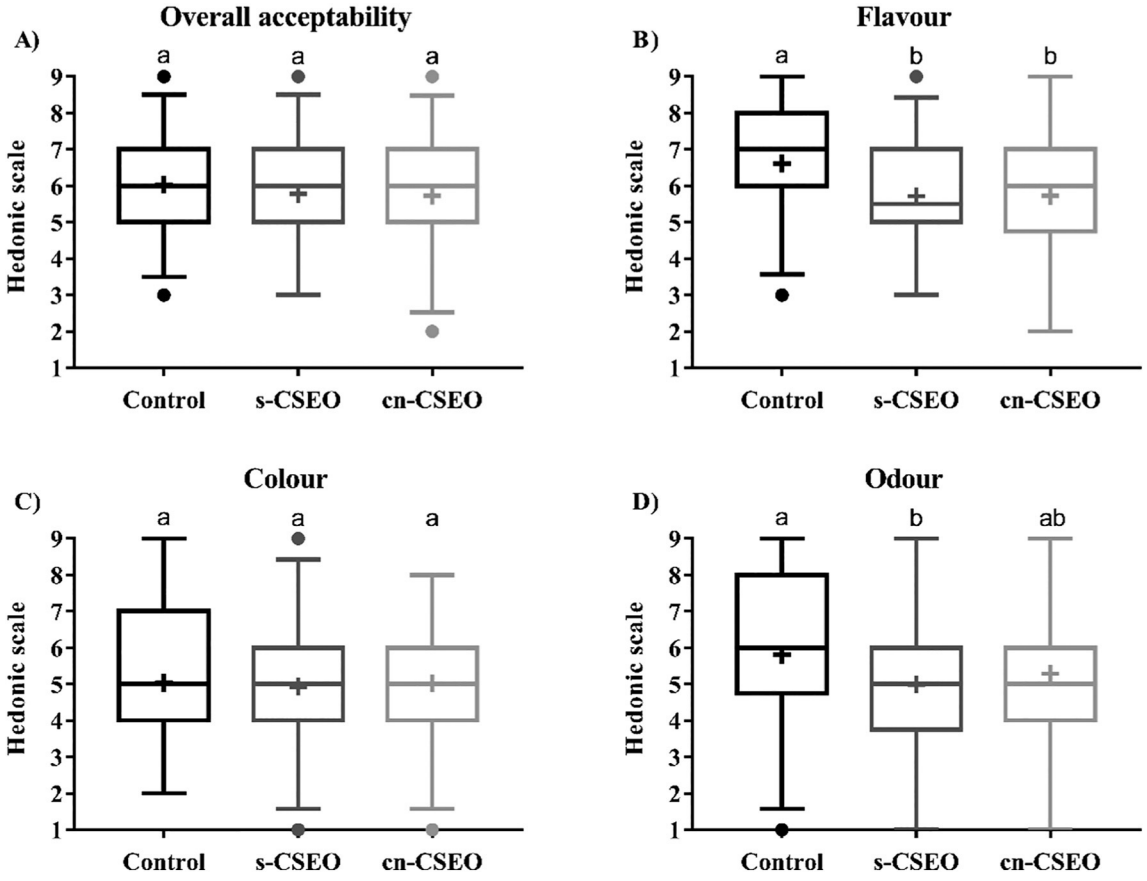

Fig. 5. Box and whisker plots displaying the hedonic data values of natural apple juice (■; control) and with 0.2 μL/mL of *Citrus sinensis* essential oil (CSEO) in form of a suspension (■) or chitosan nanoemulsion (■) added for overall acceptability (A), flavor (B), color (C) and odor (D). The cross represents the hedonic mean, the single points represent the extreme values, the whiskers correspond to 2.5 and 97.5 percentiles, the box correspond to the 25 and 75 percentiles and the median is represented by the central bar in the box. Hedonic values range from 1 to 9. Different letters over the bars represent statistically significant differences ($p < 0.05$) among the means of each group as determined by one-way ANOVA followed by Tukey's multiple pairwise comparison post hoc test.

On the other hand, chitosan has been proposed not only as an emulsifier to increase the stability of EOs, but also to reduce their strong sensory properties (Rocha et al., 2017). For example, the application of clove [*Syzygium aromaticum* (L.) Merr. & L.M. Perry] EO loaded in chitosan nanoparticles by immersion increased the shelf life of pomegranate arils without affecting their sensory properties (Hasheminejad and Khodaiyan, 2020). However, as shown by Figs. 4 and 5, not only did the addition of cn-CSEO, not mask the flavoring properties of the CSEO in orange and apple juices, but it also seemed to slightly decrease the juices' sensory properties. To the best of our knowledge, no previous studies have been conducted on the sensory effect of the use of chitosan as an emulsifier of EOs in fruit juices.

Overall, our results suggest that the use of CSEO at 0.2 μL/mL, either as s-CSEO or cn-CSEO in combination with mild heat treatment is sensorially accepted by consumers when added to orange as well as apple juices, thereby ensuring microbial safety. According to the sensory analysis carried out herein, s-CSEO use would be recommended for orange juice, whereas cn-CSEO is preferable for apple juice preservation.

4. Conclusions

This study has shown chitosan to be an excellent emulsifier that can be incorporated via a simple and reproducible methodology to prepare stable nanoemulsions of *Citrus sinensis* essential oil (cn-CSEO), which is made up of more than 90% of the monoterpene hydrocarbon limonene. The proposed method allowed to achieve a reduced droplet size (< 60 nm). Thus, nanoemulsions remained stable at 4 ± 2 °C for at least 3 months and maintained their antimicrobial activity constant against *E. coli* O157:H7 Sakai in combination with mild heat.

The use of CSEO in the form of nanoemulsions stabilized by chitosan might represent a better alternative to the use of CSEO in suspension to achieve antimicrobial synergistic effects in combination with mild heat. The synergism was greater at acid than at neutral pH, and validated in orange and apple juices. According to the antimicrobial efficacy results and the sensory analysis, the use of CSEO (0.2 μL/mL) in the form of s-CSEO is more highly indicated for the design of an alternative hygienization for orange juice, whereas the form of cn-CSEO is mainly recommendable for apple juice.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper

Acknowledgements

This study was financially supported by Agencia Estatal de Investigación (Spain) (Project No. PGC2018-093789-B-I00), by the European Social Fund, and by the Aragonese Office of Science, Technology and University Research.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijfoodmicro.2020.108786.

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