TITLE:

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

Author names: Roberta Bento[a], Elisa Pagán[b], Daniel Berdejo[b], Rayssa Julliane de Carvalho[c], Sonia García-Embid[d], Filippo Maggi[e], Marciane Magnani[c], Evandro Leite de Souza[f], Diego García-Gonzalo[b] and Rafael Pagán[b*].

Affiliation:

[a]Academic Center in Vitória de Santo Antão, Federal University of Pernambuco

[b]Departamento de Producción Animal y Ciencia de los Alimentos, Facultad de Veterinaria, Instituto Agroalimentario de Aragón-IA2 (Universidad de Zaragoza-CITA), Zaragoza, Spain

[c]Laboratory of Microbial Processes in Foods, Department of Food Engineering, Technology Center, Federal University of Paraíba, João Pessoa, Paraíba, Brazil

[d]Instituto de Ciencia de Materiales de Aragón, CSIC/University of Zaragoza, and CIBER-BBN, Zaragoza, Spain

[e]School of Pharmacy, University of Camerino, Camerino, Italy

[f]Laboratory of Food Microbiology, Department of Nutrition, Health Sciences Center, Federal University of Paraíba, João Pessoa, Paraíba, Brazil

***Corresponding author**: Dr. Rafael Pagán Tomás

Address: Dpto. PACA. Facultad de Veterinaria. Universidad de Zaragoza.

C/ Miguel Servet, 177, 50013, Zaragoza, Spain.

Phone number: 34-976-762675 **Fax. number:** 34-976-761590

E-mail: pagan@unizar.es

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 analysed 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.

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

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).

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

76 delivery systems for food preservation include starch, alginate, gellan gum, chitosan, zein,
77 gellatin, and cyclodextrin (Froiio et al., 2019). However, under certain conditions, certain
78 emulsifiers could reduce the antimicrobial activity of encapsulated EOs as compared to
79 free EOs (Salvia-Trujillo et al., 2014).

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

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

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

96 The antimicrobial properties of encapsulated EOs for purposes of food preservation
97 have been extensively demonstrated in diverse fresh foods, such as fruits, sausages,
98 cheese, and chicken fillet, mainly applied during food storage [see the review by Ju et al.
99 (2019)]. However, there is still a lack of knowledge regarding the effect of food
100 processing conditions, e.g. heat treatments, on the antimicrobial effects of encapsulated

101 EOs. In this regard, Amiri et al. (2020) noted that the thermal resistance of EOs increased
102 when they were loaded into chitosan particles.
103 We therefore carried out this research with the following goals: (i) to evaluate the
104 chemical composition of CSEO; ii) to obtain and characterize chitosan nanoemulsions of
105 CSEO (cn-CSEO); iii) to assess the antimicrobial efficacy of cn-CSEO as a single hurdle
106 or in combination with mild heat in laboratory media at pH 7.0 and 4.0; iv) to assess the
107 antimicrobial efficacy of cn-CSEO in combination with mild heat for the inactivation of
108 5 Log_{10} cycles of *Escherichia coli* O157:H7 in orange juice and apple juices; and (v) to
109 evaluate the acceptability of orange and apple juices with added cn-CSEO by means of
110 sensory analysis.
111
112 **2. Material and Methods**
113
114 *2.1. Citrus sinensis essential oil (CSEO)*
115 Sweet orange (*Citrus sinensis*) essential oil (CSEO) used in this investigation was
116 kindly provided by Indulleida S.A. (Lérida, Spain). This commercial EO was prepared
117 using a mixture of different orange varieties ('Washington Navel', 'Navelate',
118 'Navelina', 'Salustiana', 'Valencia Late') by cold press system extraction. The peels of
119 fresh fruits were cold-pressed, the EO was separated from the crude extract by
120 centrifugation, and stored in the dark in sealed glass vials at 4°C until use.
121 Following the method described by Friedman et al. (2002), a vigorous shaking
122 procedure was applied to prepare CSEO suspensions (s-CSEO) either in citrate-phosphate
123 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
124 + 12.9 g/L citric acid), or in squeezed orange and apple juices (0.2 µL of CSEO/mL),
125 prepared as described below.

126

127 *2.2. Chemical analysis of Citrus sinensis essential oil (CSEO)*

128 CSEO was diluted 1:100 in n-hexane (Carlo Erba, Milan, Italy) then injected (1
129 μL, split ratio: 1:50) into a GC-MS system consisting in an Agilent 6898N gas
130 chromatograph equipped with an autosampler and fitted with a 5973N mass spectrometer.
131 The stationary phase was composed of an HP-5MS capillary column (5%
132 phenylmethylpolysiloxane, 30 m length x 0.25 mm i.d., 0.1 μm film thickness, Agilent,
133 Folsom, CA), while the mobile phase was helium (99.999%) at 1 mL/min. Oven
134 temperature was programmed from 60°C to 220°C at 4°C/min, then raised to 280°C at
135 11°C/min. The mass spectra were acquired in electron impact mode (EI, 70 eV) in the
136 range 29-400 m/z. Qualitative and quantitative analysis was performed by using the MSD
137 ChemStation software (Agilent, Version G1701DA D.01.00) (Maggi et al., 2010). A
138 mixture of n-alkanes (C8-C24) was purchased from Supelco (Bellefonte, CA) and used
139 to calculate the linear retention indices (RIs). As components, α-Pinene, sabinene, β-
140 pinene, myrcene, α-phellandrene, n-octanal, δ-3-carene, limonene, terpinolene, linalool,
141 citronellal, α-terpineol, neral, geranial, and (E)-caryophyllene were identified by
142 comparing retention times (RTs), RIs, and mass spectra (MS) of chromatographed peaks
143 with those of authentic standards purchased from Sigma-Aldrich (Milan, Italy). n-
144 Nonanal, n-decanal, α-copaene and γ-murolene were identified by interactive
145 combination of RI and MS of peaks with those recorded in ADAMS, NIST 17, and
146 FFNSC3 libraries. Relative peak area percentages were obtained by peak area
147 normalization without applying correction factors. Values are the mean of three
148 independent injections (three different preparations of CSEO solution).

149

150 *2.3. Nanoemulsion preparation, droplet characterization and stability*

151 Nanoemulsions of CSEO (cn-CSEO) were prepared by catastrophic phase inversion
152 method (also known as the emulsion phase inversion, or EPI method; see (Zhang et al.,
153 2017) according to Pagán et al. (2018), with adaptations. Aqueous and oil phase solutions
154 were produced. Chitosan (medium molecular weight [190,000-310,000 Da, deacetylation
155 degree 75–85%, Sigma-Aldrich] solution [0.5% (w/v)]) was prepared by agitating
156 chitosan in an aqueous acetic acid solution (1%, v/v [Panreac]) at 40°C overnight.
157 The aqueous phase was prepared by mixing 1.5 mL of ethanol (Sigma-Aldrich)
158 with 35.5 mL of sterile distilled water and 5 mL of chitosan solution. The oily phase was
159 prepared by mixing 3 mL of Tween 80 (Panreac, Barcelona, Spain) with 5 mL of CSEO.
160 Nanoemulsions were prepared from a mixture of the oily phase by slowly adding the
161 aqueous phase with gentle magnetic agitation. The addition rate of aqueous phase was
162 kept constant at approximately 1.0 mL/min. A water-in-oil (W/O) emulsion with a high
163 oil-to-water ratio was formed, after which increasing amounts of water were added to the
164 system by continuous stirring. The amount of water added to a W/O emulsion was
165 progressively increased until a phase inversion occurred and an oil-in-water (O/W)
166 emulsion was formed. Final concentration of CSEO in the nanoemulsion was 10%,
167 determined by calculation.
168 The emulsion droplet size and size distribution (polydispersity index, PDI) was
169 determined using a particle size analyser (Brookhaven, 90 Plus, New York, NY). Droplet
170 size was analyzed using dynamic light scattering (DLS). Prior to all experiments, the
171 nanoemulsion formulations were diluted with water to eliminate multiple scattering
172 effects. Emulsion droplet size was estimated by an average of three measurements, and is
173 presented as the mean diameter of volume distribution.
174 Droplet size was evaluated just after preparation, and then after 1, 2, and 3 months
175 of storage under refrigeration (4±2°C). The reproducibility of the protocol for preparing

176 nanoemulsions, as well as their stability for 3 months, were likewise evaluated by
177 comparing the survival curves of *E. coli* O157:H7 Sakai obtained after a heat treatment
178 (at 52°C for 30 min) in the presence of cn-CSEO at pH 4.0, as described below.
179 .
180 *2.4. Fruit juices*
181 Oranges ('Valencia') and apples ('Golden') were purchased at a local supermarket
182 (Zaragoza, Spain) in the commercial maturation stage, and selected for similar shape and
183 uniform color, with absence of mechanical damages and no visible signs of infection. The
184 fruits were surface-disinfected by immersion in a sodium hypochlorite solution (0.15
185 μL/mL, pH 7.2 adjusted using 1 M NaOH) for 5 min, then washed with sterile distilled
186 water, and dried for 30 min in a biosafety cabinet. Oranges were subsequently squeezed
187 (mod. WDF-OJ150; Mizumo S.L., Elche, Spain), and apples were aseptically peeled, cut
188 into small pieces, and crushed using a food processor (Robot-Coupe, Blixer 6 V.V.,
189 Burgundy, France). Strained orange and apple juices were sealed and stored at -18°C in
190 20 mL plastic tubes. The final pH of the orange and apple juices was 3.8±0.1 and 4.1±0.1,
191 respectively.
192 For sensory analysis, the fruits were purchased in the same week of their evaluation,
193 transformed into juice following the procedure described above, and stored at 0-4°C until
194 sensory evaluation within the following 6 h.
195
196 *2.5. Micro-organisms and growth conditions*
197 *E. coli* O157:H7 Sakai Δstx1A/ Δstx2A- (Kim et al., 2010) was kindly provided by
198 Prof. Kyu-Tae Chang, a strain isolated from an outbreak associated with white radish
199 sprout (Michino et al., 1999), and genetically modified thereafter in order to remove Shiga
200 toxin genes. Mutant strain was obtained following the one-step PCR mutagenesis method

(Datsenko & 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 x 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

226 suspension was added to a final concentration of 3 x 10^7 CFU/mL. Treatment temperature
227 was kept constant at 52±0.2°C, and at preset intervals, samples were taken and survivors
228 were counted.
229
230 *2.8. Counts of viable cells*
231 Samples were diluted after treatment in 0.1% w/v peptone water (Oxoid).
232 Subsequently, 0.1-mL samples were pour-plated onto a recovery medium (TSAYE).
233 Plates were incubated at 37°C for 24 h. An image analyzer automatic counter (Protos;
234 Analytical Measuring Systems, Cambridge, United Kingdom) was used in order to count
235 the colony-forming units (CFUs.) Inactivation was expressed in terms of the extent of
236 reduction in Log_{10} counts (CFU) after each type of treatment, and the detection limit was
237 -5.0 Log_{10}.
238
239 *2.9. Statistical analysis for microbial experiments*
240 In order to evaluate the efficacy of lethal treatments, results were obtained from at
241 least three independent experiments carried out on separate working days with different
242 microbial cultures. Results were represented as the mean ± standard deviation using the
243 PRISM® program (GraphPad Software, Inc., San Diego, USA). Data were analyzed and
244 submitted to comparison of averages via ANOVA followed by a *post-hoc* Tukey´s test
245 and *t*-tests with GraphPad PRISM®. Differences were considered significant if $p<0.05$.
246
247 *2.10. Sensory analyses procedure*
248 The sensory portion of the study was performed in the sensory laboratory at the
249 Pilot Plant of Food Science and Technology (University of Zaragoza). A total of 65
250 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. 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 analysed and submitted to comparison of averages using analysis of variance (ANOVA) followed by *post-hoc* Tukey test, considering differences as significant if p<0.05.

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.

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

301 the main component in all cultivars was limonene, at a percentage ranging from 73.9 to
302 97%.
303
304 *3.2. Characterization and stability of chitosan nanoemulsion of CSEO (cn-CSEO)*
305 The most common method to obtain chitosan nanoparticles is by ionic gelation with
306 tripolyphosphate (TPP) (Feyzioglu and Tornuk, 2016; Ghaderi-Ghahfarokhi et al., 2017);
307 however, with the aim of simplifying the production method, we fabricated cn-CSEO
308 with an oil-in-water emulsion technique, thereby avoiding the use of TPP and other
309 conditioning stages.
310 DLS technique was applied to evaluate the hydrodynamic diameter and
311 polydispersity index (PDI) of nanoemulsions (Table 2). PDI is a measure of the size
312 distribution of particles in cn-CSEO. The PDI values obtained here, lower than 0.3,
313 indicate that the droplets have a narrow size distribution, which is related to the stability
314 of the nanoemulsions (Dickinson, 2003). In this regard, as shown in Table 2, size
315 distribution remained stable over 3 months, as well as the hydrodynamic diameter of the
316 nanoparticles (55.5-59.2 nm), without any significant differences during storage time at
317 4 ± 2°C ($p > 0.05$). This method is thus advantageous, since it provides stable solutions
318 for at least 3 months under refrigeration.
319 The particle size obtained with this methodology was very small in comparison to
320 other studies that also used chitosan as nanocarrier, and which achieved a droplet size of
321 around 100 (Woranuch and Yoksan, 2013), 300 (Hasani et al., 2018; Hasheminejad et al.,
322 2019) and 500 nm (Keawchaoon and Yoksan, 2011). This difference may be due to the
323 method we followed to prepare the emulsions, the degree of acetylation of chitosan, or
324 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, 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). 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; Ait-Ouazzou et al., 2013; Espina et al., 2011) and because of its successful sensorial acceptance in fruit juices (Espina et al., 2014). Thus, Fig. 1 shows

350 the inactivation curves obtained by the heat treatment and by the EO treatments (s-CSEO
351 or cn-CSEO) at 20°C acting separately at pH 7.0 (Fig. 1A) or pH 4.0 (Fig. 1B). As shown
352 in the figure, after a 30-min treatment the sum of the lethality caused by the heat treatment
353 and by the EO treatments effect scarcely inactivated 0.2 Log_{10} cycles at pH 7.0, both with
354 s- or cn-CSEO, and up to 1 Log_{10} cycles when s-CSEO was applied at pH 4.0. At pH 4.0,
355 cn-CSEO displayed a lower (p<0.05) antimicrobial activity as compared to s-CSEO.
356 Monoterpenes from CSEO might interact with chitosan molecules, e.g. by hydrogen
357 bonds, thereby controlling the release of CSEO during treatment time (Yuan et al., 2016).
358 Consequently, a lower release of CSEO from cn-CSEO when short treatments are applied
359 might contribute to this result. This could be associated with the lower bioavailability of
360 EOs to interact against the bacterial population when short treatments are applied (Merino
361 et al., 2019). In the case of prolonged bacteriostatic activity, however, other authors have
362 shown an increase of the antibacterial efficacy of different EOs after their emulsification
363 (Komaiko and McClements, 2016; Moghimi et al., 2016).

364 Fig. 1 shows the survival curves of *E. coli* O157:H7 Sakai after a combined
365 treatment at 52°C for 30 min in the presence of s-CSEO or cn-CSEO (0.2 μL/mL of
366 CSEO) in buffers of pH 7.0 and 4.0. As can be observed, the inactivation of *E. coli*
367 O157:H7 Sakai by the combined treatment always occurred more rapidly than the
368 additive effect. An outstanding synergism was observed between heat and cn-CSEO,
369 rather than with s-CSEO. At pH 7.0 (Fig. 1A), the heat treatment in the presence of s-
370 CSEO scarcely increased the degree of inactivation up to 1 Log_{10} cell cycle after 30 min;
371 however, a reduction of 4 Log_{10} cycles was achieved with cn-CSEO after 15 min,
372 followed by a prolonged tail for at least 15 min. At pH 4.0 (Fig. 1B), the combined
373 treatment was more effective than at pH 7.0, achieving almost 4 and 5 Log_{10} cycles of
374 inactivation after 30 min in the presence of s-CSEO and 20 min in the presence of cn-

375 CSEO, respectively. The results obtained at the two pH levels suggest that the preparation
376 of nanoemulsions with chitosan is the most efficient method to enhance synergism
377 between heat and CSEO. In fact, these results showed that the antimicrobial efficacy of
378 CSEO in the combined treatment increased with the manner of preparation of the EO (cn-
379 CSEO vs s-CSEO), with treatment temperature (52°C vs room temperature), and with the
380 acidification of the treatment medium pH (pH 4.0 vs pH 7.0). The smaller droplet size
381 achieved with chitosan (>60 nm) in comparison with other nanoemulsions of D-limonene
382 (Garre et al., 2020; Mehanna, 2020) or *Thymbra capitata* EO (Merino et al., 2019) (>100
383 nm) prepared with lecithin or zein, respectively, might be responsible for the remarkable
384 antimicrobial synergistic effect exerted by cn-CSEO in this study.
385 The greater antimicrobial activity of EOs in the form of nanoemulsions has been
386 explained by their increased polarity, thanks to the coating of the surfactants that reduces
387 the oil droplets' surface tension (Piorkowski and McClements, 2014). Thus, the
388 emulsification of hydrophobic substances might reduce their immiscibility in aqueous
389 solutions, making them more readily dispersible in the treatment media. In this regard,
390 Moghimi et al. (2016) proved that conversion of sage EO into a nanoemulsion improved
391 its antibacterial activity by enhancing its ability to promote the destruction of bacterial
392 cell membranes. Moreover, the synergism observed when combining heat with EOs has
393 been directly associated with the detection of injured cells in the cytoplasmic and outer
394 membranes of Gram-negative bacteria after the application of physical technologies as a
395 single agent (Arroyo et al., 2010; Espina et al., 2012; Somolinos et al., 2010). The greater
396 antimicrobial effect of cn-CSEO as compared with s-CSEO at 52°C might be explained
397 by several factors: a) chitosan coating prevents the degradation of CSEO at mild
398 temperatures (Amiri et al., 2020); and/or b) temperature rise destabilizes weak
399 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). 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

425 different as a function of the fruit juice assayed: while the use of s-CSEO or cn-CSEO in
426 combination with mild heat described a similar survival curve (p<0.05) in orange juice
427 (Fig. 3A), causing the inactivation of 5 Log_{10} cycles of *E. coli* O157:H7 Sakai cells after
428 25 min, cn-CSEO was much more effective in apple juice (Fig. 3B) than s-CSEO
429 (p<0.05). The lower synergism when combining heat and cn-CSEO in orange juice than
430 when doing so in apple juice might be related to the higher pectin concentration in orange
431 juice. Amine groups of chitosan present a pKa value ~6.5, meaning that in acid beverages,
432 such as fruit juices, chitosan nanoemulsions are positively charged, thereby enabling their
433 solubility as a function of pH (Abdelmalek et al., 2017; Szymańska and Winnicka, 2015).
434 Positively charged chitosan has been found to be effective as a clearing agent to
435 precipitate negatively charged pectins (Chatterjee et al., 2004). Electrostatic interaction
436 between chitosan and pectins might limit the antimicrobial activity of cn-CSEO in orange
437 juice. In apple juice, cn-CSEO caused an extra 1.5 Log_{10} cycles of inactivation after 30
438 min at 52°C, almost reaching 5 Log_{10} cycles of *E. coli* O157:H7 Sakai, which is a
439 requirement established by FDA regulation for the hygienization of fruit juices (FDA,
440 2001). Therefore, the preparation of nanoemulsions of CSEO with chitosan might
441 represent an advantageous alternative for the preservation of fruit juices when combined
442 with mild heat since, as has been shown in apple juice, it would allow for the destruction
443 of the pathogenic microorganisms at reduced treatment intensity while minimizing the
444 loss of nutritional and sensory characteristics of the fresh fruit juices.
445
446 *3.5. Determination of the hedonic acceptability of orange and apple juices with s-CSEO*
447 *and cn-CSEO added*
448 Once the antimicrobial activity of CSEO applied in suspension (s-CSEO) and
449 nanoemulsion (cn-CSEO) had been assayed, a sensory analysis was conducted with an

450 untrained panel to determine the acceptability of s-CSEO and cn-CSEO in apple and
451 orange juices.

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

461 Fig. 4 depicts the box and whisper plots corresponding to the hedonic data for the
462 overall acceptability, flavor, color, and odor of orange juice. As observed in Fig. 4, the
463 hedonic data for orange juice show that the panelists gave a mean score higher than 6 for
464 all the tested parameters. The concentration of 0.2 μL/mL of CSEO in suspension (s-
465 CSEO) as well as in nanoemulsion (cn-CSEO) would thus be accepted by the consumer
466 in orange juice. However, the samples with added CSEO showed lower ratings than
467 control for all parameters. In the case of s-CSEO, although the average values of the 4
468 parameters were slightly lower than control, the only significant observed differences
469 (p<0.05) were associated with color. On the other hand, cn-CSEO-added samples were
470 sensorially rated lower than control in terms of acceptability, taste and color (p<0.05),
471 although mean scores were always above 6; overall acceptability changed from 7.0 to 6.0
472 when cn-CSEO was added to orange juice. Comparing the addition of CSEO in the form
473 of suspension or nanoemulsion, overall acceptability and flavor were rated higher in
474 orange juice with s-CSEO than with cn-CSEO (p<0.05). Taking into account that the

475 efficacy of the combined application of heat and s-CSEO or cn-CSEO was exactly the
476 same (Fig. 3A) (p<0.05) under the treatment conditions tested, the results of the sensory
477 test would point toward s-CSEO as the best option for designing a combined treatment
478 with mild heat to preserve orange juice.

479 Fig. 5 shows the box and whisper plots corresponding to the hedonic data collected
480 for the overall acceptability, flavor, color and odor of apple juice. As shown in Fig. 5,
481 apple juice also achieved a mean score higher than 5.0 (acceptable) for both control and
482 for juice with added cn-CSEO, but not for juice with added s-CSEO in the parameters of
483 color (4.9) and odor (4.9). As in the orange juice, the best hedonic scores for the 4 tested
484 parameters were obtained in the apple control juice but, in this case, the only significant
485 differences observed (p<0.05) were in the parameters of flavor and odor. While the
486 average odor score of control apple juice was 5.8, the odor of apple juice with s-CSEO
487 and cn-CSEO was rated at an average of 4.9 and 5.2, respectively. In contrast with the
488 hedonic data of orange juice, apple juice with cn-CSEO achieved a better flavor rating
489 than apple juice with s-CSEO; the 50-percentile was 6.0 for cn-CSEO juice in comparison
490 to 5.5 for s-CSEO juice. As a result, the use of cn-CSEO would be the best option when
491 combining mild heat and CSEO to preserve apple juice not only because of the better
492 sensory results obtained in comparison with the application of s-CSEO, but also because
493 of its higher antimicrobial efficacy in combination with heat as applied to apple juice (Fig.
494 3B) (p<0.05).

495 It is known that most EOs have strong odors and flavors that prevent their direct
496 use as a sole method of food preservation because of the high concentrations required for
497 this purpose (Burt, 2004; Hyldgaard et al., 2012; Mani-López, 2017). Thus, in order to
498 reduce EO doses and, consequently, their sensory impact, several studies seek to apply
499 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).

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.

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.

CRediT author statement

Roberta Bento: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Writing - review & editing. **Elisa Pagán:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Roles/Writing - original draft. **Daniel Berdejo:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Roles/Writing - original draft, Writing - review & editing. **Rayssa Julliane de Carvalho:** Writing - review & editing. **Sonia García-Embid:** Data curation, Investigation, Methodology. **Filippo Maggi:** Data curation, Investigation, Methodology, Roles/Writing - original draft, Writing - review & editing. **Marciane Magnani:** Writing - review & editing. **Evandro Leite de Souza:** Writing - review & editing. **Diego García-Gonzalo:** Conceptualization, Data curation, Funding acquisition, Resources, Supervision, Roles/Writing - original draft, Writing - review & editing. **Rafael Pagán:** Conceptualization, Data curation, Funding acquisition, Project administration, Resources, Supervision, Roles/Writing - original draft, Writing - review & editing.

References

Abdelmalek, B.E., Sila, A., Haddar, A., Bougatef, A., Ayadi, M.A., 2017. β-Chitin and chitosan from squid gladius: Biological activities of chitosan and its application as clarifying agent for apple juice. Int. J. Biol. Macromol. 104, 953-962.

Aboudaou, M., Ferhat, M.A., Hazzit, M., Ariño, A., Djenane, D., 2019. Solvent free-microwave green extraction of essential oil from orange peel (*Citrus sinensis* L.): effects on shelf life of flavored liquid whole eggs during storage under commercial retail conditions. J. Food. Meas. Charact. 13, 3162-3172.

574 Ait-Ouazzou, A., Cherrat, L., Espina, L., Lorán, S., Rota, C., Pagán, R., 2011. The
575 antimicrobial activity of hydrophobic essential oil constituents acting alone or in
576 combined processes of food preservation. Innov. Food Sci. Emerg. Technol. 12, 320-329.
577 Ait-Ouazzou, A., Espina, L., Gelaw, T.K., de Lamo-Castellvi, S., Pagán, R.,
578 García-Gonzalo, D., 2013. New insights in mechanisms of bacterial inactivation by
579 carvacrol. J. Appl. Microbiol. 114, 173-185.
580 Amiri, A., Mousakhani-Ganjeh, A., Amiri, Z., Guo, Y.G., Pratap Singh, A.,
581 Esmaeilzadeh Kenari, R., 2020. Fabrication of cumin loaded-chitosan particles:
582 Characterized by molecular, morphological, thermal, antioxidant and anticancer
583 properties as well as its utilization in food system. Food Chem. 310, 125821.
584 Arroyo, C., Somolinos, M., Cebrián, G., Condón, S., Pagán, R., 2010. Pulsed
585 electric fields cause sublethal injuries in the outer membrane of *Enterobacter sakazakii*
586 facilitating the antimicrobial activity of citral. Lett Appl Microbiol. 51, 525-531.
587 Berdejo, D., Pagán, E., García-Gonzalo, D., Pagán, R., 2019. Exploiting the
588 synergism among physical and chemical processes for improving food safety. Curr. Opin.
589 Food Sci. 30, 14-20.
590 Bica, K., Gaertner, P., Rogers, R.D., 2011. Ionic liquids and fragrances - direct
591 isolation of orange essential oil. Green Chem. 13, 1997-1999.
592 Burt, S., 2004. Essential oils: their antibacterial properties and potential
593 applications in foods - a review. Int. J. Food Microbiol. 94, 223-253.
594 Charara, Z.N., Williams, J.W., Schmidt, R.H., Marshall, M.R., 1992. Orange
595 Flavor Absorption Into Various Polymeric Packaging Materials. J. Food Sci. 57, 963-968.
596 Chatterjee, S., Chatterjee, S., Chatterjee, B.P., Guha, A.K., 2004. Clarification of
597 fruit juice with chitosan. Process Biochem. 39, 2229–2232.

598 Datsenko, K.A., Wanner, B.L., 2000. One-step inactivation of chromosomal
599 genes in *Escherichia coli* K-12 using PCR products. Proc. Natl. Acad. Sci. U.S.A. 97,
600 6640-6645.
601 de Carvalho, R.J., de Souza, G.T., Pagán, E., García-Gonzalo, D., Magnani, M.,
602 Pagán, R., 2018. Nanoemulsions of Mentha piperita L. essential oil in combination with
603 mild heat, pulsed electric fields (PEF) and high hydrostatic pressure (HHP) as an
604 alternative to inactivate *Escherichia coli* O157: H7 in fruit juices. Innov. Food Sci.
605 Emerg. Technol. 48, 219-227.
606 de Souza, E.L., da Cruz Almeida, E.T., de Sousa Guedes, J.P., 2016. The Potential
607 of the Incorporation of Essential Oils and Their Individual Constituents to Improve
608 Microbial Safety in Juices: A Review. Compr. Rev. Food Sci. Food Saf. 15, 753-772.
609 de Souza Pedrosa, G.T., de Carvalho, R.J., Berdejo, D., de Souza, E.L., Pagán, R.,
610 Magnani, M., 2019. Control of Autochthonous Spoilage Lactic Acid Bacteria in Apple
611 and Orange Juices by Sensorially Accepted Doses of *Citrus* spp. Essential Oils Combined
612 with Mild Heat Treatments. J. Food Sci. 84, 848-858.
613 Dickinson, E., 2003. Hydrocolloids at interfaces and the influence on the
614 properties of dispersed systems. Food Hydrocoll. 17, 25-39.
615 Donsì, F., Annunziata, M., Sessa, M., Ferrari, G., 2011. Nanoencapsulation of
616 essential oils to enhance their antimicrobial activity in foods. LWT. 44, 1908-1914.
617 Espina, L., Condon, S., Pagán, R., García-Gonzalo, D., 2014. Synergistic effect
618 of orange essential oil or (+)-limonene with heat treatments to inactivate *Escherichia coli*
619 O157:H7 in orange juice at lower intensities while maintaining hedonic acceptability.
620 Food and Bioprocess Tech. 7, 471-481.

621 Espina, L., Somolinos, M., Lorán, S., Conchello, P., García, D., Pagán, R., 2011.
622 Chemical composition of commercial citrus fruit essential oils and evaluation of their
623 antimicrobial activity acting alone or in combined processes. Food Control 22, 896-902.
624 Espina, L., Somolinos, M., Ouazzou, A.A., Condón, S., García-Gonzalo, D.,
625 Pagán, R., 2012. Inactivation of *Escherichia coli* O157:H7 in fruit juices by combined
626 treatments of citrus fruit essential oils and heat. Int. J. Food Microbiol. 159, 9-16.
627 FDA, 2001. Hazard analysis and critical control point (HACCP); procedures for
628 the safe and sanitary processing and importing of juice, in: Food and Drug Administration
629 (Ed.), pp. 20450-20486.
630 Feyzioglu, G.C., Tornuk, F., 2016. Development of chitosan nanoparticles loaded
631 with summer savory (*Satureja hortensis* L.) essential oil for antimicrobial and antioxidant
632 delivery applications. LWT. 70, 104-110.
633 Friedman, M., Henika, P.R., Mandrell, R.E., 2002. Bactericidal activities of plant
634 essential oils and some of their isolated constituents against *Campylobacter jejuni*,
635 *Escherichia coli*, *Listeria monocytogenes*, and *Salmonella enterica*. J. Food Prot. 65,
636 1545-1560.
637 Froiio, F., Mosaddik, A., Morshed, M.T., Paolino, D., Fessi, H., Elaissari, A.,
638 2019. Edible Polymers for Essential Oils Encapsulation: Application in Food
639 Preservation. Ind. Eng. Chem. Res. 58, 20932-20945.
640 Garre, A., Espin, J.F., Huertas, J.P., Periago, P.M., Palop, A., 2020. Limonene
641 nanoemulsified with soya lecithin reduces the intensity of non-isothermal treatments for
642 inactivation of *Listeria monocytogenes*. Sci. Rep. 10, 3656.
643 Geraci, A., Di Stefano, V., Di Martino, E., Schillaci, D., Schicchi, R., 2017.
644 Essential oil components of orange peels and antimicrobial activity. Nat. Prod. Res. 31,
645 653-659.

Ghaderi-Ghahfarokhi, M., Barzegar, M., Sahari, M.A., Ahmadi Gavlighi, H., Gardini, F., 2017. Chitosan-cinnamon essential oil nano-formulation: Application as a novel additive for controlled release and shelf life extension of beef patties. Int. J. Biol. Macromol. 102, 19-28.

Gonçalves, D., Costa, P., Rodrigues, C.E.C., Rodrigues, A.E., 2018. Effect of *Citrus sinensis* essential oil deterpenation on the aroma profile of the phases obtained by solvent extraction. J. Chem. Thermodyn. 116, 166-175.

Guevara, L., Antolinos, V., Palop, A., Periago, P.M., 2015. Impact of Moderate Heat, Carvacrol, and Thymol Treatments on the Viability, Injury, and Stress Response of *Listeria monocytogenes*. Biomed Res. Int. 2015, 548930-548930.

Guinebretière, S., Briançon, S., Fessi, H., Teodorescu, V.S., Blanchin, M.G., 2002. Nanocapsules of biodegradable polymers: preparation and characterization by direct high resolution electron microscopy. Mater. Sci. Eng. C. 21, 137-142.

Hasani, S., Ojagh, S.M., Ghorbani, M., 2018. Nanoencapsulation of lemon essential oil in Chitosan-Hicap system. Part 1: Study on its physical and structural characteristics. Int. J. Biol. Macromol. 115, 143-151.

Hasheminejad, N., Khodaiyan, F., 2020. The effect of clove essential oil loaded chitosan nanoparticles on the shelf life and quality of pomegranate arils. Food Chem. 309, 125520.

Hasheminejad, N., Khodaiyan, F., Safari, M., 2019. Improving the antifungal activity of clove essential oil encapsulated by chitosan nanoparticles. Food Chem. 275, 113-122.

Hyldgaard, M., Mygind, T., Meyer, R.L., 2012. Essential oils in food preservation: mode of action, synergies, and interactions with food matrix components. Front. Microbiol. 3, 12.

671 Isman, M.B., 2017. Bridging the gap: Moving botanical insecticides from the
672 laboratory to the farm. Ind. Crop. Prod. 110, 10-14.
673 Ju, J., Xie, Y., Guo, Y., Cheng, Y., Qian, H., Yao, W., 2019. Application of edible
674 coating with essential oil in food preservation. Crit. Rev. Food Sci. Nutr. 59, 2467-2480.
675 Keawchaoon, L., Yoksan, R., 2011. Preparation, characterization and in vitro
676 release study of carvacrol-loaded chitosan nanoparticles. Colloids Surf. B 84, 163-171.
677 Kim, S.-H., Lee, S.-R., Kim, K.-S., Ko, A., Kim, E., Kim, Y.-H., Chang, K.-T.,
678 2010. Shiga toxin A subunit mutant of *Escherichia coli* O157:H7 releases outer
679 membrane vesicles containing the B-pentameric complex. FEMS Immunol. Med.
680 Microbiol. 58, 412-420.
681 Komaiko, J.S., McClements, D.J., 2016. Formation of Food-Grade
682 Nanoemulsions Using Low-Energy Preparation Methods: A Review of Available
683 Methods. Compr. Rev. Food Sci. Food Saf. 15, 331-352.
684 Lubbe, A., Verpoorte, R., 2011. Cultivation of medicinal and aromatic plants for
685 specialty industrial materials. Ind. Crop. Prod. 34, 785-801.
686 Luis-Villaroya, A., Espina, L., García-Gonzalo, D., Bayarri, S., Pérez, C., Pagán,
687 R., 2015. Bioactive properties of a propolis-based dietary supplement and its use in
688 combination with mild heat for apple juice preservation. Int. J. Food Microbiol. 205, 90-
689 97.
690 Maggi, F., Cecchini, C., Cresci, A., Coman, M.M., Tirillini, B., Sagratini, G.,
691 Papa, F., Vittori, S., 2010. Chemical composition and antimicrobial activity of the
692 essential oils from several Hypericum taxa (Guttiferae) growing in central Italy
693 (Appennino Umbro-Marchigiano). Chem. Biodiversity 7, 447-466.

694 Mahato, N., Sharma, K., Koteswararao, R., Sinha, M., Baral, E., Cho, M.H., 2019.
695 Citrus essential oils: Extraction, authentication and application in food preservation. Crit.
696 Rev. Food Sci. Nutr. 59, 611-625.
697 Mani-López, E., Lorenzo-Leal A.C., Palou, E., López-Malo, A., 2017. Principles
698 of Sensory Evaluation in Foods Containing Essential Oil. In: S.M.B. Hashemi, A.
699 Mousavi Khaneghah and A. de Souza Sant'Ana (Eds.), Essential Oils in Food Processing.
700 John Wiley & Sons Ltd., pp. 293-325.
701 Mate, J., Periago, P.M., Palop, A., 2016. Combined effect of a nanoemulsion of
702 D-limonene and nisin on *Listeria monocytogenes* growth and viability in culture media
703 and foods. Food Sci. Technol. Int. 22, 146-152.
704 Matica, A., Menghiu, G., Ostafe, V., 2017. Toxicity of chitosan based products.
705 New Front. Chem. 26, 65-74.
706 McClements, D.J., 2013. Nanoemulsion-based oral delivery systems for lipophilic
707 bioactive components: nutraceuticals and pharmaceuticals. Ther. Deliv. 4, 841-857.
708 Mehanna, M., 2020. Limonene-based Self-nanoemulsifying System:
709 Formulation, Physicochemical Characterization and Stability Int. J. Pharm. Investig. 10,
710 64-69.
711 Merino, N., Berdejo, D., Bento, R., Salman, H., Lanz, M., Maggi, F., Sánchez-
712 Gómez, S., García-Gonzalo, D., Pagán, R., 2019. Antimicrobial efficacy of *Thymbra*
713 *capitata* (L.) Cav. essential oil loaded in self-assembled zein nanoparticles in combination
714 with heat. Ind. Crop. Prod. 133, 98-104.
715 Merzendorfer, H., Zimoch, L., 2003. Chitin metabolism in insects: structure,
716 function and regulation of chitin synthases and chitinases. J. Exp. Biol. 206, 4393.
717 Michino, H., Araki, K., Minami, S., Takaya, S., Sakai, N., Miyazaki, M., Ono, A.,
718 Yanagawa, H., 1999. Massive outbreak of *Escherichia coli* O157:H7 infection in

719 schoolchildren in Sakai City, Japan, associated with consumption of white radish sprouts.
720 Am. J. Epidemiol. 150, 787-796.
721 Moghimi, R., Ghaderi, L., Rafati, H., Aliahmadi, A., McClements, D.J., 2016.
722 Superior antibacterial activity of nanoemulsion of *Thymus daenensis* essential oil against
723 *E. coli*. Food Chem. 194, 410-415.
724 Morin-Crini, N., Lichtfouse, E., Torri, G., Crini, G., 2019. Applications of
725 chitosan in food, pharmaceuticals, medicine, cosmetics, agriculture, textiles, pulp and
726 paper, biotechnology, and environmental chemistry. Environ. Chem. Lett. 17, 1667-1692.
727 Oboh, G., Ademosun, A.O., Olumuyiwa, T.A., Olasehinde, T.A., Ademiluyi,
728 A.O., Adeyemo, A.C., 2017. Insecticidal activity of essential oil from orange peels
729 (*Citrus sinensis*) against *Tribolium confusum*, *Callosobruchus maculatus* and *Sitophilus*
730 *oryzae* and its inhibitory effects on acetylcholinesterase and Na+/K+-ATPase activities.
731 Phytoparasitica. 45, 501-508.
732 Pagán, E., Berdejo, D., Espina, L., García-Gonzalo, D., Pagán, R., 2018.
733 Antimicrobial activity of suspensions and nanoemulsions of citral in combination with
734 heat or pulsed electric fields. Lett. Appl. Microbiol. 66, 63-70.
735 Piorkowski, D.T., McClements, D.J., 2014. Beverage emulsions: Recent
736 developments in formulation, production, and applications. Food Hydrocoll. 42, 5-41.
737 Rocha, M.A.M., Coimbra, M.A., Nunes, C., 2017. Applications of chitosan and
738 their derivatives in beverages: a critical review. Curr. Opin. Food Sci. 15, 61-69.
739 Salvia-Trujillo, L., Rojas-Graü, M.A., Soliva-Fortuny, R., Martín-Belloso, O.,
740 2014. Formulation of Antimicrobial Edible Nanoemulsions with Pseudo-Ternary Phase
741 Experimental Design. Food and Bioprocess Tech. 7, 3022-3032.

742 Shao, Y., Wu, C., Wu, T., Li, Y., Chen, S., Yuan, C., Hu, Y., 2018. Eugenol-
743 chitosan nanoemulsions by ultrasound-mediated emulsification: Formulation,
744 characterization and antimicrobial activity. Carbohydr. Polym. 193, 144-152.
745 Somolinos, M., García, D., Mañas, P., Condón, S., Pagán, R., 2010. Organic acids
746 make *Escherichia coli* more resistant to pulsed electric fields at acid pH. Int. J. Food
747 Microbiol. 136, 381-384.
748 Sreekumar, S., Goycoolea, F.M., Moerschbacher, B.M., Rivera-Rodriguez, G.R.,
749 2018. Parameters influencing the size of chitosan-TPP nano- and microparticles. Sci. Rep.
750 8, 4695.
751 Szymańska, E., Winnicka, K., 2015. Stability of chitosan-a challenge for
752 pharmaceutical and biomedical applications. Mar. Drugs. 13, 1819-1846.
753 Uprety, B.K., Rakshit, S.K., 2017. Compositional Shift in Fatty Acid Profiles of
754 Lipids Obtained from Oleaginous Yeasts upon the Addition of Essential Oil from *Citrus*
755 *sinensis* L. Appl. Biochem. Biotechnol. 183, 1158-1172.
756 Viacava, G.E., Ayala-Zavala, J.F., González-Aguilar, G.A., Ansorena, M.R.,
757 2018. Effect of free and microencapsulated thyme essential oil on quality attributes of
758 minimally processed lettuce. Postharvest Biol. Technol. 145, 125-133.
759 Woranuch, S., Yoksan, R., 2013. Eugenol-loaded chitosan nanoparticles: I.
760 Thermal stability improvement of eugenol through encapsulation. Carbohydr. Polym. 96,
761 578-585.
762 Yuan, G., Chen, X., Li, D., 2016. Chitosan films and coatings containing essential
763 oils: The antioxidant and antimicrobial activity, and application in food systems. Food
764 Res. Int. 89, 117-128.
765 Zhang, S., Zhang, M., Fang, Z., Liu, Y., 2017. Preparation and characterization of
766 blended cloves/cinnamon essential oil nanoemulsions. LWT. 75, 316-322.

767 **Figure legends**

768

769 **Fig. 1.** Survival curves of *Escherichia coli* O157:H7 Sakai (initial concentration: 3 x 107
770 CFU/mL) after heat treatment at 52ºC (○), *Citrus sinensis* essential oil (CSEO) treatment
771 in form of a suspension (■) or chitosan nanoemulsion (■) (0.2 µL/mL of CSEO) and
772 combined treatment with heat and CSEO in form of a suspension (●) or chitosan
773 nanoemulsion (●) (0.2 µL/mL of CSEO), for 30 min in phosphate-citrate buffers of pH
774 7.0 (A) and 4.0 (B). Data represent the mean ± standard deviation (error bars) of at least
775 three independent experiments. The dotted line represents the detection limit (-5.0 Log_{10}).

776

777

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

785

786 **Fig. 3.** Survival curves of *Escherichia coli* O157:H7 Sakai (initial concentration: 3 x 10^7
787 CFU/mL) after heat treatment at 52ºC (○), *Citrus sinensis* essential oil (CSEO) treatment
788 in form of a suspension (■) or chitosan nanoemulsion (■) (0.2 µL/mL of CSEO) and
789 combined treatment with heat at 52°C and *Citrus sinensis* essential oil (CSEO) in form
790 of a suspension (●) or chitosan nanoemulsion (●) (0.2 µL/mL of CSEO) for 30 min in
791 natural orange (A) and apple (B) juices. Data represent the mean ± standard deviation

792 (error bars) of at least three independent experiments. The dotted line represents the
793 detection limit (-5.0 Log_{10}).
794
795 **Fig. 4.** Box and whisker plots displaying the hedonic data values of natural orange juice
796 (■; control) and with 0.2 µL/mL of *Citrus sinensis* essential oil (CSEO) in form of a
797 suspension (■) or chitosan nanoemulsion (■) added for overall acceptability (A), flavor
798 (B), color (C) and odor (D). The cross represents the hedonic mean, the single points
799 represent the extreme values, the whiskers correspond to 2.5 and 97.5 percentiles, the box
800 correspond to the 25 and 75 percentiles and the median is represented by the central bar
801 in the box. The hedonic values range from 1 to 9. Different letters over the bars represent
802 statistically significant differences ($p < 0.05$) among the means of each group as
803 determined by one-way ANOVA followed by Tukey's multiple pairwise comparison *post*
804 *hoc* test.
805
806 **Fig. 5.** Box and whisker plots displaying the hedonic data values of natural apple juice
807 (■; control) and with 0.2 µL/mL of *Citrus sinensis* essential oil (CSEO) in form of a
808 suspension (■) or chitosan nanoemulsion (■) added for overall acceptability (A), flavor
809 (B), color (C) and odor (D). The cross represents the hedonic mean, the single points
810 represent the extreme values, the whiskers correspond to 2.5 and 97.5 percentiles, the box
811 correspond to the 25 and 75 percentiles and the median is represented by the central bar
812 in the box. Hedonic values range from 1 to 9. Different letters over the bars represent
813 statistically significant differences ($p < 0.05$) among the means of each group as
814 determined by one-way ANOVA followed by Tukey's multiple pairwise comparison *post*
815 *hoc* test.
816

Figure 1.

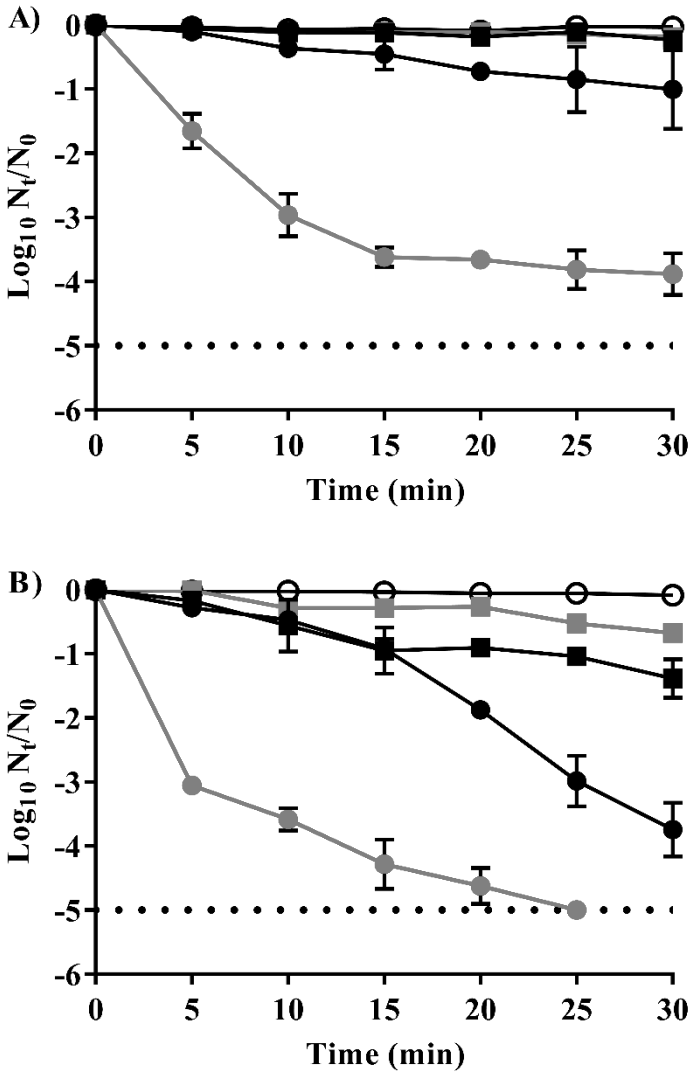

A)
0
-1
-2
-3
-4
-5
-6
Log10 Nt/N0
0
5
10
15
20
25
30
Time (min)
B)
0
-1
-2
-3
-4
-5
-6
Log10 Nt/N0
0
5
10
15
20
25
30
Time (min)

Figure 2.

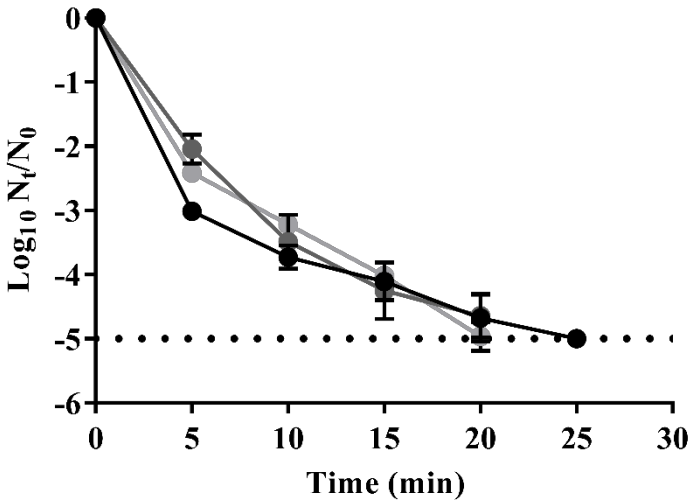


Figure 3.

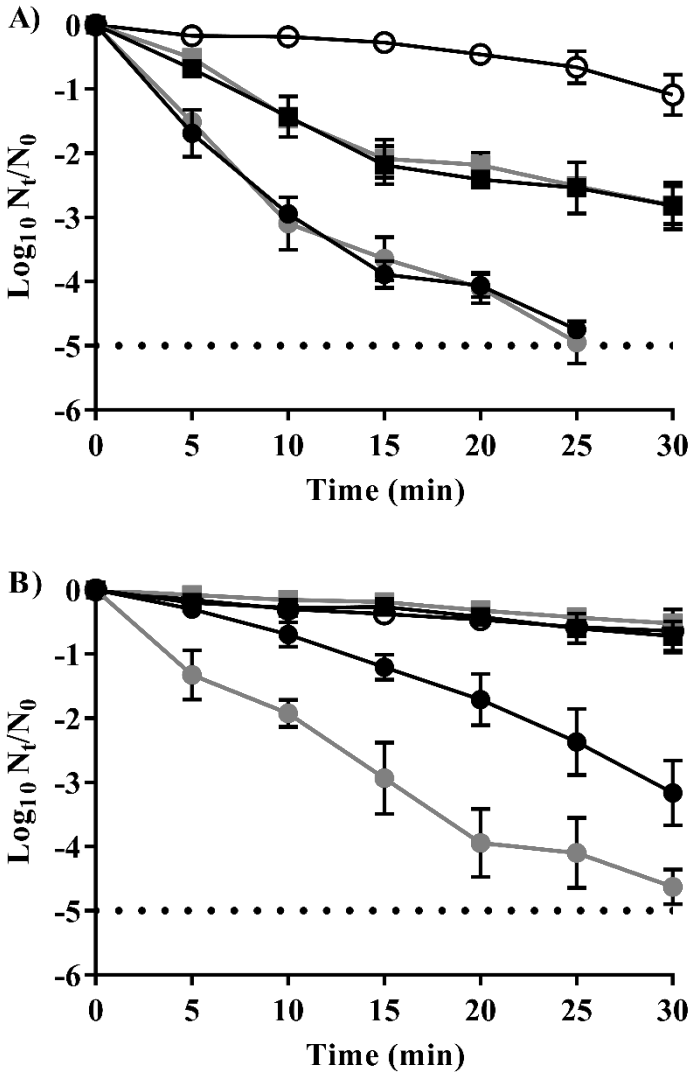
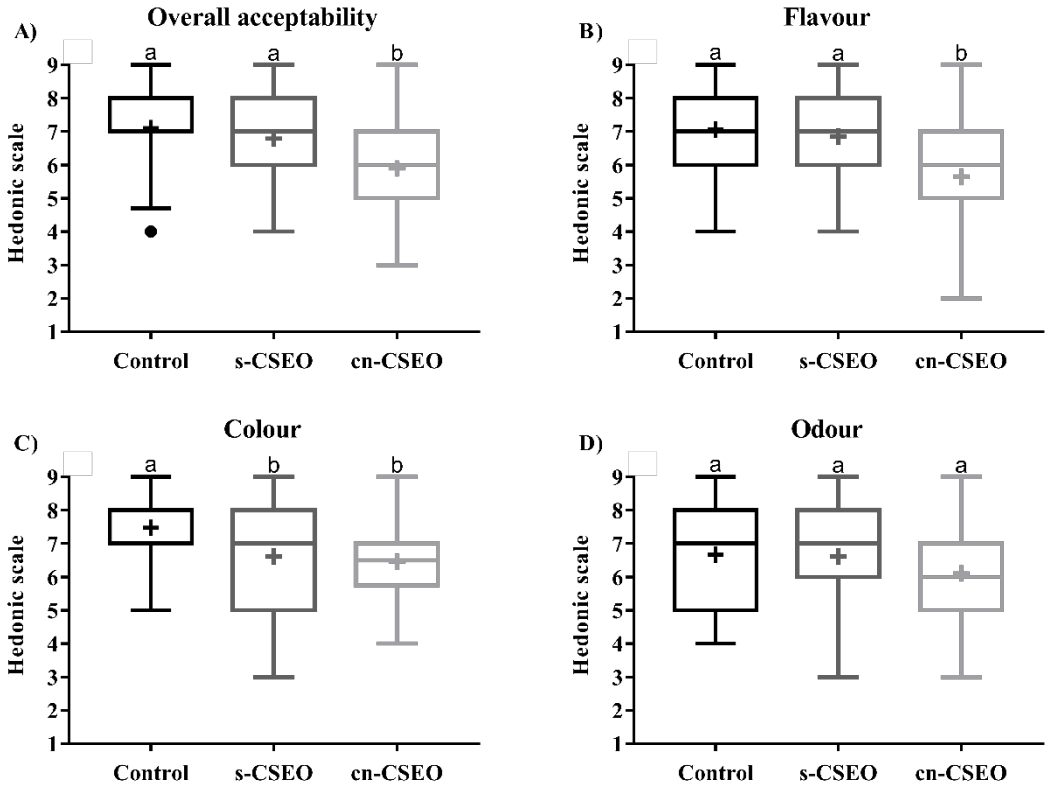
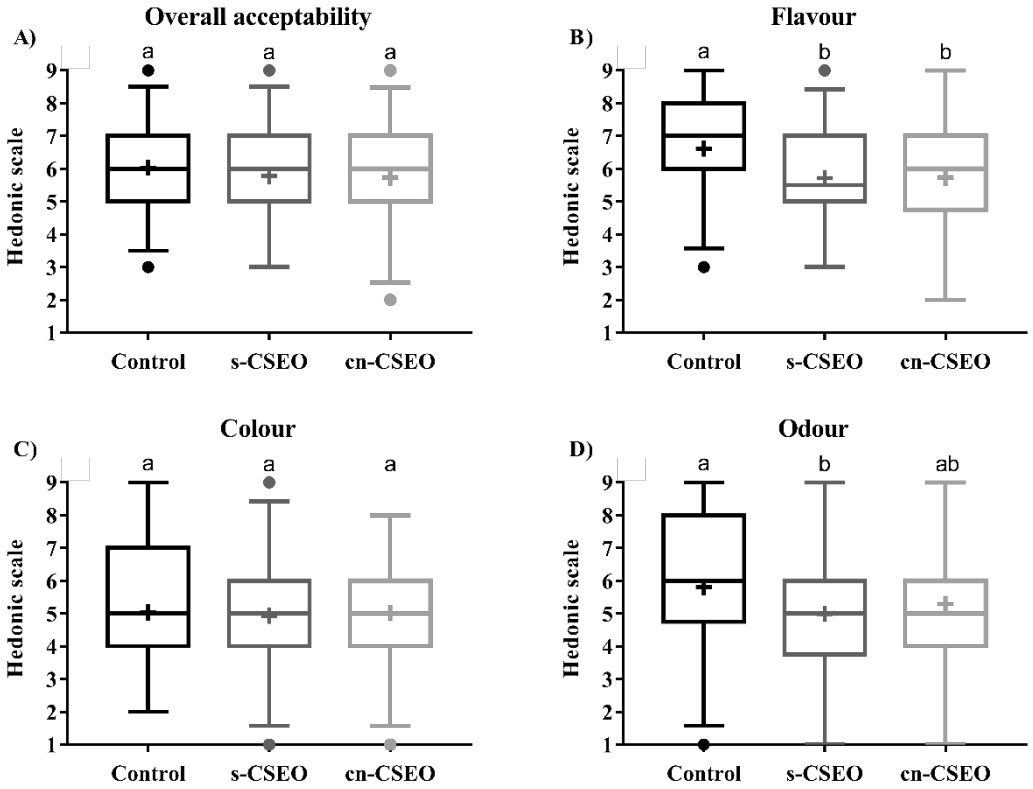


Figure 4.


A)
Overall acceptability
B)
Flavour
C)
Colour
D)
Odour
Hedonic scale
Control
s-CSEO
cn-CSEO
a
b

921 **Figure 5.**



922

923
924

925 **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	

926 [a] The order of compounds is consistent with elution from a HP-5MS column (30 m x 0.25 mm i.d., 0.1
927 µm f.t.). [b] RI, temperature-programmed retention index calculated using a mixture of *n*-alkanes (C_8-C_{24}).
928 [c] Literature retention index taken from ADAMS library. [d] Relative percentage values are mean of three
929 replicates ± standard deviation. [e] ID, identification method: Std, comparison of retention time, retention
930 index and mass spectrum with those of analytical standard (Sigma-Aldrich, Milan, Italy); RI, coherence
931 of the calculated RI with respect to those reported in ADAMS library; MS, marching with spectra stored
932 in ADAMS, NIST 17 and FFNSC2 libraries. [e] tr, traces, % < 0.1.
933

934

935

936

937

938 **Table 2.** Droplet size and polydispersity index (PDI) of nanoemulsions of *Citrus sinensis*
939 essential oil storage under refrigeration. Data represent the mean ± standard error of the
940 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]

941 Different superscript letters in the same column show a significant difference ($p < 0.05$)
942
943

Suplemental materials

Figure S1.

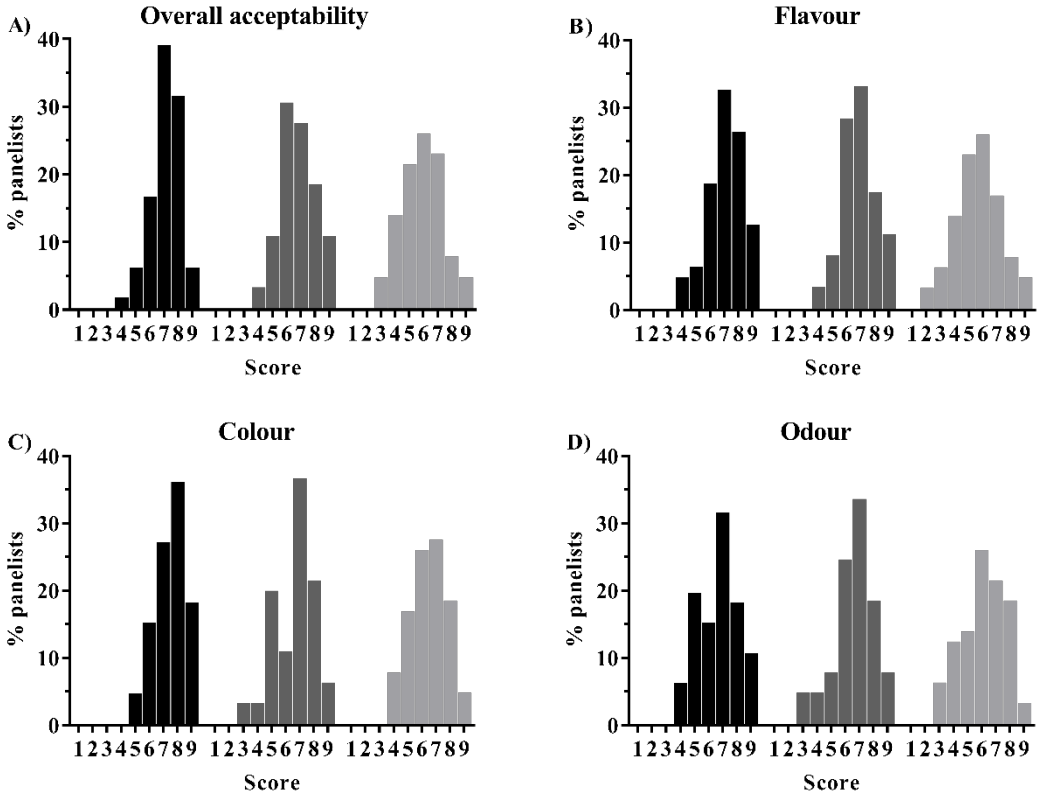
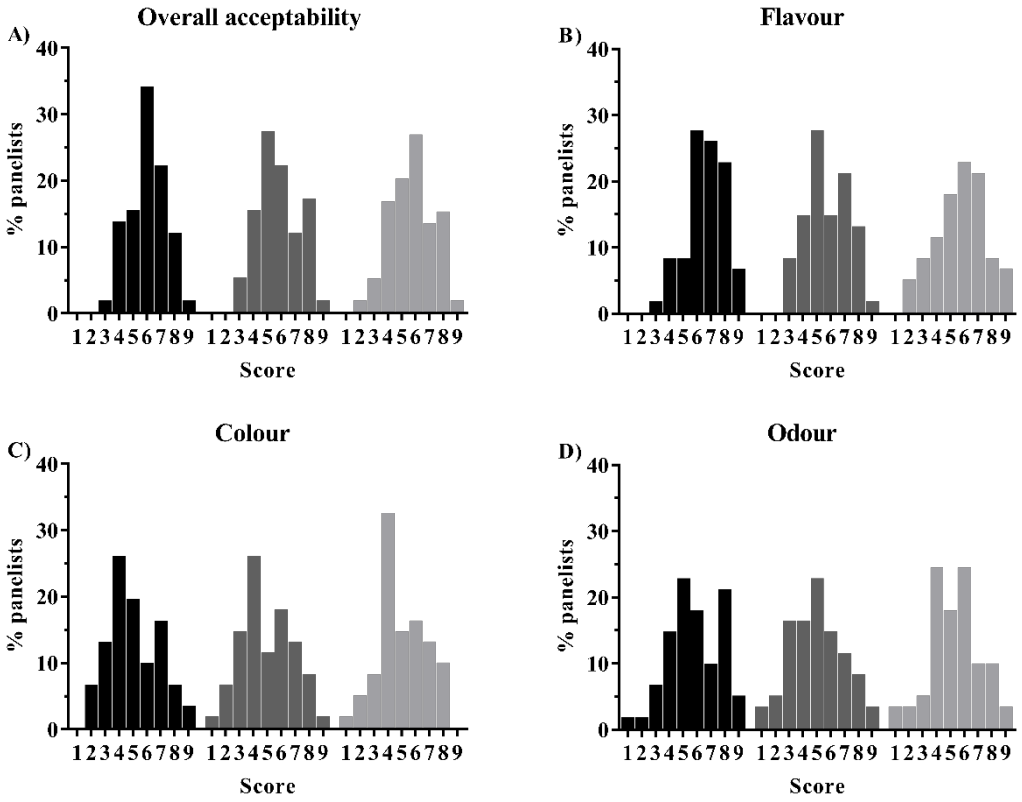


Fig. 1S. Histograms depicting the hedonic data of natural orange juice (■; control) and with 0.2 µL/mL of s-CSEO (■) or cn-CSEO (■) added for overall acceptability (A), flavor (B), color (C) and odor (D). The hedonic values range from 1 to 9 (from 'dislike extremely' to 'like extremely').

967 **Figure S2.**
968



969
970
971 **Fig. 2S.** Histograms depicting the hedonic data of natural apple juice (■; control) and
972 with 0.2 µL/mL of s-CSEO (■) or cn-CSEO (■) added for overall acceptability (A), flavor
973 (B), color (C) and odor (D). The hedonic values range from 1 to 9 (from 'dislike
974 extremely' to 'like extremely').

975
976