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

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Abstract: A possible way to valorize citrus peels, which are byproducts of the juice extraction industry, is to use them as natural biopreservatives. In this paper we present early results from a compared Solvent Free Microwave Extraction (SFME) with Hydro-Distillation (HD) and Cold Pressing (CP) of essential oils (EOs) using fresh orange peel (*Citrus sinensis* L. var. Valencia late), a by-product in the production of orange juice in Algeria. The EOs were analyzed by gas chromatography coupled to mass spectrometry (GC-MS). All extracted *C. sinensis* EOs were chemotype limonene (94.64 to 95.48%). SFME is performed without added any solvent or water. SFME increases EO yield and eliminate wastewater treatment, resulting in a great progress in terms of time and cost efficiency. In its second part, the present study was conducted to evaluate "*in vitro*", the antioxidant activities of Solvent Free Microwave (SFM) extracted orange EO by using the DPPH• (2,2-di-phenyl-1-picrilhydrazyl)

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free radical scavenging assay. The ability of orange EO to scavenge the free radical DPPH• was high, exceeding 80%. The result of the DPPH assay gives an IC_{50} range value of 89.25 µg/mL (0.09 mg/mL) for the studied sample. Accordingly to the scientific literature, *C. sinensis* EO tested in the present study presented strong antioxidant activity, when looking to its values of AAI = 1.12 µg/mL. The feasibility of biopreservation used EOs as an alternative to synthetic techniques for liquid whole egg (LWE) stored under commercial retail conditions was investigated. The orange EO extracted by SFM was screened for its antibacterial and antioxidant activities in LWE at concentrations of 0.1, 0.3 and 0.5%. The TBA-RS results showed that the EO treatments significantly ($p < 0.05$) reduced the lipid oxidation in LWE. The long term oxidative, microbial and organoleptical stability of the LWE during display was positively influenced by orange EO treatments. Therefore, the results obtained here confirm that EO treatment as a promising technology to extend the commercial shelf-life of liquid egg products during retail/display.

Keywords orange peel essential oil; green extraction; liquid whole eggs; biopreservation; shelf-life

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1. Introduction

In terms of international trade of orange fruits, the main citrus-producing countries, such as Brazil, the United States, China, India, Spain, Mexico, Egypt and Turkey [1] were produced in 2014, 140 Mt of these fruits, about 60% of world production. Algerian production in orange fruits estimated at 571 000 tons/year, which is the 3rd in the Arab Maghreb Union and 19th producers in the world [2]. The climatic conditions of Algeria are favorable for the production of most citrus fruits.

Citrus by-product resulting from the citrus processing represents a source of various bioactive molecules such as EOs [3]. Interest in taking profit from waste products of the juice industry, permit to minimize adverse effects of by-products in the environment. Generally, the pharmaceutical and food industries are using citrus oils as flavoring for masking other added ingredients in abundant quantity in the processed product.

Worldwide, consumers' enthusiasm for natural products and increasingly severe legislations in respect of synthetic substances, EOs can be a good alternative for the food industry. The citrus EOs and their constituents are suitable for use in foods as biological agents or for their flavor, in pharmaceutical industries for its anti-inflammatory and antimicrobial properties. In addition, great amount of these oils is also used in cosmetic industries.

During the last few years, a great expectation for green chemistry was recorded by various industries. Environmentally friendly and low cost ecological extraction techniques are becoming more attractive. Therefore, in recent years, conventional techniques, such as HD, tend to be replaced by various novel extraction techniques, such as microwave-assisted extraction (MAE). This technique is largely focused on finding technological solutions to

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diminish or even prevent the use of organic solvents in extraction processes to obtain more products with higher added value. Meanwhile, this technique is considered a novel with better extraction yields, decreased extraction times and prevents the degradation of thermolabile compounds and thus prevents the antioxidant activity loss of the plant extract [4].

Recently, LWE is preferred for food industry and for households for his easy and commode use. According to its biological nature, is an easily perishable animal food under conventional cooled storage, its shelf life is limited due to the oxidation and microbial development. Both considered a major concern for food technologists due to the loss of quality associated with those processes [5]. The citrus EOs represents a significant source of bioactive compounds with potential prophylactic properties for the development of functional foods [6]. Generally, to destroy microorganisms in liquid egg products, heat treatments were commonly employed that can improve microbial safety and increase shelf life of products, but can have negative effects on the functional properties of egg proteins, which results in technologically unattractive products [7]. In this scenario, the search for new strategies and new biopreservative agents for stabilization of liquid eggs has become a central goal for food industry. EOs can be an interesting alternative to improve traditional heat treatments to reduce their intensity and therefore reduce their adverse effects on food quality. To the best of our knowledge, there is no published report regarding the biological effectiveness of SFM orange peel EO in egg products. The aims of this study were (**i**) to describe a fast solvent free microwave extraction of orange EO, (**ii**) to assess "*in vitro*" its antioxidant activity (DPPH method) of extracted EO, (**iii**) The application of this EO in a food matrix for biopreservative purposes.

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2. Materials and Methods

2.1. Collection of fresh peel orange fruit

Fresh oranges, variety Valencia (*Citrus sinensis* L.) were collected from healthy trees in full bloom during sunny days at the end of January of 2015, in province of Metidja (Algeria). The whole fresh fruits were then extensively washed with distilled water (20 °C). Only the fresh orange peels were recovered for subsequent extraction. Citrus fruits were peeled to separate the external part of the orange (flavedo), giving a yield of ~ 15% (w/w) of the peel with respect to the whole fruit.

2.2. List of chemicals and reagents

All reagents and solvents used in the extractions were of analytical grade. All chemicals were purchased from represented Algerian Chemical Society (Sigma–Aldrich, Chemistry-GmbH, Germany; Biochem, Chemopharma, Spain; Merck; Darmstadt, Germany).

2.3. Essential oils extraction

SFME has been performed using the "DryDist" microwave oven illustrated in **Figure 1**. In a typical microwave accelerated distillation **(**MAD**)** procedure performed at atmospheric pressure, 200 g of fresh orange peels were heated using a fixed power of 200 W for 10 minutes. The extraction was continued at 100 °C until no more EO was obtained. SFME is based on a relatively simple principle; this method involves placing plant material in a microwave reactor, without any added solvent or water. The internal heating of the *in situ*

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water within the plant material distends the plant cells and leads to rupture of the glands and oleiferous receptacles. This process thus frees EO which is evaporated by the *in situ* water of the plant material. A cooling system outside the microwave oven condensed the distillate continuously. The excess of water was refluxed to the extraction vessel in order to restore the *in situ* water to the plant material. For comparison, 200 g of fresh orange peels were submitted to HD with a Clevenger-type apparatus according to the European Pharmacopoeia and extracted with 2 L of water for 3 hours (until no more EO was obtained). Another comparison has been made by CP procedure. EO is collected from 1 kg of whole orange fruit using an automated cold pressing machine. The epidermis and oil glands were lacerate by a needle, creating areas of compression in the peel, surrounded by areas of lower pressure, across which the oil flows to the exterior. The oil is carried down to a decantation vessel in a stream of water, the emulsion being collected and then separated by centrifugation. The orange peel extractions provide a liquid limpid, mobile EO of yellowish color with a very strong and persistent odor of orange. Its density at 20 ºC was ~ 0.82 g/mL, its refraction index at 20 ºC was ~ 1.49, while its boiling point was ~ 52 ºC. The EOs were collected and stored in darkness in opaque sealed vials at 4 °C until further analysis and use in bioassays.

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141 *2.4. GC-FID and GC-MS analysis*

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143 The EOs were analyzed by gas chromatography coupled to mass spectrometry (GC–MS)
144 (Hewlett–Packard computerized system comprising a 6890 gas chromatograph coupled to a
145 5973A mass spectrometer) using two fused-silica-capillary columns with different stationary
146 phases. The non-polar column was HP5MS™ (30 m × 0.25 mm × 0.25 μm film thickness) and
147 the polar one was a Stabilwax™consisting of Carbowax™-PEG (60 m × 0.2 mm × 0.25 mm
148 film thickness). GC–MS spectra were obtained using the following conditions: carrier gas He;
149 flow rate 0.3 mL/min; split-less mode; injection volume 1 μL; injection temperature 250 ºC;
150 the oven temperature programme was 60 ºC for 8 min increased at 2 ºC/min to 250 ºC and
151 held at 250 ºC for 5 min; the ionisation mode used was electronic impact at 70 eV. The
152 relative percentage of the components was calculated from GC-FID peak areas. Most
153 constituents were tentatively identified by comparison of their GC Kovats retention indices
154 (RI), determined with reference to an homologous series of C5–C28 n-alkanes and with those
155 of authentic standards available in the authors' laboratory. Identification was confirmed when
156 possible by comparison of their mass spectral fragmentation patterns with those stored in the
157 MS database (National Institute of Standards and Technology and Wiley libraries) and with
158 mass spectra literature data [8].Then listed according to Kovat's retention index calculated in
159 GC on apolar HP-5MS column.

160

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161 *2.5. In vitro assay for antioxidant activity*

162 *2.5.1. DPPH radical-scavenging activity*

163

164 The ability of the SFM extracted EO to scavenge 1,1-diphenyl-2 picrylhydrazyl Radical
165 (DPPH•) was estimated. Pure EO was dissolved in 5% (vol/vol) Dimethyl Sulfoxide (DMSO),
166 and then EO dilutions were made to obtain different concentrations (50 to 450 µg/mL).
167 Aliquots 4 ml of DPPH (100 µg/L) methanol solution was taken in a test tube, and then mixed
168 well with 4 ml of EO DMSO solution. Then, the mixture was vortexed using a vortex mixer
169 (Cyclo-mixer) and incubated in the dark at ambient temperature (25 ± 1 ºC) for 30 min.
170 Decreasing of absorbance of tested mixtures was monitored every 1 min for 30 min at 517 nm
171 using a Perkin–Elmer Lambda 25 UV/Vis spectrophotometer. The absorbance was read
172 against pure methanol at 517 nm and the percentage of DPPH radical scavenging activity
173 (RSA) was calculated using the following equation:

174

$$RSA\ (\%) = [(Abs_{(DPPH)} - Abs_{(EO)})/Abs_{(DPPH)}] \times 100 \quad (1)$$

175

176 where $Abs_{(DPPH)}$ is the absorbance value at 517 nm of the methanolic solution of DPPH and
177 $Abs_{(EO)}$ is the absorbance value at 517 nm for the EO extracts.

178 The RSA was also expressed as the IC_{50} value (µg/mL), the concentration required to
179 cause 50% of DPPH inhibition. The percentage of scavenged DPPH• was plotted against the
180 EO extract concentration, and that required to quench 50% of initial DPPH radical was
181 obtained from the graph by linear regression. A lower IC_{50} value indicates greater antioxidant
182 activity. Synthetic antioxidant reagent butylated hyroxytoluene (BHT) was used as the
183 positive control. To standardize DPPH results, the antioxidant activity index (AAI), proposed
184 by Scherer and Godoy [9] was calculated as follows Eq. (4):

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185

$$AAI = \text{DPPH in reaction mixture } (\mu g/mL)/IC_{50}\ (\mu g/mL) \quad (2)$$

186

187 Studied EO was classified as showing poor antioxidant activity when AAI < 0.5, moderate
188 antioxidant activity when 0.5 < AAI < 1.0, strong antioxidant activity when 1.0 < AAI < 2.0,
189 and very strong when AAI > 2.0.

190

191 *2.6. Biopreservative effects in LWE*

192 *2.6.1. Preparation of LWE*

193

194 Eggs were surface-disinfected by immersion for 1 min in a dilute solution of ethanol
195 (70%), washed twice by immersion in distilled and sterile water, and left in a dry place to
196 remove excess water on the surface until using for "*in vivo*" assays. Just before experiments
197 were carried out, the eggs content (separately, egg whites and egg yolks) were removed under
198 aseptic conditions, and collected in sterile containers. When albumen was separated from
199 yolk, care was taken to ensure that albumen was not contaminated by the yolk content. The
200 chalaza was removed and the separated egg fractions were then homogenised for 2 min at
201 4000 rpm, using a commercial blender (31BL44, Waring, USA). To prepare 100 mL of LWE
202 samples, 33.25 mL of egg yolk were mixed with 66.75 mL of egg white; this proportion is the
203 normal average composition of whole egg and was used to attain a constant white to yolk
204 relationship. The proportion of albumen and yolk greatly affects processing characteristics of
205 LWE products and this proportion is mainly characterized by different moisture percentages,
206 total solids content and fat content [10,11].

207 The total LWE obtained were centrifuged at 103 × g for 2 min using a Heraeus
208 Megafuge 1.0R to eliminate any residual air. EO was added directly into LWE at a final

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concentration between 0.1-0.5%. All samples were placed under continuous lighting (1000 lux) in display cases at 4 ± 1 °C for 8 days, simulating commercial retail/display conditions. The positions of the samples in display cases (~ 80 cm under fluorescent tube) were rotated to minimize abuse light and temperature intensities at the surface of product.

Fat contents are analyzed according to the method of Folch et al. [12]. The pH of the LWE was measured using a micro pH-meter model 2001 (Crison Instruments, Barcelona, Spain) after homogenizing 3 g of the product in 27 mL of distilled water for 10 s at 1300 rpm using a Ultra-Turrax T25 macerator (Janke & Kunkel, Staufen, Germany).

2.6.2. Thiobarbituric Acid Assay

To measure the potential antioxidant capacity of studied EO and to evaluate the extension of the lipid oxidation on the LWE samples, the determination of the amount of the formed 2-thiobarbituric acid-reactive substances (TBARS) was undertaken, according to protocol developed by Djenane et al. [13]. The results were expressed as mg of MDA/kg of LWE and calculated using a standard curve prepared with 1,1,3,3-tetramethoxypropane. The Percentage inhibition rate (IR%) respect to the control was calculated as follows:

$$IR\% = [(C_{(sample)} - T_{(sample)})/C_{(sample)}] \times 100 \quad (3)$$

Where C is the number of TBA-RS in the untreated samples (LWE prepared without EO addition: control) and T is the number of TBA-RS in the treated samples.

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231 *2.6.3. Psychrotrophic bacteria analysis in LWE*

232

233 A sample of 10 g was removed aseptically and transferred to a stomacher bag containing
234 90 mL of sterile peptone water (PW) solution (0.1%), and was homogenized at room
235 temperature. Further serial decimal dilutions were prepared in PW solution (0.1%). The
236 appropriate dilutions were subsequently used for enumeration of microorganisms. Total
237 Psychrotrophic bacteria counts were determined using plate count agar (PCA) after incubation
238 at 7 ºC for 10 days [14]. The $\log_{10}$ CFU/mL of mean values for the counts were recorded.

239

240 *2.6.4. Sensory Analysis*

241

242 The attribute "EO odor" referred to the intensity of perceptible orange EO odor after
243 container sample opening: 1 = none; 2 = slight; 3 = small; 4 = moderate; and 5 = extreme.
244 Scores for "*off-odor*" referred to the intensity of odors associated to product spoilage: 1 =
245 none; 2 = slight; 3 = small; 4 = moderate; and 5 = extreme. A score of 3 or higher (≥ 3) in
246 both attributes denoted that LWE was unacceptable for sale or consumption.

247

248 *2.6.5. Instrumental color evaluation*

249

250 The color profile was measured using color space coordinates CIE (L*, a*, and b*)
251 (Mini XE, Portable type, USA) in accordance with the recommendations of the International
252 Commission on Illumination [15]. L (lightness), a (redness), b (yellowness) values were
253 recorded on the LWE emulsion of all samples. The instrument was calibrated using black and
254 white tile, provided with the instrument. The LWE samples were kept inside the glass Petri
255 dishes (diameter and height is 100 × 15 mm) in triplicate, and then instrument was directly

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256 put on the surface of the LWE emulsion at six different points for each Petri dishes. The mean
257 (n = 18) and standard error for each parameter were estimated.
258
259 *2.6.6. LWE Shelf life extension*
260
261 For shelf life study, chemical guidelines were used following the recommendations of
262 Djenane et al. [5] for animal foods: TBA-RS value: limit ~ 2 mg MDA/kg. The additional
263 microbial guidelines were also used following the recommendations of Garcia-Gonzalez et al.
264 [16] for whole egg: ~ 6 $\log_{10}$ CFU/mL for total aerobic psychrotrophic counts.
265
266 *2.7. Statistical analysis*
267
268 All data were analyzed by the general linear model (GLM) procedure of the Statistical
269 Package for the Social Sciences software (SPSS version 21, IBM Corporation, USA). Results
270 are presented as the means of three independent experiments ± standard deviation with three
271 replicates. Student's t-test was used to compare the efficacy of EO treatment and to determine
272 any significant differences among the treatments at a 95% confidence interval ($p < 0.05$).
273

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274 # 3. Results and Discussion

275

276 *3.1. Extraction and analysis of orange EO*

277 *3.1.1. Kinetics extraction yield*

278

279 The choice of the technique is the result of a compromise between efficiency and
280 reproducibility of extraction, ease of procedure, together with considerations of cost, time, the
281 degree of automation and safety. These shortcomings have led to the consideration of the use
282 of new "clean" technique in EO extraction, which typically use less solvent and energy. The
283 yields obtained by the both methods, SFME and HD, are similar but the difference is related
284 to the extraction time. With SFME, 10 min provides yields comparable to those obtained after
285 3 h by HD. The values obtained are respectively 0.40% for both methods (SFME and HD),
286 and 0.16% for CP (**Table 1**). The SFME is clearly quicker than its conventional counterparts.
287 The extraction takes 10 min., whilst 1 hour and 3 hours were required by CP and HD,
288 respectively. For the case of CP, an additional time of centrifugation (30 min.) of the
289 emulsion containing EO could be added to the real extraction time (1 h.). For HD or SFME,
290 the extraction temperature is equal to water boiling temperature at atmospheric pressure (100
291 °C). To reach this extraction temperature (100 °C) and thus obtain the distillation of the first
292 EO droplet, it is necessary to heat only 2 min. with SFME against 30-40 min. for HD. From
293 point of view early formation of first droplets, the first EO droplets were observed after 3.0
294 min in SFME and 23.0 min in HD. Heat transfer within the samples was the most important
295 factor for theses differences [17]. The rapid increase in temperature in the case of SFME
296 extraction is the main reason for this time reduction [18]. The obtained yield of peel EO from
297 *C. sinensis* was 1.89% [19]. This is not in agreement with Minh Tu et al. [20] who reported a
298 yield of 0.13%. Moreover, it has to be highlighted that one of the highest yields obtained was

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that in the case of Valencia Late oranges (2.3%) [21]. A yield of 0.79% has been recorded for Colombian orange peel oils [22]. Fresh peels of the Valencia late cultivar from Algeria yielded 0.39% of EOs [23]. EOs were extracted from epicarp (waste product from India) of *C. sinensis* (L.) Osbeck by hydro-distillation yielded 1.8% of EO [24]. Boukroufa et al. [25] found that the EO yields obtained from orange peel by Microwave Hydrodiffusion and Gravity (MHG) and steam distillation (SD) processes are comparable but a difference is only observed in the extraction time. Indeed, 15 min of extraction with this process are sufficient to extract totality from oil whereas it takes 240 min with SD, which is the one of the reference methods in EO extraction (gain of time of more than 93%). Advantages in terms of reducing time due to microwaves were also reported by other studies such as Ferhat et al. [26] for orange peel EO (12 min with microwave steam diffusion (MSD) vs. 40 min for steam diffusion (SD), Sahraoui et al. [27] for Citrus EO extraction using MSD (3 h with SD vs. 6 min with MSD), Perino- Issartier et al. [28] for lavender EO (15 min with MHG vs. 120 min with SD), and Sahraoui et al. [29] for EO extraction from lavender with MSD (6 min vs. 40 min for SD). The SFME was also reported to be the best compared to conventional HD for extracting EO from *Citrus grandis* peels [30]. Luciardi et al. [31] found that mandarin EOs obtained by CP/SD contains more oxygenated monoterpenes and sesquiterpenes fractions than mandarin EOs obtained by CP. Probably, the SD, could remove the oils that remains in the peel and effluent after pressing and separation.

SFME is proposed an “environmentally friendly” extraction method. SFME is a very clean method, which avoids residue generation (vs. CP) and the use of large quantity of water and voluminous extraction vessels (vs. HD). The reduced cost of extraction is clearly advantageous for the proposed SFME method in terms of energy and time. Regarding environmental impact, the calculated quantity of carbon dioxide (CO_2) rejected in the atmosphere is higher in the case of HD (3464 g CO_2/ g of EO) than for SFME (70 g CO_2/ g of

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EO). These calculations have been made according to literature: to obtain 1 kW h from coal or fuel, 800 g of CO_2 will be rejected in the atmosphere during combustion of fossil fuel [32,33].

3.1.2. Orange EOs composition

Table 1 lists the grouped compounds in EO: oxygenated and non-oxygenated fractions and composition of chemical families of orange EOs obtained by different extraction methods. The oxygenated fraction in EO samples from SFME (1.6%) was 40% higher than HD (0.82%) and 30% higher than CP (0.95%). The monoterpene hydrocarbons (i.e., limonene) are present in larger amounts in the hydro-distilled EO (95.48%) than the SFME EO (94.64%), but the extract obtained by SFME is more concentrated in oxygenated compounds (1.6 vs. 0.85-0.95%). The greater proportion of oxygenated compounds in the SFME EO is probably due to the diminution of thermal and hydrolytic effects, compared with CP which uses a large quantity of water and with HD which is time and energy consuming. Water is a polar solvent, which accelerates many reactions, especially reactions via carbocation as intermediates. Linalool was the main oxygenated component in the EO extracted from orange peels but the relative amounts differed for the three extraction methods.

Evaluating the results of the present study with those on orange EO, the percentages of the majority of the identified compounds in the orange species EOs differ in the literature. It is therefore possible that genetic differences within species determine the expression of different metabolic pathways regardless of geographic location [34]. The EOs of orange peels isolated by SFME, HD and CP are rather similar in their composition. Limonene is the main component with 94.64% for SFME, 95.48% for SD and 95.06% for CP. It is clear that microwave methods greatly accelerates the extraction process, but without causing considerable changes in the volatile oil composition. Bustamante et al. [35] found that no

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remarkable difference was observed in the composition of orange peel EOs extracted by HD and Microwave Assisted Hydrodistilation (MAHD). D-limonene was the most abundant chemical in both extracted EOs (96.75 and 97.38% for HD and MAHD, respectively). The yields for both samples were similar. In terms of energy consumption, the same authors found that a significant reduction was observed with comparing conventional HD to the microwave extraction (3.2 and 0.5 kWh, respectively), resulting in a great progress in terms of cost efficiency. A microwave heating causes a superheating phenomenon which facilitates the distension of the plant cells and leads to liberate the EO more quickly than in the case of conventional extraction methods [27].

However, it is worthy to note that limonene which was reported as the main component in *C. sinensis* EO was found in our samples. Previous studies regarding the EO of *C. sinensis* from different regions of the world were focused on the peel oil composition of val. cultivar. For example, limonene (94.95%) and myrcene (1.87%) were reported as the main components of the Italian val. cultivar [36]. Limonene (96.0–97.3%) was the main component of orange EO from Tunisia [19]. A study of Ferhat et al. [23] from Algeria reported that limonene (78.5%), myrcene (5.3%) and β-pinene (2.7%) were the typical components of the val. cultivar peel oil. Limonene (94.7%) and myrcene (2%) were reported as the basic components of the Vietnamese val. cultivar [20].

3.2. DPPH scavenging capacity

The activity of the *C. sinensis* EO was found to be dose dependent and the DPPH scavenging capacity of the tested oil augmented by increasing the concentration of EO and the inhibition ranged from 21 to 81% according to the tested concentrations (**Figure 3**). The lowest IC_{50} value (highest antioxidant activity) of 89.25 µg/L was obtained (**Table 2**), eight

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374 times greater than the value found for the reference compound.; BHT that was used as
375 positive control (IC_{50} = 11.37 µg/mL). Accordingly to the categories defined by Scherer and
376 Godoy [9], *C. sinensis* EO tested in the present study presented strong antioxidant activity,
377 when looking to its values of AAI = 1.12 µg/mL. The aptitude of orange EO to scavenge free
378 radicals could be attributed to the phenolic constituents as monoterpenes are responsible for
379 the overall reactivity of the orange oil towards DPPH radical. Our findings are similar to those
380 reported previously by Singh et al. [37] who examined the antioxidant activity of orange EO
381 using the DPPH way. The relationship among the antioxidant activity and their chemical
382 profiles was previously reported. Thus, the intensity of the antioxidant activity for the studied
383 specie may be related mainly to the major components which are limonene (94.64-95.48%),
384 and β-myrcene (1.64-1.87%). Even the cited compounds could explain in a part the elevated
385 antioxidant activities for this specie; it is difficult to attribute the antioxidant effect of the
386 whole EO to one or few active compounds. Both minor and major compounds should make a
387 significant role to the oil's activity which is the interaction result of their chemical
388 composition. Given that limonene is the main compound of orange EO, our results confirm
389 the study by Bacanli et al. [38] and Fancello et al. [39] which describes the antioxidant
390 activity of this compound. In the same order of ideas, Junior et al. [40] and Singh et al. [37]
391 found that the antioxidant nature of the EOs in terms of free radical scavenger may be due
392 also to DL-limonene mostly present in the citrus EO.
393
394 *3.3. Application in LWE*
395
396 The pH values of non-treated and treated LWE were not significantly modified during
397 chilled display (Results not shown, $p > 0.05$). Rossi et al. [41] and Monfort et al. [42] reported
398 mean values of 7.5 and 7.64, respectively for raw LWE. The lipid analysis of LWE showed

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399 9.78% (w/w) value. Jin et al. [11] and Ragni et al. [43] reported 9.88 and 10.09%,
400 respectively. The pH and lipid are an essential attributes for obtaining adequate LWE
401 functional properties [44].
402
403 *3.3.1. Lipid oxidation (TBARS-RS)*
404
405 It would seem interesting to say that EOs which are able of scavenging free radicals may
406 play an important role in food processing industry as natural additives to replace synthetic
407 antioxidants with natural ones**.** Synthetic antioxidants have successfully been used to prevent
408 lipid oxidation in animal food products but latest statement on health declares of these
409 synthetic chemicals have necessitated research on alternative successful compounds
410 particularly from natural sources. EOs have been qualified as natural bioactive agents due to
411 their ability to delay lipid oxidation in various food systems [13,45].
412 A large number of compounds are generated from lipid oxidation which adversely affect
413 quality and this limits the shelf-life of food. Lipid oxidation in animal food products it is
414 influenced by various factors such amount of polyunsaturated fatty acids (PUFA), O_2, metal
415 ions, temperature, and lighting. To prevent or delay lipid oxidation in foods, antioxidants can
416 be applied. Today, food industry search new economical and effective natural antioxidants
417 that can replace synthetic antioxidants.
418 The initial TBA-RS value of fresh whole eggs was 0.05 mg MDA/kg (**Table 3**). The
419 activity of the orange EO was found to be dose dependent. The TBA values of control
420 samples increased rapidly throughout the display. Results shows that at the 5^{th} day of display,
421 samples containing higher level of orange EO (0.5%) had TBA values 69.73% lower than the
422 TBA values of control samples. However, at the end of display, the degree of inhibition was
423 55.2% lower. During all period of storage, the lower treated samples (0.1%) showed a

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424 significant increment, and were more similar to the untreated controls. Our results are in
425 conformity with those of Viuda-Martos et al. [46] who studied the effect of various plant
426 extracts at different concentrations on lipid oxidation in egg products during storage. It was
427 reported that the lipid oxidation values was lower at higher concentration of plant extract.
428 Fernandez-Lopez et al. [47] was tested the orange powder for its potential as antioxidants in
429 beef meatballs and found them very effective against rancidity more than 12 days during
430 storage. Lowering the concentration of EOs without compromising their antioxidant activity
431 can also be obtained by applying them in combination with other antioxidant compounds that
432 provide a synergistic effect [45].
433
434 *3.3.2. Psychrotrophic bacteria analysis*
435
436 Whole eggs are prone to be contaminated by Gram-positive and Gram-negative
437 microorganisms [48], being microbial growth considerably decelerated due to the presence of
438 natural antimicrobials.; in particular lysozyme and conalbumin, in the egg white fraction
439 [49,50]. The changes in microbiological quality in the treated LWE samples during display, at
440 4 ºC are presented in **Figure 4.** The initial Psychrotrophic bacteria counts of samples reach
441 2.5 log CFU/mL. Garcia-Gonzalez et al. [16] reported 4.1 log CFU/g values for the initial
442 microbial loads of unprocessed LWE. In the first batches LWE0.1% and LWE0.2%, EO
443 treatment was less effective in decreasing the number of Psychrotrophic bacteria counts
444 compared to LWE0.5% treatments ($p < 0.05$). However, as display continued, significant
445 changes were observed: the Psychrotrophic counts continuously increased in control and 1st
446 batch (0.1%) and 2nd batch (0.2%) samples, whereas slower microbial development was
447 observed for the 3rd batch (0.5%). At the end of the shelf life study, the number of
448 psychrotrophic bacteria in the LWE treated samples reached 5.63 to 7.27 $\log_{10}$ CFU/mL.

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However, at the same time values of 7.5 $\log_{10}$ CFU/mL were obtained for untreated samples. High treatment (LWE0.5%) was as effective to ensure microbial stability during the entire display study. However, Psychrotrophic bacteria limit was not exceeded at the end of the storage study (< 7 $\log_{10}$ CFU/g) and thus acceptable for consumption even after one weeks of display for both higher treatments (0.2 and 0.5%) in accordance with the microbiological guidelines**.** A number of studies have demonstrated the wide spectrum of antimicrobial activity of citrus species, especially orange EO against a wide range of microorganisms [51], which has been confirmed and extended in this study. Luciardi et al. [31] showed that citrus EOs can be used to inhibit quorum sensing and virulence factors of *Pseudomonas aeruginosa* by producing a biofilm in food systems. Orange and lemon dry powder at 5% (w/w) were tested by Fernandez-Lopez et al. [47] for their potential as antimicrobials in beef meatballs and found them to be very effective against LAB over a 12-day period. Citrus EOs were also investigated for their ability to reduce total aerobic bacteria and psychrotrophic number in comparison to controls over a period of 90 days at refrigerated beef storage [52]. Callaway et al. [53] and Muthaiyan et al. [54]. have studied the antimicrobial effects against common foodborne pathogens of citrus by-products industry EOs and found that several of them have been shown to possess antimicrobial properties. Several studies have shown that Gram-positive bacteria are more susceptible to Citrus EO than Gram-negative bacteria [55]. Contrary to all expectations, Lin et al. [56] showed that the orange EOs could effectively inactivate some Gram negative bacteria such as *V. parahaemolyticus*, *S.* Typhimurium, and *E. coli* but not *S. aureus* (Gram positive), on the food contact surfaces.

Higher contents of phenolic compounds such as major monoterpene hydrocarbons present in the EO could be responsible for the higher antimicrobial activity. The antibacterial activity of studied orange EO can be assigned to several molecules, amongst other the

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limonene, major constituent of orange EO. It has been reported that limonene is more effective on bacterial strains [57].

3.3.3. Sensorial odor analysis

The production of *off-flavor* or strong odor limits the use of EOs as food preservatives to increase the shelf life of food products. The use of EOs as biological agents in food industry could affect the organoleptic properties of the products, so that obtaining a better biological effect in the lower concentration is essential. Thus, sensory tests by a trained panel would be necessary in some cases to elucidate this aspect. Regarding the intensity of the orange smell (**Table 5**), LWE 0.5% samples were given a initial score of 3 (Day 0), while LWE subjected to low treatment with 0.2 and 0.1% orange EO were given a score < 2.2 during all period of display, representative of acceptable orange smell. It appears evident that the intensity of the orange smell decreases with time of display; no more perceptible after 5 days ($p < 0.05$) and reached acceptable values for all samples. From a practical point of view, the dose used for processing LWE is low and acceptable in this product. The treatment with orange EO × display times had an effect on *off-odor* attribute sensory scores ($p < 0.05$). At all period of display, the untreated samples had higher ($p < 0.05$) off-odor scores. Generally, TBA-RS values have been correlated with consumer perception of lipid oxidation in animal products [5]. Increased *off-odor* of untreated samples was probably due to oxidative rancidity by increased lipid oxidation as shown by the TBARS values, which could be easily perceived by the panelists'. It would also be probable that the *off-odor* perceived by panelists' is also due to microbial development as shown by the psychrotrophic bacteria's counts. Untreated samples were assessed by the panelists with scores rejection limit (score ~ 4) at 5rd day of display, whereas higher treated samples (0.2 and 0.5%) were assessed by the panelists with scores

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498 below ($P < 0.05$) the rejection limit during all period of display. The higher treatments with
499 orange EO significantly ($P < 0.05$) extended LWE odor shelf life attributes. The antioxidant
500 activity may be ascribed to the presence of chemical components; monoterpenes found in this
501 EO may act as bioactive agents and to avoid loss in sensory quality. The extreme aroma of
502 various EOs, even at low concentrations, can cause negative organoleptic effects exceeding
503 the threshold acceptable to consumers. In the last decades, different strategies can be used to
504 resolve this problem. One option is to use plant extracts in active packaging rather than as an
505 ingredient in the product itself. EOs can be encapsulated in edible and biodegradable coatings
506 polymer that provide a slow release to the headspace of food packages and consequently to
507 the food surface [5].
508 The edible gelatin coating enriched with orange EO has perceptible effects on the quality and
509 shelf life of shrimps [58].
510
511 *3.3.4. Instrumental color*
512
513 At positive values, the CIE a* index indicates reddish colors, however, at negative
514 values, colors are green. CIE b* index take positive values for yellow and negative for blue.
515 Finally, the CIE L* parameter is an approximate measure of luminosity. Each of the color
516 parameters mentioned above is directly associated with certain quality criteria of the product
517 in question, for example, the presence of fundamental compounds in the product, pH, water
518 retention capacity and texture. The color of the LWE is a subject of practical importance for
519 the egg-processing industry, which requires egg products with an appropriate and a
520 homogeneous distribution of the color to satisfy the demand of the food industry. Customers
521 may not accept discoloration caused by long term storage, considering the LWE as being of
522 low-quality. For this reason, synthetic antioxidants are usually added to the egg products to

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reach the desired color and make the products more attractive for consumers and more appropriate for the egg-processing industry. Color perception highly depends on the chemical and microbial properties of the LWE components. It has been described that denaturation of certain proteins.; lipids and pigments, which occurs during storage, explains the main color changes that occurred in stored animal food products [5,59].

The most obvious changes during the storage of liquid egg products are typically related to color. Results concerning orange EO treatments are recorded on **Table 6**. After the treatments, red (CIE a*) and yellow (CIE b*) coordinates increased moderately for LWE0.5% during display if compared with other treatments, resulting in more orange products. Also, the CIE L* value decreased with the exposure time, and results for untreated samples are comparatively darker than the others. In similar products, de Souza and Fernandez [59] found a comparable tendency for the CIE Lab color coordinates. The results obtained instrumentally would justify the physical- chemical and microbial tests results for displayed fresh LWE, being the oxidation reaction and microbial development likely responsible for the observed differences.

3.3.5. Shelf-life determination

The shelf life of LWE is determined based on sensory analysis (attribute scoring: A score < **3** in "*off odor*" parameter denoted that product was acceptable, chemical (TBA-RS value: limit ~ **1.5** mg MDA/kg), and microbiological (psychrotrophic aerobic count: limit ~ **7** $\log_{10}$ CFU/g) properties. The shelf life is defined as "the period between raw LWE scale laboratory preparation and the storage sampling, during which the product is in a state of satisfactory quality in terms of chemical, physical, microbiological and sensory attributes".

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The display of LWE demonstrated that orange EO was needed for obtaining a significant increase of retail shelf life. It was evident that the LWE quality attributes during the display period depended on the concentration of orange EO added in the product. The long term chemical, physical, microbial and sensory stabilities of the raw LWE during display were positively influenced by EO treatments and shelf-life was up to 1 week. The quality stability of LWE during display was higher at higher orange EO treatment.

It is evident that the constituents separately isolated from EOs may play an important role in the biological activity of the latter. However, the main obstacle for using separated constituents as food preservatives is that they are most frequently not potent as separate use, and they cause negative organoleptic effects when added in sufficient amounts to provide a biological effect.

The food industry primarily uses EOs as flavorings. However, application of EOs as food preservatives requires detailed knowledge about their properties, i.e., the range of target organisms, the mode of action, and the effect of food matrix components on their antimicrobial and antioxidant properties. A range of EO components have been accepted by the European Commission for their intended use as flavorings'' in food products such as limonene which is considered to present no risk to the health of the consumer.

The United States Food and Drug Administration (FDA) also classify these substances as generally recognized as safe (GRAS). The high value of LD_{50} of the *C. sinensis* oil through oral administration on mice indicates their non-mammalian toxicity [37] and recommended that EO of *C. sinensis* and DL-limonene can be used as potent shelf life enhancement of stored food. In addition, the various constituents may interact, causing synergistic, antagonistic, indifferent and additive effects. A comparative study of EO constituents may help to understand the key points of the biological activity of EOs, acting alone or in combination with other food preservation techniques.

572

573 4. Conclusion

574

575 Microwave energy is a key enabling technology in achieving the objective of sustainable
576 "clean" production for research, teaching and commercial applications. It has been shown that
577 solvent-free conditions are especially suited to microwave-assisted organic synthesis, as
578 reactions can be run safely under atmospheric pressure in the presence of significant amounts
579 of products. SFME has been conceived following the concepts of SFM synthesis. When
580 coupled to microwave radiation, solvent free techniques have proved to be of special
581 efficiency as clean and economic procedures. Major improvements and simplifications over
582 conventional methods originate from their rapidity, their enhancement in yields and product
583 purities. Orange EO is also shown to be a particularly interesting field for applications within
584 the food industries. The long term oxidative, microbial and sensory stability of the LWE was
585 positively influenced by orange EO treatments and shelf-life was up to 1 week of refrigerated
586 display. Therefore, the results obtained confirm EO treatment as a promising technology to
587 lengthen the commercial shelf-life of liquid egg products.

588

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591
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593
594 Author **Contributions**: A.M designed and performed the experiments, and analyzed the data, and wrote the
595 manuscript. F.M.A., H.M., provide citrus samples and materials. A.A and D.D conceived
596 designed, revised the manuscript, and supported the project.
597

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781 Figure captions
782
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784
785 **Fig. 1** Solvent-Free Microwave Extraction System

786 **Fig. 2** Characteristics of the microwave heating
787
788 **Fig. 3** Free radical scavenging activity (%) of the orange EO
789
790 **Fig. 4** Total psychrotrophic bacteria counts ($\log_{10}$ CFU/mL ± SD) of LWE treated with orange EO during cold
791 storage. Letters show significant differences among the groups at $p < 0.05$
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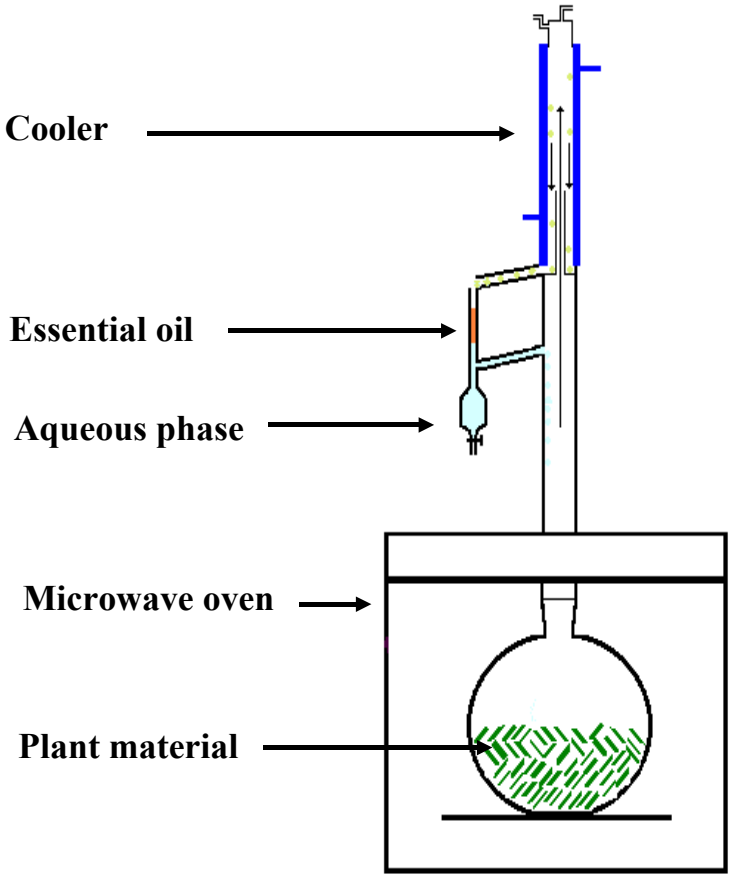
796 **Figure 1**



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800 **Figure 2**

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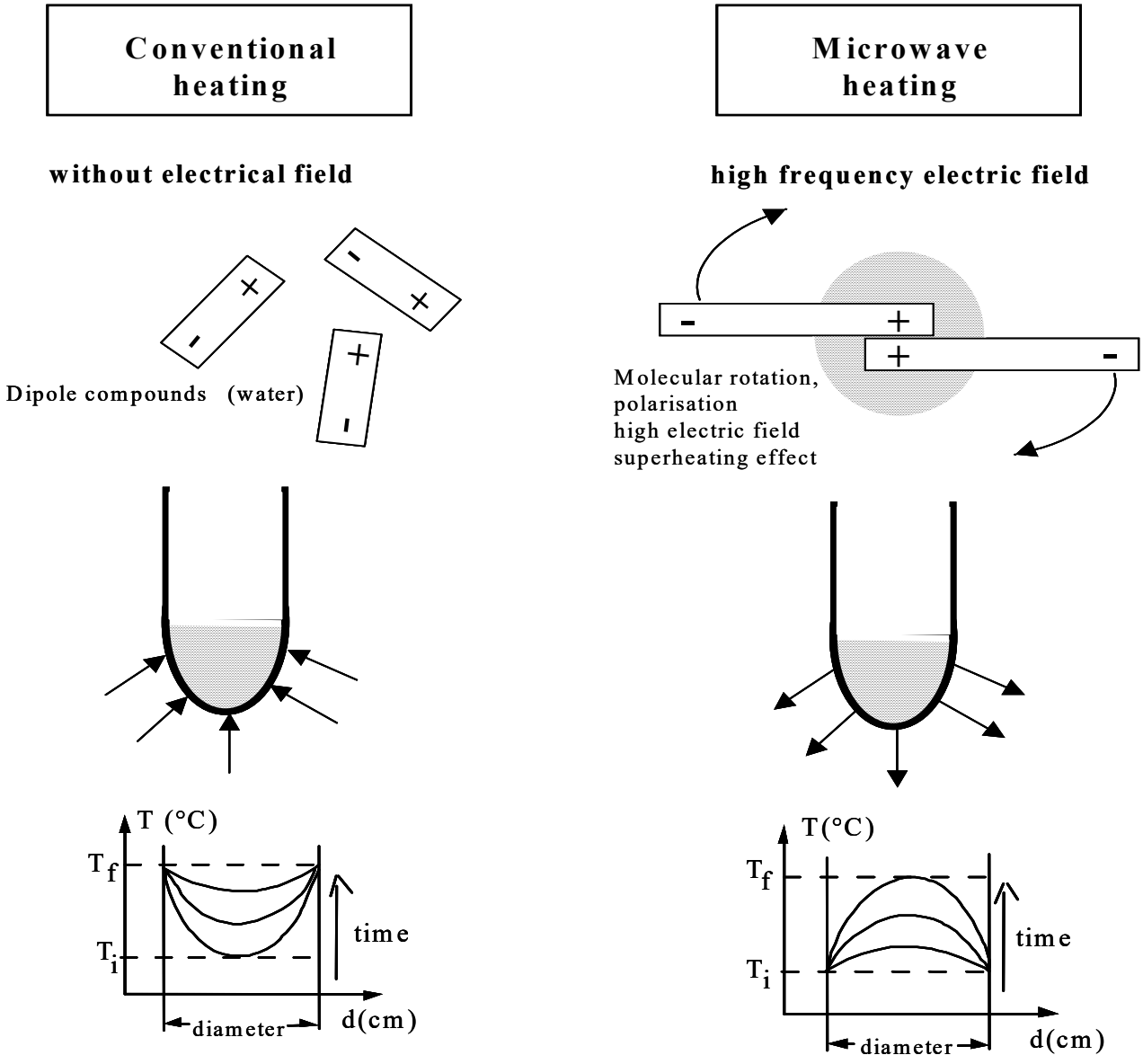

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Figure 3

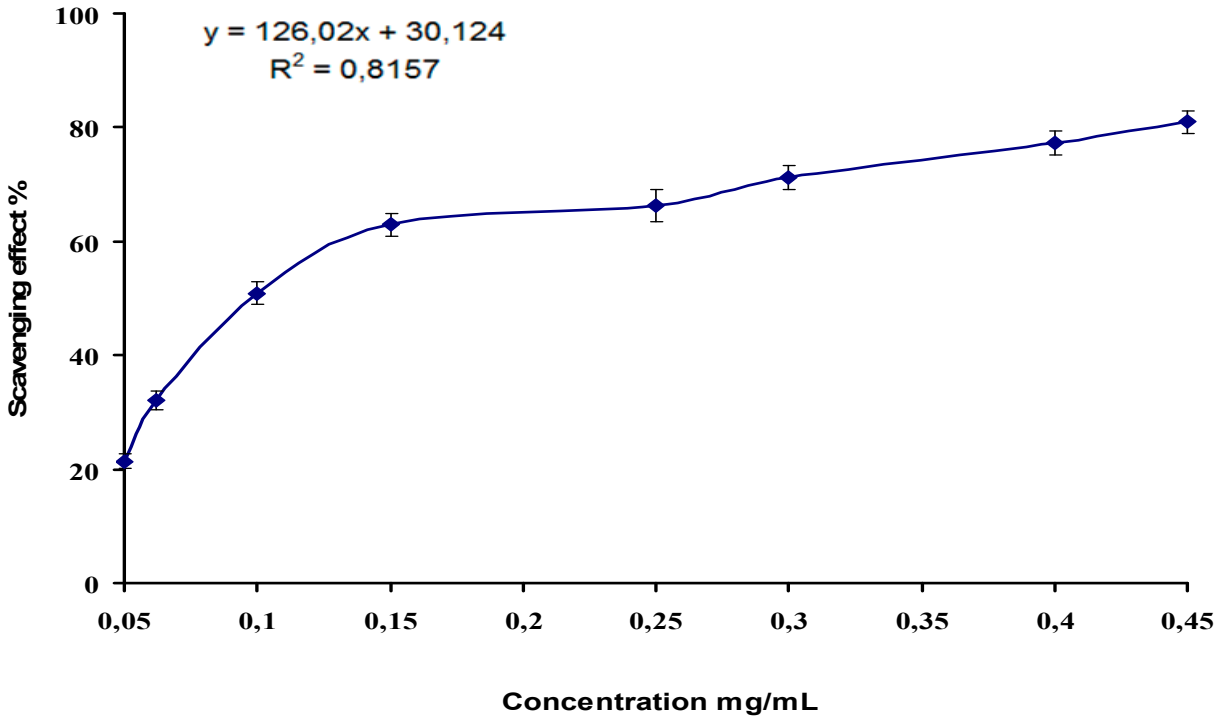

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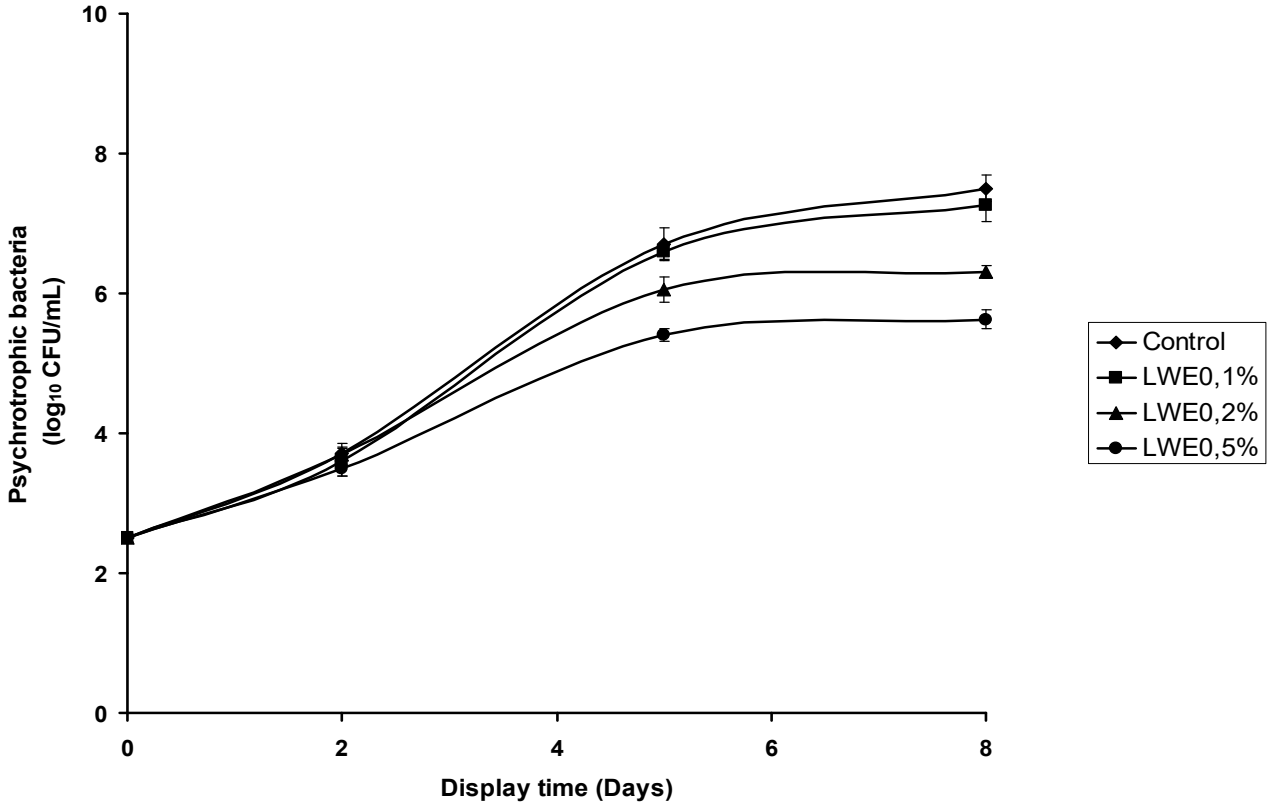
Figure 4



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812 **Table 1** Extraction time, yields and chemical compositions of EOs obtained by SFME, HD,
813 and CP extraction from Valencia late (*Citrus sinensis* L.) peels.

N°	Compounds [a]	R.I. [b]	R.I .[c]	Valencia late		
				SFME[e]	HD[f]	CP[h]
				Percentage (%)		
	Monoterpene Hydrocarbons			**97.48**	**98.61**	**98.32**
1	Thujene<Alpha->	920	1036	-	-	-
2	Pinene<Alpha->	926	1023	0.43	0.53	0.51
3	Sabinene	961	1121	0.54	0.49	0.54
4	Pinene<Beta->	974	1109	-	-	-
5	Myrcene<Beta->	988	1165	1.64	1.87	1.82
6	Phellandrene<Alpha->	1001	1177	0.15	0.17	0.36
7	Terpinene<Alpha->	1020	1083	-	-	-
8	Limonene	1030	1206	**94.64**	**95.48**	**95.06**
9	Ocimene<(E)-Beta->	1048	1282	0.02	0.02	0.02
10	Terpinene<Gamma->	1103	1285	0.05	0.03	0.01
11	Terpinolene	1120	1304	0.01	0.02	0.01
	Oxygenated Monoterpenes			**1.09**	**0.58**	**0.55**
12	Linalool	1125	1538	0.62	0.30	0.30
13	Citronellal	1167	1478	0.06	0.03	0.04
14	Terpin-4-ol	1191	1590	0.03	0.06	0.02
15	Terpineol<Alpha->	1203	1677	0.10	0.06	0.05
16	Nerol	1237	1781	0.10	0.03	0.08
17	Neral	1268	1670	0.05	0.03	0.06
18	Geraniol	1271	1828	0.04	0.01	-
19	Geranial	1284	1714	0.09	0.06	0.11
20	Thymol	1288	2107	-	-	-
	Sesquiterpene Hydrocarbons			**0.06**	**0.10**	**0.15**
21	Caryophellene<E->	1391	1594	0.02	0.02	0.01
22	Humulene<Alpha->	1450	1657	0.01	0.03	0.03
23	Germacrene D	1477	1696	-	-	-
24	Valencene	1488	1705	0.02	0.05	0.02
25	Bisabolene(Beta-)	1508	1718	-	-	-
26	**Oxygenated Sesquiterpenes**			**0.01**	**-**	**0.09**
27	Elemol	1540	1381	0.01	-	0.09
28	Caryophellene Alcohol	1560	1398	-	-	-
29	Nootkatone	1799	2250	-	-	-
30	**Other Oxygenated Compounds**			**0.50**	**0.27**	**0.31**
31	Octanol<N->	1102	1544	0.15	0.05	-
32	Nonanal<N->	1126	1400	0.01	0.01	0.02
33	Decanal	1210	1497	0.31	0.19	0.27
34	Linalyl acetate	1255	1553	-	-	-
35	Citronellyl Acetate	1342	1645	0.02	0.02	0.01
36	Neryl Acetate	1351	1706	0.01	-	0.01
37	Geranyl Acetate	1366	1742	-	-	-
38	Dodecanal	1391	1691	-	-	-
	Extraction time (min)			**10**	**180**	**60**
	Yield %			**0.40**	**0.40**	**0.16**
	Total Oxygenated Compounds %			**1.60**	**0.85**	**0.95**
	Total non- Oxygenated Compounds %			**97.54**	**98.71**	**98.47**

814 [a] *Essential oil compounds sorted by chemical families and percentages calculated by GC-FID on non-polar HP5MS™*
815 *capillary column.*
816 [b] *Retention indices calculated on non-polar HP5MS™ capillary column.*
817 [c] *Retention indices calculated on polar Carbowax™-PEG capillary column.*
818 [e] *SFME: solvent free microwave extraction*
819 [f] *HD: hydro-distillation*
820 [h] *CP: cold pressing*
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822 **Table 2** DPPH scavenging method of SFME EO of *Citrus sinensis* L. (Mean values ± standard deviation)
823

Method	Parameters	*Citrus sinensis* L.
DPPH scavenging assay	- IC_{50} (µg/ml)*	89.25 ± 3.45
	- Antioxidant Activity Index (AAI)**	1.12 ± 0.08
	- Antioxidant activity	Strong

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826 **IC50 value (µg/ml) of BHT: 11.37 ± 0.35*
827 ***AAI < 0.5 (Poor); 0.5 < AAI < 1.0 (moderate); 1.0 < AAI < 2.0 (strong) and AAI > 2.0 (very strong).*
828 *- IC_{50} values are defined as the concentration of test material which is able to decrease the initial concentration of DPPH to*
829 *half of its initial value.*
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Table 3 TBA values (mg MDA/kg of meat) and IR%* (values between parenthesis) of LWE containing orange EO during cold display.

Display (Days)	Control	LWE 0.1%	LWE 0.3%	LWE 0.5%
0	0.05 ± 0.006^{aW}	0.05 ± 0.006^{aW}	0.05 ± 0.006^{aW}	0.05 ± 0.006^{aW}
3	0.50 ± 0.09^{aX}	0.41 ± 0.06^{abX} (18.00%)	0.45 ± 0.04^{bX} (10.00%)	0.25 ± 0.06^{cX} (50.00%)
5	1.85 ± 0.11^{aY}	1.60 ± 0.08^{abY} (13.51%)	1.09 ± 0.06^{cY} (41.08%)	0.56 ± 0.08^{dXY} (69.72%)
8	2.5 ± 0.07^{aYZ}	2.05 ± 0.13^{abYZ} (18.00%)	1.58 ± 0.09^{bYZ} (36.80%)	1.12 ± 0.12^{cYZ} (55.20%)

**The Percentage inhibition rate (IR%) respect to the control was calculated as follows:*
$IR\% = [(TBA\text{-}RS_C - TBA\text{-}RS_T)/TBA\text{-}RS_C] \times 100$. Where C is the number of TBA-RS in the untreated samples (control) and T is the number of TBA-RS in the treated samples.

$^{a-d}$Within each row, different superscript lowercase letters show differences between treatment groups ($p < 0.05$).
$^{W-Z}$Within each column, different superscript uppercase letters show differences between the storage times within same treatment group ($p < 0.05$).
LWE0.1%: Liquid Whole Eggs treated with 0.1%of orange EO.
LWE0.3%: Liquid Whole Eggs treated with 0.3%of orange EO.
LWE0.5%: Liquid Whole Eggs treated with 0.5% of orange EO.

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852
853 **Table 4** CIELAB L*, a* and b* color coordinates in LWE treated with orange EO. Each sample was measured
854 in 10 different positions; results are the mean of three independent replications.
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856

LWE samples	*Days of storage*	*L**	*a**	*b**
Control	0	65.12 ± 1.52[a]	13.50 ± 2.01[a]	41.21 ± 0.99[a]
	3	64.45 ± 0.88[a]	11.10 ± 1.11[b]	40.61 ± 1.07[a]
	5	60.72 ± 1.01[a,b]	09.20 ± 2.01[c]	36.58 ± 0.89[b]
	8	59.52 ± 0.68[b]	09.04 ± 0.99[c]	36.02 ± 0.25[b]
LWE 0.1%	0	65.52 ± 0.54[a]	13.80 ± 1.01[a]	41.19 ± 0.99[a]
	3	64.92 ± 1.88[a]	13.40 ± 1.31[a]	40.42 ± 0.49[a]
	5	63.72 ± 1.31[a]	11.10 ± 0.05[b]	37.98 ± 0.69[b]
	8	63.08 ± 0.81[a]	10.03 ± 199[b]	36.10 ± 0.15[b]
LWE 0.2%	0	65.35 ± 0.32[a]	13.90 ±1.01[a]	41.20 ± 0.21[a]
	3	65.12 ± 1.08[a]	13.25 ±1.41[a]	40.88 ± 0.32[a]
	5	65.08 ± 1.25[a]	13.08 ± 0.55[a]	40.08 ± 0.60[a]
	8	63.73 ± 0.51[a]	12.23 ± 0.95[a,b]	39.10 ± 0.05[a]
LWE 0.5%	0	65.12 ± 1.52[a]	13.50 ±2.01[a]	41.28 ± 0.99[a]
	3	66.22 ± 0.48[a]	13.62 ± 1.12[a]	41.42 ± 0.69[a]
	5	65.09 ± 0.23[a]	13.59 ± 0.05[a]	41.55 ± 1.08[a]
	8	64.69 ± 0.05[a]	13.50 ± 2.11[a]	40.89 ± 2.19[a]

857
858 *L** (*lightness*); *a** *(redness); b** (*yellowness*)
859 [a-c]*Within each column for each color coordinates, different superscript uppercase letters show differences*
860 *between the storage times within treatment groups (p < 0.05).*
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Table 5 Sensory scores* (Mean ± SD) for EO *Citrus sinensis* L. odor and *off-odor* of LWE during display.

Parameters		Days of display 0	3	5	8
Citrus sinensis L. odor	Control	1.00 ± 0.00^{aA}	1.00 ± 0.00^{aA}	1.00 ± 0.00^{aA}	1.00 ± 0.00^{aA}
	LWE 0.1%	1.00 ± 0.00^{aA}	1.00 ± 0.00^{aA}	1.00 ± 0.00^{aA}	1.00 ± 0.00^{aA}
	LWE 0.2%	2.17 ± 0.41^{aB}	2.17 ± 0.41^{aB}	1.17 ± 0.41^{bA}	1.00 ± 0.00^{bA}
	LWE 0.5%	**3.00 ± 0.00^{aC}**	**2.83 ± 0.41^{aC}**	1.33 ± 0.52^{bAB}	1.17 ± 0.41^{bA}
*Off-odor***	Control	1.00 ± 0.00^{aw}	2.33 ± 0.52^{bw}	**3.67 ± 0.52^{cw}**	**4.17 ± 0.41^{cdw}**
	LWE 0.1%	1.00 ± 0.00 ^{aw}	2.17 ± 0.41^{aw}	**3.00 ± 0.00^{bwx}**	**3.33 ± 0.00^{bwx}**
	LWE 0.2%	1.00 ± 0.00 ^{aw}	2.17 ± 0.52^{bw}	2.33 ± 0.52^{bcy}	2.33 ± 0.52^{bcy}
	LWE 0.5%	1.00 ± 0.00 ^{aw}	2.00 ± 0.00^{bx}	2.17 ± 0.41^{by}	2.33 ± 0.52^{bcy}

A score < 3 in any of the parameters denoted that LWE was acceptable. When the score ≥ 3 in any of the parameters denoted that LWE can be rejected (End of the shelf life).

$^{a–d}$Means of the same row (between days of display) with different letters differ significantly ($p < 0.05$).

$^{A–C}$Means for Citrus sinensis L. odor of the same column (between treatments) with different letters differ significantly ($p < 0.05$).

$^{w–y}$Means for off-odor of the same column (between treatments) with different letters differ significantly ($p < 0.05$).

***Off-odor: referred to the intensity of odors associated to LWE oxidation and microbial development: 1 = none; 2 = slight; 3 = small; 4 = moderate; and 5 = extreme.*

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877
878
879 **Table 6** Proposed criteria for determining the expiry date (shelf life)* of LWE
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Oxidative parameters	End of shelf life (mg MDA/kg	Odor parameters	End of shelf life (off-odor score)	Microbiological parameters	End of shelf life (log_{10} CFU/mL)
TBA-RS	≥ 1.5	*Off-odor*	≥ 3	- Total psychrotrophic aerobic bacteria	≥ 7

881
882 *When the oxidative, odour sensorial, microbiological and colour instrumental parameters at the end of the shelf
883 life exceeds the guide values the products can only be rejected.