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1 **Essential oil-based nano-emulsions: effect of different surfactants, sonication and plant species on**
2 **physicochemical characteristics**

3

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10

11 **Abstract**

12 Essential oils (EOs) are promising active ingredients for biopesticides, although their use under field conditions is
13 limited by several criticisms concerning their high volatility and degradability. To overcome these negative qualities,
14 EOs can be encapsulated inside nanostructures (i.e. nanoparticles and nano-emulsions), which can guarantee the
15 preservation of the insecticidal properties. In the current study, oil in water (O/W) nano-emulsions of seven commercial
16 EOs (15% w/w of anise, artemisia, fennel, lavender, peppermint, rosemary, sage) were developed using different non-
17 ionic surfactants and formulation processes, to identify the best possible surfactant/process to produce stable nano-
18 formulations. The EOs were firstly examined by gas-chromatography analyses to identify their chemical constituents.
19 Sage, rosemary, peppermint, lavender and artemisia EOs were characterized by high percentage (up to 50% of the
20 detected compounds) of oxygenated monoterpenes, while fennel and anise EOs are mainly constituted by
21 phenylpropenes (e.g. anethol). Then, nano-emulsions were developed via the self-emulsifying process alone or in
22 combination with sonication, using four surfactants with different Hydrophilic Lipophilic Balance (HLB) index (5%
23 w/w of Tween 20, Tween 80, Span 20 or Span 80). The physical characteristics (droplet size and surface charge) of
24 nano-emulsions were analyzed using the dynamic light-scattering technique. Sonicated nano-formulations presented
25 smaller and more homogeneous size of the micelles than the non-sonicated ones, resulting in more stable nano-
26 emulsions. Furthermore, usually emulsions produced using Tween 80 as surfactant gave the best results in terms of
27 droplet size and polydispersity index (PDI) values. Therefore, Tween 80 sonicated nano-emulsions were examined
28 during a storage period of 28 weeks to determine their stability over time and possible alteration of their physical
29 characteristics. Results suggest that these nano-formulations had a good stability over time, since relatively small
30 increases in PDI and size values were recorded. Formulation stability is a key issue to consider when proposing
31 botanical biopesticides for agricultural applications. Our study reports the first step in the introduction of EO-based
32 nano-emulsions into practical application.

33

34 **Key words:** Bioinsecticide formulation, Botanicals, Dynamic Light Scattering, Essential oil, Non-ionic surfactant.

35

36 **1. Introduction**

37 Essential Oils (EOs) are hydrophobic natural materials containing secondary metabolites (i.e. monoterpenes,
38 sesquiterpenes and phenylpropenes). They are mainly produced by higher plants, especially aromatic ones (over 17000
39 species) (Asbahani et al., 2015). To be defined as EOs, the matrices must be obtained by hydrodistillation, steam
40 distillation, dry distillation or mechanical extraction from plant organs (Do et al., 2015). The historical sources of EOs
41 originates in the Middle Ages from Arabs, who first developed the extraction techniques for these natural substances
42 (i.e. steam and hydro-distillation) (Burger et al., 2019). EOs are synthesized by endocrine and/or exocrine plant glands,
43 as well as epidermal cells (Svoboda and Greenaway, 2003), and can be stored in all plant organs such as flowers,
44 leaves, roots, bark or seeds. EOs play a key role in plant defense and they can be produced in response to abiotic and
45 biotic stress, but they can additionally serve as attractive allelochemicals for pollinators and other beneficial insects
46 (Regnault-Roger et al., 2012). EOs have been widely used in the traditional pharmacopeia and as food preservers due to
47 their antimicrobial properties (Bakkali et al., 2008). Today, their use is constantly increasing because of the strong
48 demand for natural ingredients in many sectors (e.g. cosmetic, flavor and fragrance industries).

49 Aside from antiseptic properties, in the last decades many studies evaluated the insecticidal activity of EOs
50 against plant pests (Ikbal and Pavela, 2019; Isman and Seffrin, 2018; Mudrončeková et al., 2019; Soares et al., 2019),
51 stored product pests (Campolo et al., 2018; Akami et al., 2019) and insect vectors (Baskar et al., 2018; Benelli et al.,
52 2019a ; Benelli, 2015; Pavela et al., 2019 ; Pavela, 2015), as well as on non-target organisms (Benelli et al. 2019b; Liu
53 et al., 2019), highlighting great perspectives for the development of EO-based biopesticides. Pest control is crucial to
54 ensure crop production, but the indiscriminate use of synthetic pesticides can cause several problems related to the
55 environmental and human health (Pavela and Benelli, 2016). In this scenario, among the alternative tools to control
56 insect pests, botanical-based pesticides are of major interest. Nevertheless, to date, few commercial products containing
57 EOs as active components are commercially available, and limited efforts have been made to evaluate their efficacy in
58 operative conditions (Isman and Grieneisen, 2014).

59 The main causes of the low conversion of laboratory studies into practical applications can be identified with a
60 series of problematics related to some physicochemical characteristics of these substances. First, EOs are lipophilic
61 liquids and they are generally poorly soluble in water, impairing an easy distribution in field conditions (Krzyżowski et
62 al., 2020). Second, many EOs are known to be phytotoxic, in fact they could be applied for weed control (Karalija et al.,
63 2020). Thus, their direct application on crops may cause severe plant desiccation and yield losses (De Almeida et al.,
64 2010; Ibáñez and Blázquez, 2020). Third, the constituents of EOs are volatile and highly degradable (i.e. they can be
65 thermolabile, oxidizable or hydrolysable) (Pavela and Benelli, 2016). The biodegradability of the EOs can be

66 considered positive for the absence of residues on the products and in the environment, but the low persistence of pure
67 EOs could reduce their efficacy against insect pests. The interest in the design of persistent EO-based formulations with
68 insecticidal properties is rising, although researches are still at an early stage.

69 The increasing number of publications about the application of nanotechnology in agriculture highlights the
70 opportunity to extend these techniques to biopesticides (He et al., 2019). The nano-encapsulation processes may
71 guarantee the stability over time of the active ingredients, as well as their constant release, decreasing the adverse
72 effects on plant tissues. Encapsulation processes require the bioactive components to be coated inside a matrix (i.e. a
73 synthetic or natural polymer), which isolates the active ingredients from the external environment (Rodríguez et al.,
74 2016). Several nano-encapsulation methods have been developed, but encapsulations inside nanoparticles or into nano-
75 emulsions seem to be the most adequate and promising (Campolo et al., 2017; Giunti et al., 2019). Apart from the
76 increased stability of the active components, nano-encapsulation ensures a good dispersibility/solubility in water.

77 Nano-emulsions are defined as kinetically stable systems, whose droplets usually present a size range from 50
78 to 200 nm (Tadros et al., 2004). Thus, nano-emulsions present a long-term physical stability, with no visible
79 flocculation or coalescence for long periods, which is a distinctive characteristic of these nanomaterials. The long-term
80 stability of nano-emulsions can be attributed to the impact of steric stabilization occurring when using non-ionic
81 surfactants or polymers (Tadros et al., 2004). Nano-emulsions present further advantages, like the lower quantity of
82 surfactants required compared to micro-emulsions, the uniform surface-coating and the good wetting, spreading and
83 penetration ability (Bouchemal et al., 2004). Furthermore, the nano-metrical size of these systems can improve the
84 efficacy of the active components. Nevertheless, the formulation of stable EO-based nano-emulsions can be dependent
85 on the EO/surfactant combination and/or their ratio, and it can require high energy inputs, such as sonication. Usually,
86 sonication and high amount of surfactant make the nano-emulsion's droplet size decrease; however, non-ionic
87 surfactants are known to cause phytotoxicity themselves (Hess and Foy, 2000; Hurtt and Hodgson, 1987; Liu, 2004) or
88 to increase the phytotoxic effect of some commercial herbicides (Liu, 2004; Mirgorodskaya et al., 2020; Niedobová et
89 al., 2019; O'Donovan et al., 1985).

90 In the current research, we investigated the role of different ingredients (i.e., various EOs and non-ionic
91 surfactants) and approaches (i.e. self-emulsification and sonication) on the EOs-based nano-emulsion outcomes in terms
92 of stability, droplet dimension, droplet homogeneity and surface charge. Different surfactants and EOs were tested and
93 the stability of the best formulations were evaluated over time. All the EOs as well as the developed nano-emulsions
94 were chemically and physically characterised. The main objective of the experiments was to develop EO nano-delivery
95 systems containing a high amount of essential oil (i.e. 15%) through a high oil:surfactant ratio (i.e. 3:1). Nano-
96 emulsions containing high percentage of EOs are preferable, since the insecticidal activity of these plant extracts is

97 generally dose-dependent (Campolo et al., 2018); therefore a concentrated formulation, due to the reduced quantity of
98 product needed, ensures an easier handling during field treatments. However, to the best of our knowledge, few studies
99 tried to develop EO-based nano-emulsions with high rate of EO (Adak et al. 2020; Lee et al. 2019), since stability may
100 be a key issue for this kind of formulation.

101

102 **2. Materials and methods**

103 ***2.1 Essential oils and chemical material***

104 Anise (*Pimpinella anisum*), artemisia (*Artemisia vulgaris*), fennel (*Foeniculum vulgare*), lavender (*Lavandula*
105 *angustifolia*), peppermint (*Mentha x piperita*), rosemary (*Rosmarinus officinalis*) and sage (*Salvia officinalis*) essential
106 oils were purchased from Esperis s.p.a (Milan, Italy). Each EO was diluted at 80 mg/ml with pentane distilled before
107 gas-chromatography analysis. Chemicals such as pentane, Tween 20, Tween 80, Span 20 and Span 80 were obtained
108 from Sigma Aldrich (Milan, Italy).

109

110 ***2.2 Essential oils chemical characterization***

111 *2.2.1 Gas Chromatography analyses*

112 GC/FID analysis.

113 The GC/FID analyses were performed using an Agilent 6890N gas chromatograph equipped with a flame-ionization
114 detector (FID), an electronic pressure control (EPC) SSL injector (Agilent Technologies, J&W Scientific Products, Palo
115 Alto, CA, USA), and an apolar HP-1 capillary column (100% polymethylsiloxane; 0.2mm x 50m; film thickness, 0.33
116 µm). The oven temperature was programmed rising from 40 °C to 200 °C at 2 °C/minute, then increased to 270 °C at 20
117 °C/minute and, finally, held isothermally at 270 °C for 20 minutes. Injector temperature was set at 220 °C and the
118 detector temperature at 280 °C. Split ratio was 1/100 and injection volume 1 µL. Samples were injected in triplicate for
119 quantitation.

120

121 GC-MS analysis.

122 The GC-MS analyses were carried out with a gas chromatograph model Agilent 6890 (Palo Alto, CA) equipped with a
123 mass selective detector MSD5975B (Agilent) and a multifunction automatic sampler (Combi-Pal, CTC Analytics,
124 Zwingen, Swiss) using an HP-1 MS capillary column (100 % polymethylsiloxane; 0.2 mm x 50 m; film thickness, 0.33
125 µm). The oven temperature was programmed rising from 40 °C to 200 °C at 2 °C/minute, from 200 to 270 °C at 20
126 °C/minute and kept isothermally at 270 °C for 20 minutes. Carrier gas was Helium (constant flow: 1 ml/minute);

127 ionization voltage, 70 eV; scan time, 1 second; mass range, 40–300 amu. Split ratio was 1/100, injector temperature 250
128 °C with an injection volume of 1 µL.

129

130 *2.2.2 Identification of volatile compounds*

131 Compounds were identified by comparison of their mass spectra with existing mass-spectral libraries (Flora97, Lca98,
132 NIST02) and of their linear retention index with those reported in the literature (NIST, Adams (2017)). Linear retention
133 indices (LRI) were determined by performing GC-MS on a set of known alkanes (C5–C20) whose LRI times were used
134 to calculate the LRI of the volatile compounds found in samples according to the definition of Van den Dool and Kratz.

135

136 *2.3 Nano-emulsion formulation and characterization*

137 All the nano-emulsions were prepared following Giunti et al. (2019), with modifications. Specifically, the nano-
138 emulsions were obtained using the spontaneous emulsification process, which can naturally occur when an organic
139 phase and an aqueous phase are mixed, followed by sonication. To evaluate the effect of surfactants on the droplet size
140 and stability of the EO-based emulsions Span 80, Span 20, Tween 80, and Tween 20, with an hydrophilic-lipophilic
141 balance value (HLB) of 4.3, 6.7, 15.0 and 16.7 respectively, were used for the nano-emulsion formulation. The process
142 consisted in mixing using a magnetic stirrer (30 min at 8000 RPM) the surfactant (5% w/w) with the essential oil (15%
143 w/w). Then, double-distilled water (80%) was dropwise ($1 \text{ ml} \cdot \text{min}^{-1}$) added to this mixture and then stirred for 60 min
144 to attain a homogeneous emulsified phase. Furthermore, double-distilled water solutions containing each surfactant
145 alone (5% w/w) were prepared and used as control for the subsequent analyses.

146 We hypothesized that the combination of self-emulsifying process and sonication may play a key role in the
147 nano-emulsion stability and droplets' size. To evaluate the effect of the sonication process on the stability and physical
148 parameters, the previously developed emulsions were sonicated for 5 min using an UP200ST ultrasonic immersion
149 homogenizer (Hielsher®, Teltow, Germany) at 100W power (frequency: 26 kHz). To avoid EO degradation due to the
150 heat generated by the sonicator, the process was carried out in an ice bath. The choice to use a high oil:surfactant ratio
151 (i.e. 3:1) was based on preliminary tests that showed a phytotoxicity of the nano-formulations proportional to the
152 amount of surfactant used.

153 The physical characteristics of the EO-based nano-emulsions were analysed by a Dynamic Light Scattering
154 (DLS) instrument (Zetasizer Nano, Malvern). These qualitative analyses assessed the droplet surface charge, indicated
155 by the zeta potential (ζ) values (electro-phoretic mobility of emulsions), and the droplets dimension, expressed in terms
156 of Z-average size (D_z) and polydispersity index (PDI), of the tested formulations at 25 °C. To avoid multiple scattering
157 effects 0.5 mL of every nano-emulsion were diluted in 100 mL of double-distilled water and aliquots (1 mL for D_z and

158 0.75 mL for ζ -potential) of the diluted emulsions were analysed. These physical characteristics are useful to determine
159 the quality of the developed nano-emulsions and to predict the stability over time of the formulations. Low Z-average
160 sizes (<200 nm) ensure that nano-metric droplets are obtained; it is commonly acknowledged that to smaller droplet
161 sizes correspond better nano-emulsions. Similarly, nano-emulsions with PDI values close to zero are preferred; actually,
162 low PDI values are characteristic of highly homogeneous nano-emulsions, which are less prone to the Ostwald ripening
163 destabilization phenomenon than non-homogeneous ones (Anarjan et al., 2014). Droplet surface charge (ζ -potential) can
164 play a role stabilizing the nano-emulsion by electrostatic repulsion (Müller et al., 2001; Tadros et al., 2004); however,
165 using non-polar surfactants, stability is mainly attributable to steric stabilisation over electrostatic one, hence ζ -potential
166 is not considered a primary parameter in the selection of optimal formulation. Nevertheless, drastic changes over time in
167 all the described parameters can be alerts of the emulsion instability. Therefore, the physical characteristics of the best
168 EO-based nano-emulsions were measured during the 1st, 2nd, 4th, 14th and 28th week of storage inside stainless steel
169 bottles (1.2 L) at controlled conditions ($T= 25 \pm 1^\circ\text{C}$; $\text{RH}= 50 \pm 5\%$), to check for significant changes.

170 For each sample, three replicates of fourteen cycles were provided. Three samples were analysed as replicates
171 for every tested parameter.

172

173 **2.4 Statistical analysis**

174 Dependent variables [Z-average size (Dz), the polydispersity index (PDI) and the zeta potential (ζ)] were subjected to
175 non-parametric Mann Whitney (U) and Kruskal–Wallis (H) tests since the ANOVA assumption (homogeneity and
176 normality of variance across the groups) were violated even after data transformation. To evaluate possible association
177 existing between two continuous variables (i.e. droplet size and PDI), the data were subjected to Pearson's correlation.
178 All statistical analyses were conducted using IBM SPSS 19 statistical software.

179

180 **3. Results**

181 **3.1 Essential oil composition**

182 All the EOs were analyzed by mean of GC-MS and GC/FID. **Table S1 to S7** detailed components identified
183 representing 90.77–98.53% of the oils from different plant species. Sage, rosemary, peppermint, lavender and artemisia
184 EOs were characterized by high percentage (up to 50%) of oxygenated monoterpenes, while fennel and anise EOs are
185 mainly constituted by phenylpropenes (**Figure 1**). Sage, rosemary and fennel EOs also contain significant amount of
186 hydrocarbon monoterpenes (between 27.68 % and 41.37 %). The most abundant compounds were eucalyptol ($52.58 \pm$
187 0.1 %) in rosemary EO; menthol (38.63 ± 0.05 %) in peppermint EO; linalol (38.13 ± 0.1 %) along with linalyl acetate
188 (33.42 ± 0.1 %) in lavender EO; (*E*)-anethol in fennel (40.58 ± 0.1 %) and anise (86.54 ± 0.2 %) EOs. Sage and

189 rosemary EOs were characterized by mix of compounds including α -pinene, eucalyptol, α -thujone, β -thujone and
190 camphor (**Table S1 and S5**).

191 192 **3.2 Effect of surfactant and sonication on physical characteristics of nano-emulsions**

193 Droplet size, PDI and ζ -potential were checked for all the developed nano-emulsions to determine the optimal
194 formulation (**Table S8**). Statistical analyses aimed to highlight possible common trends across all the tested EOs and to
195 select the best formulation process (i.e. sonication/no sonication) and composition (i.e. surfactant). Overall, the
196 sonication and the surfactant seem to be the most important variables affecting the physical characteristics of the nano-
197 emulsions. Sonication played a key role to reduce the droplet size and the PDI ($U_{1,154}=923.00$; $P<0.01$ - $U_{1,154}=561.00$;
198 $P<0.01$) regardless the surfactant and the EO used to develop the nano-emulsions. Conversely, the sonication process
199 did not affect the ζ -potential values ($U_{1,154}=3318$; $P = 0.328$). The different surfactants influenced all the measured
200 parameters (Size: $H_{3,152}=54.16$; $P<0.01$ - PDI: $H_{3,152}=25.64$; $P<0.01$ - ζ -potential: $H_{3,152}=89.44$; $P<0.01$). The
201 formulations developed using the different EOs highlighted statistical differences in the PDI ($H_{6,149}=18.45$; $P<0.01$),
202 whereas non statistical differences were registered for the ζ -potential values ($H_{6,149}=4.60$; $P>0.59$) and the droplet size
203 ($H_{6,149}=5.70$; $P>0.46$).

204 In terms of stability, many of non-sonicated emulsions showed a separation into two different phases just 24
205 hours after the production (**Figure 2**). Only the emulsions made with Tween 80 appeared quite stable. Anise and Fennel
206 EO-based formulations produced with Span 80 had a foamy consistency which made the sonication process impossible,
207 therefore these two emulsions were discarded from subsequent analyses. In the non-sonicated emulsions, the droplets
208 sizes were often within the micrometric range and presented high PDI values (0.25-1).

209 Among the sonicated formulations the smallest particle size was obtained using Tween 80 as surfactant
210 ($H_{3,74}=52.11$; $P<0.01$), followed in order by Tween 20, Span 20 and Span 80. In terms of PDI values, Tween 80 and
211 Tween 20 generated lower PDI values than the other two surfactants ($H_{3,74}=45.56$; $P<0.01$). Similar results were
212 obtained considering the surface charge (ζ -potential); the nano-formulations containing Tween 80 and Tween 20
213 obtained ζ -potential values statistically different ($H_{3,74}=50.62$; $P<0.01$) from Span surfactants. Disregarding the
214 different surfactants, the EOs used to develop the different sonicated formulations had a strong impact on particle size
215 ($H_{6,71}=24.07$; $P<0.01$), ζ -potential ($H_{6,71}=24.48$; $P<0.01$), and PDI values ($H_{6,71}=19.45$; $P<0.01$).

216 The control solutions presented droplet size typical for every surfactant (Tween 20: 8.9 ± 0.3 nm; Tween 80:
217 10.3 ± 0.4 nm; Span 20: 207 ± 3.8 nm; Span 80: 239.5 ± 10.5 nm). PDI values and ζ -potentials were similar for all the
218 control solutions (PDI: 0.09-0.19; ζ -potential: from -4 to 0 mV). The physical characteristics of the controls did not
219 change after sonication, neither over time.

220 The best combination in terms of particle size and PDI values were obtained when using Tween 80. This
221 surfactant, coupled with sonication, contributed to produce particle size ranged from 116 ± 2 nm (Artemisia) to 188 ± 2
222 nm (Sage). Nevertheless, the PDI values (from 0.14 to 0.20) and the size were not correlated ($\rho=0.364$; $P = 0.11$); the
223 lowest PDI value was recorded for the fennel EO-based formulation (0.14 ± 0.01), whereas the highest one was detected
224 in the artemisia one (0.20 ± 0.01). Among the sonicated nano-emulsions made with Tween 80, the employed EO affected
225 all the physical parameters measured (Size: $H_{6,14}=19.36$; $P<0.01$ – PDI: $H_{6,14}=12.66$; $P<0.05$ – ζ -potential: $H_{6,14}=18.92$;
226 $P<0.01$)

227

228 *3.3 Evolution of nano-emulsion formulations over time*

229 Tween 80 sonicated EO-based nano-emulsions were identified as the optimal formulations. Different physical
230 parameters related to the Tween 80-based formulations were measured for 28 weeks from their production, to
231 understand the evolution of the size, the PDI and the surface charge during storage. The developed nano-emulsions
232 retained an average size of the droplets within the nanometre range either after 28 weeks of storage (**Figure 3**). Droplet
233 size increased in all the evaluated formulations according to the storage duration ($H_{4,100}=24.48$; $P<0.01$) and it was
234 affected by the EO used to develop the formulation ($H_{6,98}=63.86$; $P<0.01$). In sage and lavender EO-based formulations
235 the recorded droplet sizes increased from the fourth week of storage, exceeding the threshold of 250 nm, while the
236 lowest increase of droplet dimension was reported for Artemisia EO-based nano-emulsion, whose average size was
237 around 130 nm after 28 weeks of storage.

238 Furthermore, the low values of the PDI, recorded just after a week from the emulsion preparation, indicated the
239 size homogeneity of the formulations, since few or no aggregates were detected (**Figure 4**). Nevertheless, PDI values
240 increased during storage in correlation with the size variations ($\rho=0.859$; $P<0.01$) and the time of storage ($H_{4,100}=51.76$;
241 $P<0.01$). Artemisia nano-emulsion, which showed slight size changes during storage, also showed slight changes for
242 PDI. In contrast, Sage formulation presented the highest PDI at 4 weeks, when the dimension of the droplets started to
243 grow. Conversely, the PDI of anise and peppermint EO-based formulations remained quite stable until 14 weeks, but
244 they presented a massive increase at 28 weeks, consistently with the increase of the droplet size.

245 All the EO nano-emulsion exhibited a negative ζ -potential, which ranged approximatively between -7 and -27
246 mV and the decline on the surface charge was time dependent ($H_{4,100}=40.48$; $P<0.01$) regardless of the EO used to
247 formulate the nano-emulsions (**Figure 5**). Generally, ζ -potential remained stable for at least 2 weeks, when it started to
248 decline. However, the decline of ζ -potential mainly depended on the EO employed for the formulation. The
249 formulations made with fennel and rosemary EOs showed a great decrease after two weeks of storage, while other EO-
250 based nano-emulsions, like anise and artemisia ones, had more constant patterns over time.

251

252 4. Discussion

253 In the current study, stable EO-based nano-emulsions with high relative amount of EO (i.e. 15%) were developed using
254 a mixed bottom up/top down process (Donsì and Ferrari, 2016). Nano-emulsions are perfect candidates for several
255 applications, since they display characteristic qualities, as their ability to dissolve non-polar active compounds in water
256 (Gutiérrez et al., 2008). The nano-dimensions generally improve both the persistence and effectiveness of botanical
257 extracts and active ingredients, boosting their gradual release (de Oliveira et al., 2014). However, the typical unstable
258 nature of plant-based formulations is the key criticism to make these materials applicable for large-scale application.

259 Generally, the self-emulsifying process, alone, did not produce nano-emulsions with small droplet size
260 (Lombardo et al. 2020). In the current study, only few formulations (i.e. some EOs formulated with Tween 80)
261 presented droplets with dimension ranging within the nanometric scale. The minimum droplet size in the non-sonicated
262 nano-emulsions was about 180 nm for the formulation containing lavender EO and Tween 80, whereas, in most of the
263 other EO/surfactant combinations, the droplet size was in the micrometric range. Several studies highlighted that the
264 droplet size of nano-emulsions decreases with the decrease of the oil:surfactant ratio (Gulotta et al., 2014; Li et al.,
265 2017; Saberi et al., 2013; Wang et al., 2009); thus, formulations containing high amount of surfactant could guarantee
266 small droplets' dimension. In insecticide formulations, the use of large amount of surfactant could have adverse effect
267 on plants (Mirgorodskaya et al., 2020; Niedobová et al., 2019); for these reason, the lowest relative amount of
268 surfactant, producing small droplets and high stability, was chosen.

269 In the current study, the different surfactants had an appreciable impact on both the droplet size and the PDI
270 values. Among the tested surfactants, Tween 80 was able to produce the smallest droplets when compared with the
271 other tested surfactants (Tween 20 Span 20 and Span 80). Similar results were reported by Chang et al. (2013), who
272 obtained the smallest droplets in carvacrol-based nano-emulsions made with a mix of food-grade non-ionic surfactants
273 (Tween 20, 40, 60, 80, and 85). Tweens, a class of non-ionic surfactants derived from sorbitan esters, are soluble or
274 dispersible in water and are oil-in-water emulsifiers frequently used in pharmaceutical, cosmetic and cleaning
275 industries. Among these surfactants, Tween 80 is one of the most commonly used to develop EO-based insecticides.
276 The EO/surfactant combination is fundamental to attain valuable formulations. As example, two of the seven tested EOs
277 (i.e. fennel and anise), when combined with Span 80, generated a dense foam impossible to sonicate. These two oils
278 were characterized by the presence of (*E*)-anethole as their main constituent, whereas this molecule was absent or
279 present only in small quantities in the other EOs; thus, (*E*)-anethole could be responsible for the formation of foam in
280 presence of Span 80. In literature, (*E*)-anethole or anise-based nano-emulsions were usually produced using Tween 80
281 alone or in combination with co-surfactants (i.e. ethanol) (Hashem et al., 2018; Pascual-Villalobos et al., 2017).

282 Furthermore, the sonication process had a strong positive impact on both the droplet size and the PDI. This
283 method is widely used for fabrication of nano-emulsions (Donsì and Ferrari, 2016). Ultrasonication for short period (i.e.
284 3 min) were found to greatly decrease droplet size (coarse: 1420.5 ± 111.0 nm; sonicated: 221.3 ± 0.8 nm) and increase
285 homogeneity (coarse: 0.709 ± 0.04 PDI; sonicated: 0.251 ± 0.00 PDI) when applied to oregano EO nano-emulsion
286 (EO=10%; Tween 80 & lecithin=10%, ratio 3:1) (Lee et al., 2019). In the current study, the comparison of various
287 surfactants and emulsification processes highlighted that the sonicated nano-emulsions produced with Tween 80 had the
288 best physical characteristics. The developed EO/Tween 80-based nano-emulsions presented small droplet size and good
289 stability over time. Nevertheless, both size and stability seemed mainly dependent on the plant-source, since every EO-
290 based formulation showed a peculiar size trend over time. The best results were obtained with artemisia EO, whose
291 nano-emulsion was characterized by a size of 116 ± 2 nm and it remained almost stable after 28 weeks of storage, with
292 a droplet dimension of 128 ± 1 nm. In contrast, results for sage EO-based nano-emulsion presented the highest droplet
293 size both at 1 week (188 ± 2 nm) and 28 weeks (260 ± 1) of storage. The difference of droplet size and droplet stability
294 can be related to the chemical composition of EOs. The molecular weight, polarity and conformation of volatile
295 compounds, as well as the presence of surface-active substances in the EOs can affect their water solubility and their
296 capacity to form droplets with Tween 80 (Acevedo-Fani et al. 2015; Ziani et al., 2012). Artemisia EO is mainly
297 constituted of oxygenated monoterpenes (82.5%), while composition of sage EO is more structurally diversified with
298 monoterpenes (33.4%), oxygenated monoterpenes (56.2%) and sesquiterpenes (7.7%). However, main compounds of
299 these two EOs are α -thujone (27.1% and 16.2%), β -thujone (13.2% and 4.4%), and camphor (21.4% and 12.6%) for
300 artemisia and sage EOs, respectively. Lavender EO also contain camphor (6.9%) and high percentage of oxygenated
301 monoterpenes (89.9%), but only artemisia based nano-emulsion is stable over 28 weeks. Finally, artemisia EO is
302 characterized by the lowest content in sesquiterpenes (0.6%) while percentage of sesquiterpenes is always over 2% in
303 other EOs. It turns out that the most unstable nano-emulsions made from sage and lavender EOs are characterised by
304 the highest content in sesquiterpenes (7.7% and 4.3%, respectively). Even if it seems difficult to correlate droplet size
305 stability to one or mix of compounds in this study, interesting results obtained with artemisia EO could be explain by a
306 high percentage of oxygenated monoterpenes (i.e. α -thujone, β -thujone and camphor) combined with a low content in
307 sesquiterpenes.

308 The good stability of the sonicated formulations was additionally supported by the low PDI values (0.13-0.20)
309 recorded during the first storage phases. This characteristic seemed strongly linked to size increase, and PDI values
310 tended to grow with increase in storage time. These results suggested these nano-emulsions are not permanently stable,
311 but a good stability of their characteristics may be guaranteed for long storage periods. The droplet dimensions obtained
312 in the present study are consistent with previous studies on EO-based nano-emulsions containing lower oil:surfactant

313 ratio (Hashem et al., 2018; Lee et al., 2019; Moghimi et al., 2016; Werdin González et al., 2014). As example, Adak et
314 al. (2020) formulated eucalyptus EO nano-emulsions containing from 6 to 10% of EO and EO:Tween 80 ratios ranging
315 from 1:05 to 1:1.5 obtaining dimensions comprised between 150-400 nm. However, when using EO:surfactant ratio
316 similar to those applied in the current study (i.e. around 1:0.3), droplet sizes of 429.6 nm and 318.0 nm and PDI values
317 of 0.490 and 0.331 were reported for the emulsions containing 8 and 10% of eucalyptus EO, respectively (Adak et al.,
318 2020). High PDI values (around 0.3) were also noted for geranium EO-emulsions, which contained oil:surfactant (i.e.
319 Tween 80) ratios of 1:05 to 1:1, and showed dispersed phase diameters of 79 to 106 nm, respectively (Jesser et al.
320 2020). However, Jesser et al. (2020) noted that these emulsions were not stable after just three days from preparation. In
321 contrast, the nano-emulsions developed in the present study, containing a higher oil:surfactant ratio (3:1), presented
322 lower PDI values and a good stability over time.

323 The developed nano-emulsion owed their stability mainly to steric-repulsion, considering that Tween 80 is a
324 non-ionic surfactant. For this kind of emulsifier, osmotic forces can be substantial at distances of close interfacial
325 approach, and they generally present greater order of magnitude than double layer repulsion and Van der Waals forces
326 (Babchin and Schramm, 2012). Thus, artemisia formulation, which was noted as the most stable nano-emulsion,
327 presented the highest (i.e. closest to 0 mV) values of ζ -potential (from -10.6 to -7.6 mV). Nevertheless, the presence of a
328 negative surface charges, which depends on the composition of oil, the pH and the electrolytes present in the water
329 phase, may help to stabilize the nano-emulsions (Müller et al., 2001; Tadros et al., 2004). A minimum of ± 30 mV of ζ -
330 potential is required for a physically stable nano-suspension solely stabilized by electrostatic repulsion (Müller et al.,
331 2001). The developed nano-emulsions in the present experimentation showed negative surface charge, which slightly
332 increased during storage. To best of our knowledge, EO-based nano-emulsions prepared with non-ionic surfactant
333 generally present negative surface charge (Acedo-Carrillo et al., 2006; Fernandes et al., 2014; Giunti et al., 2019;
334 Hashem et al., 2018; Salvia-Trujillo et al., 2015). It has been acknowledged that this kind of surfactant can slightly alter
335 the charge of the dispersed phase, depending on the covering surface created by the non-ionic surfactants (Li et al.,
336 2016; Martins et al., 2012; Zhao et al., 2010). Hsu and Nacu (2003) demonstrated that an increasing concentration of
337 non-ionic surfactant leads to ζ potential of the nano-emulsion nearest to zero, due to the highest coverage of the droplet
338 surface by the non-charged surfactant molecules. According to several studies, the negative surface charge of EO-based
339 nano-emulsions could be attributed to the dissociation of ionizable compound of the oils, which can be adsorbed on the
340 droplet surface by the surfactant (Bonilla et al., 2012; Ge and Ge, 2016; Stachurski and Michalek, 1996). Furthermore,
341 the absorption of negative ions (-OH) in the oil-water interface can change depending on the affinity between surfactant
342 and oil, generating different ζ -potentials for different surfactant (Salvia-Trujillo et al., 2015). Hence, it is not surprising
343 that, similar to size trends, ζ -potentials behaved differently on the basis of the plant species. Therefore, anise

344 formulation had quite stable surface charge (between -20.57 and -17.4 mV), while fennel and rosemary displayed
345 fluctuating ζ -potential (from about -26 to -12 mV).

346 The EO-based nano-formulations proposed in this paper showed good physical characteristics and excellent
347 stability over time, making them suitable for industrial applications. To the best of our knowledge, this is the first time
348 that EO-based nano-emulsion characteristics were reported during a medium-long storage period. The stability of
349 formulations over time is a key issue for consideration in these EO-based nano-emulsions as biopesticides. This
350 research highlighted the potential application of this methodology to pest control although further studies are required to
351 evaluate the use of EO-based nano-emulsions under field conditions.

352

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358 **CRedit authorship contribution statement**

359 **Orlando Campolo:** Conceptualization, validation, writing, data analysis. **Giulia Giunti:** Metodology, formal analysis,
360 validation, writing. **Vincenzo Palmeri:** Conceptualization, project administration, funding acquisition, writing.
361 **Thomas Michel:** Conceptualization, writing, project administration, funding acquisition. **Maryne Laigle:** Metodology,
362 formal analysis, validation, writing.

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552 **Figure Captions**

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554 **Figure 1:** Percentage (Mean \pm SE) of main classes of volatile compounds characterized in the essential oils of different
555 species (n = 3)

556 **Figure 2:** Essential oil-based nano-emulsions. A) Before sonication (24h from preparation); B) After sonication (24h
557 from sonication). Surfactant: T20= Tween 20; T80= Tween 80; S20=Span 20; S80=Span 80. Some not sonicated
558 formulations (Anise-S80 and Fennel-S80) were not suitable for the sonication process.

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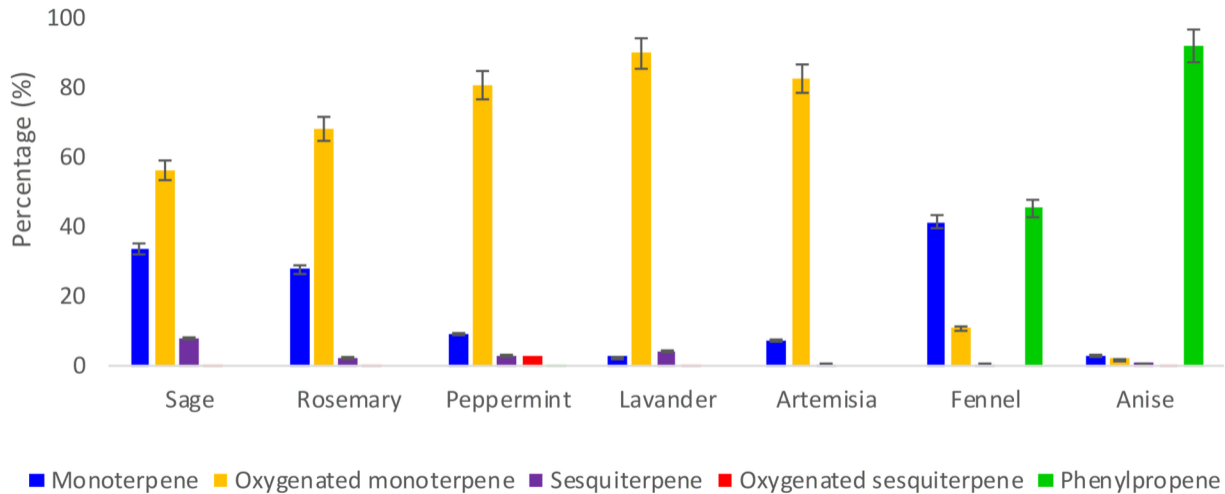
560 **Figure 3** Mean Z-average (\pm SE) of Tween 80 sonicated EO-based nano-emulsions over 28 weeks of storage (n=3).
561 Values are means of triplicates. Vertical bars indicate standard errors.

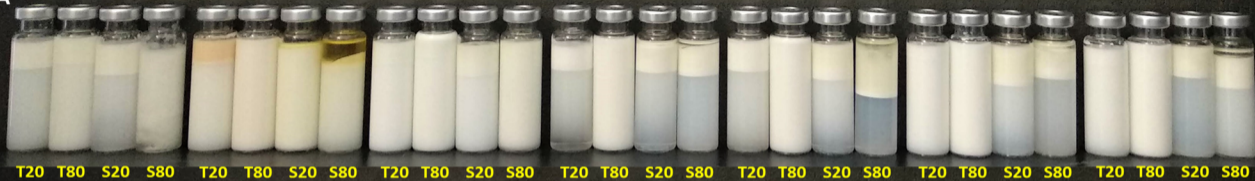
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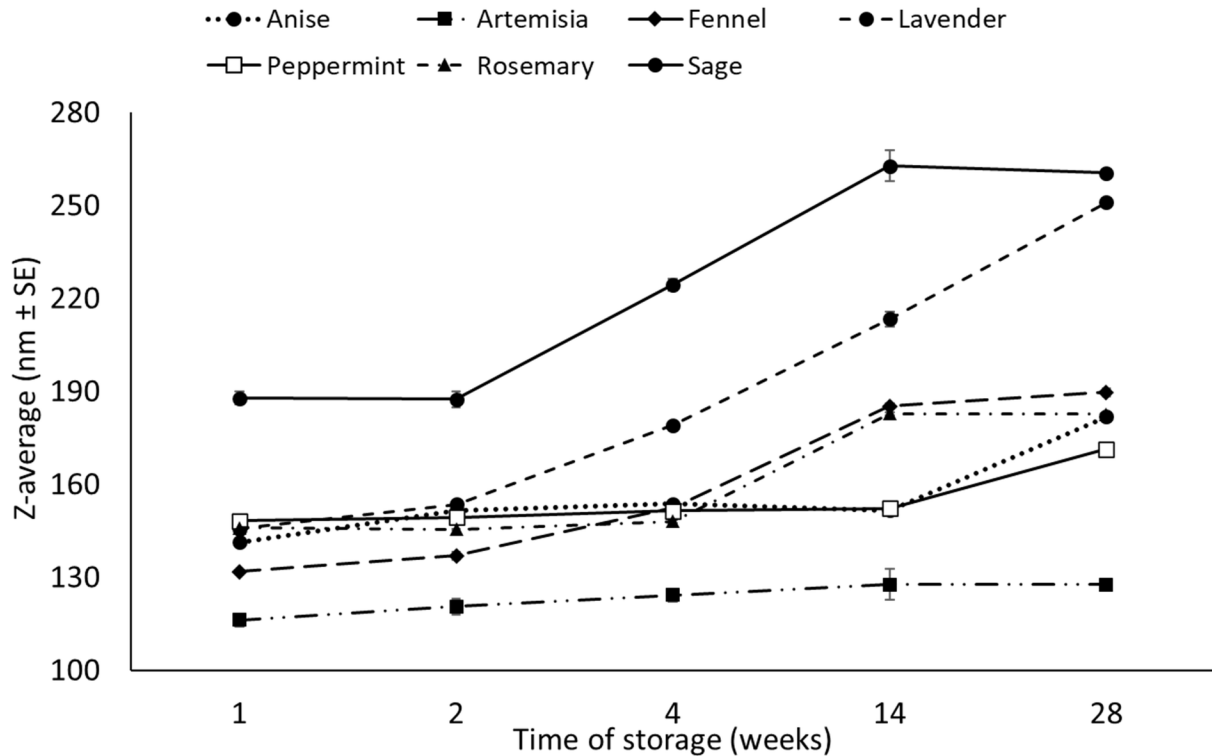
563 **Figure 4** Mean PolyDispersion Index (\pm SE) of Tween 80 sonicated EO-based nano-emulsions over 28 weeks of
564 storage (n=3). Values are means of triplicates. Vertical bars indicate standard errors.

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566 **Figure 5** Mean ζ -potential (\pm SE) of Tween 80 sonicated EO-based nano-emulsions over 28 weeks of storage (n=3).
567 Values are means of triplicates. Vertical bars indicate standard errors.



A**T20 T80 S20 S80****T20 T80 S20 S80****T20 T80 S20 S80****T20 T80 S20 S80****T20 T80 S20 S80****T20 T80 S20 S80****T20 T80 S20 S80****B****ANISE****ARTEMISIA****FENNEL****LAVANDER****PEPPERMINT****ROSEMARY****SAGE**



●●● Anise ■· Artemisia ◆ Fennel -●- Lavender
□ Peppermint ▲ Rosemary ● Sage

