

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

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4    Orlando Campolo<sup>1</sup>, Giulia Giunti<sup>1</sup>, Maryne Laigle<sup>2</sup>, Thomas Michel<sup>2\*</sup>, Vincenzo Palmeri<sup>1</sup>

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6    <sup>1</sup>Department of Agriculture, University Mediterranea of Reggio Calabria, Loc. Feo di Vito, 89122, Reggio Calabria,  
7    Italy

8    <sup>2</sup>Université Côte d'Azur, CNRS, Institut de Chimie de Nice UMR 7272, 06108 Nice, France

9    \*Corresponding Author: Thomas.MICHEL@univ-cotedazur.fr

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## 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.

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

## 1. Introduction

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

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

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

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

## **2. Materials and methods**

### **2.1 Essential oils and chemical material**

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

### **2.2 Essential oils chemical characterization**

#### **2.2.1 Gas Chromatography analyses**

##### GC/FID analysis.

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

##### GC-MS analysis.

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

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

### 2.2.2 Identification of volatile compounds

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

### 2.3 Nano-emulsion formulation and characterization

All the nano-emulsions were prepared following Giunti et al. (2019), with modifications. Specifically, the nano-emulsions were obtained using the spontaneous emulsification process, which can naturally occur when an organic phase and an aqueous phase are mixed, followed by sonication. To evaluate the effect of surfactants on the droplet size and stability of the EO-based emulsions Span 80, Span 20, Tween 80, and Tween 20, with an hydrophilic-lipophilic balance value (HLB) of 4.3, 6.7, 15.0 and 16.7 respectively, were used for the nano-emulsion formulation. The process consisted in mixing using a magnetic stirrer (30 min at 8000 RPM) the surfactant (5% w/w) with the essential oil (15% 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 to attain a homogeneous emulsified phase. Furthermore, double-distilled water solutions containing each surfactant alone (5% w/w) were prepared and used as control for the subsequent analyses.

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

The physical characteristics of the EO-based nano-emulsions were analysed by a Dynamic Light Scattering (DLS) instrument (Zetasizer Nano, Malvern). These qualitative analyses assessed the droplet surface charge, indicated by the zeta potential ( $\zeta$ ) values (electro-phoretic mobility of emulsions), and the droplets dimension, expressed in terms of Z-average size ( $D_z$ ) and polydispersity index (PDI), of the tested formulations at 25 °C. To avoid multiple scattering 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

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

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

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## 173 **2.4 Statistical analysis**

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

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## 180 **3. Results**

### 181 **3.1 Essential oil composition**

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

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

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

193 Droplet size, PDI and  $\zeta$ -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  $\zeta$ -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:  $H_{3,152}=25.64$ ;  $P<0.01$  –  $\zeta$ -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  $\zeta$ -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 ( $\zeta$ -potential); the nano-formulations containing Tween 80 and Tween 20  
213 obtained  $\zeta$ -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 ( $H_{6,71}=24.07$ ;  $P<0.01$ ),  $\zeta$ -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:  $8.9 \pm 0.3$  nm; Tween 80:  
217  $10.3 \pm 0.4$  nm; Span 20:  $207 \pm 3.8$  nm; Span 80:  $239.5 \pm 10.5$  nm). PDI values and  $\zeta$ -potentials were similar for all the  
218 control solutions (PDI: 0.09-0.19;  $\zeta$ -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  $116 \pm 2$  nm (Artemisia) to  $188 \pm 2$   
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 ( $0.14 \pm 0.01$ ), whereas the highest one was detected  
224 in the artemisia one ( $0.20 \pm 0.01$ ). 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:  $H_{6,14}=12.66$ ;  $P<0.05$  –  $\zeta$ -potential:  $H_{6,14}=18.92$ ;  
226  $P<0.01$ )

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### 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  $\zeta$ -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,  $\zeta$ -potential remained stable for at least 2 weeks, when it started to  
248 decline. However, the decline of  $\zeta$ -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.

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**4. Discussion**

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

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

In the current study, the different surfactants had an appreciable impact on both the droplet size and the PDI values. Among the tested surfactants, Tween 80 was able to produce the smallest droplets when compared with the other tested surfactants (Tween 20 Span 20 and Span 80). Similar results were reported by Chang et al. (2013), who obtained the smallest droplets in carvacrol-based nano-emulsions made with a mix of food-grade non-ionic surfactants (Tween 20, 40, 60, 80, and 85). Tweens, a class of non-ionic surfactants derived from sorbitan esters, are soluble or dispersible in water and are oil-in-water emulsifiers frequently used in pharmaceutical, cosmetic and cleaning industries. Among these surfactants, Tween 80 is one of the most commonly used to develop EO-based insecticides. The EO/surfactant combination is fundamental to attain valuable formulations. As example, two of the seven tested EOs (i.e. fennel and anise), when combined with Span 80, generated a dense foam impossible to sonicate. These two oils were characterized by the presence of (*E*)-anethole as their main constituent, whereas this molecule was absent or present only in small quantities in the other EOs; thus, (*E*)-anethole could be responsible for the formation of foam in presence of Span 80. In literature, (*E*)-anethole or anise-based nano-emulsions were usually produced using Tween 80 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:  $1420.5 \pm 111.0$  nm; sonicated:  $221.3 \pm 0.8$  nm) and increase  
285 homogeneity (coarse:  $0.709 \pm 0.04$  PDI; sonicated:  $0.251 \pm 0.00$  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  $116 \pm 2$  nm and it remained almost stable after 28 weeks of storage, with  
292 a droplet dimension of  $128 \pm 1$  nm. In contrast, results for sage EO-based nano-emulsion presented the highest droplet  
293 size both at 1 week ( $188 \pm 2$  nm) and 28 weeks ( $260 \pm 1$ ) 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  $\alpha$ -thujone (27.1% and 16.2%),  $\beta$ -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.  $\alpha$ -thujone,  $\beta$ -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

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

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

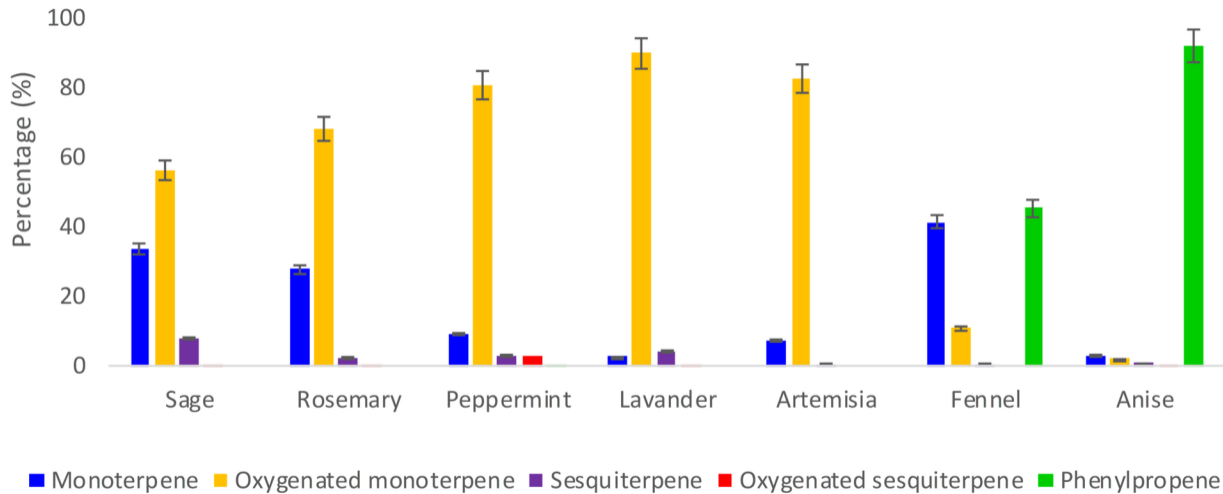
**Figure 1:** Percentage (Mean  $\pm$  SE) of main classes of volatile compounds characterized in the essential oils of different species (n = 3)

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

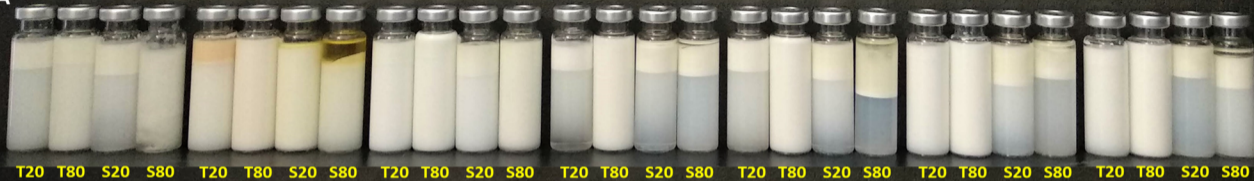
**Figure 3** Mean Z-average ( $\pm$  SE) of Tween 80 sonicated EO-based nano-emulsions over 28 weeks of storage (n=3). Values are means of triplicates. Vertical bars indicate standard errors.

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

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

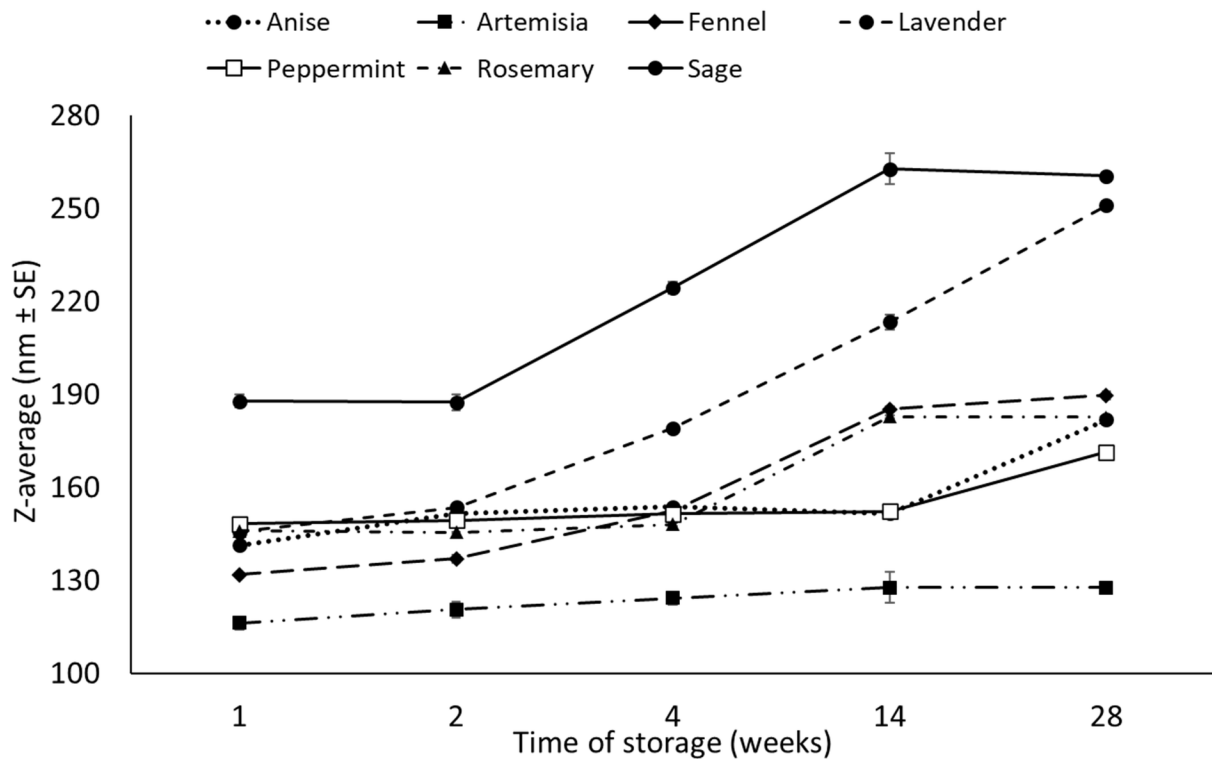


A



B





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

