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**Melaleuca alternifolia Essential Oil: Evaluation of Skin Permeation and Distribution from Topical Formulations with a Solvent-Free Analytical Method**

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1 **Tea tree (*Melaleuca alternifolia*) essential oil: evaluation of skin permeation and distribution**  
2 **from topical formulations with a solvent-free analytical method.**

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10

11 **Abstract**

12 *Melaleuca alternifolia* essential oil (Tea Tree Oil, TTO) is widely used as an ingredient in skin-care  
13 products because of its recognized biological activities. The European Scientific Committee on  
14 Consumer Products (SCCP) constantly promotes research and collection of data on both skin  
15 distribution and systemic exposure to TTO components after the application of topical formulations.  
16 This study quantitatively evaluates permeation, skin-layer distribution (*stratum corneum*, epidermis  
17 and dermis) and release into the surrounding environment of bioactive TTO markers (i.e.,  $\alpha$ -pinene,  
18  $\beta$ -pinene,  $\alpha$ -terpinene, 1,8-cineole,  $\gamma$ -terpinene, 4-terpineol,  $\alpha$ -terpineol) when a 5% TTO formulation  
19 is applied at a finite dosing regimen. Permeation kinetics were studied *in vitro* on pig-ear skin using  
20 conventional static glass Franz diffusion cells and cells *ad hoc* modified to monitor the release of  
21 markers into the atmosphere. Formulation, receiving phases and skin-layers were analyzed using a  
22 fully automatic and solvent-free method based on Headspace Solid Phase Microextraction/Gas  
23 Chromatography-Mass Spectrometry. This approach affords for the first time to quantify TTO  
24 markers in the different skin layers while avoiding using solvents and overcoming the existing  
25 methods based on solvent extraction.

26 The skin-layers contained less than 1% of each TTO marker in total. Only oxygenated terpenes  
27 significantly permeated across the skin, while hydrocarbons were only absorbed at trace level.  
28 Substantial amounts of markers were released into the atmosphere.

29

30 **KEYWORDS:** tea tree oil; headspace solid phase microextraction; GC-MS; *in vitro* permeation  
31 kinetics; skin-layer distribution

32

## 33 **1. Introduction**

34 Interest in the skin permeation of the volatile bioactive components in topical formulations is  
35 constantly increasing, not only from scientists, but also from regulatory authorities and, thereby,  
36 industries of the field. This interest has resulted in the 2003 EU directive that sets the no-declaration  
37 limits at 10 ppm for leave-on and at 100 ppm for rinse-off products for 24 volatile allergens in  
38 perfumes and cosmetics [1]. In 2011, the Scientific Committee on Consumer Safety (SCCS) proposed  
39 to extend the list of “established contact allergens in humans” to 54 chemicals and 28 natural extracts  
40 [2].

41 Tea tree essential oil (TTO) is widely used because of its recognized biological activities [3-5], in  
42 particular as ingredient in formulations to treat skin diseases, including acne, seborrheic dermatitis  
43 [6], scabies [7], and dandruff. It is obtained through steam distillation of the aerial parts of *Melaleuca*  
44 *alternifolia* (Maiden & Betche) Cheel, *Melaleuca dissitiflora* F.Muell. and *Melaleuca linariifolia* Sm.  
45 [8] (Myrtaceae family), TTO has been the object of interest from the European Scientific Committee  
46 on Consumer Products (SCCP), which remarked that the correct evaluation of both skin distribution  
47 and systemic exposure to its markers after treatment with topical formulations is not possible because  
48 of the limited number and inadequate nature of TTO dermal-penetration studies [9]. The SCCP  
49 thereby hoped that this lack of data on TTO would be quickly resolved, especially because some of  
50 its monoterpenoid markers are on the list of allergens updated in 2011 [2].

51 To the best of the authors’ knowledge, only a few studies are available on TTO permeation [10-12],  
52 all of them investigating markers accumulated in the skin by solvent extraction. In 2006, Reichling  
53 *et al.* [5], compared several formulations, and studied the permeation of the major TTO component,  
54 i.e., 4-terpineol, without measuring its distribution in the skin layers. In 2008, Cross *et al.* [10] studied  
55 the epidermal retention of neat TTO and of a 20% TTO solution in ethanol by submitting skin layers  
56 to extraction with acetonitrile. In 2016, Sgorbini *et al.* studied the influence of both the formulation  
57 and partition coefficient of TTO bioactive components on their permeation at an “infinite dosing

58 regimen” on pig-ear skin slices in an occluded system to evaluate both their systemic and overall skin  
59 bioavailability [13].

60 The present study reports the results of a project carried out on pig-ear skin slices and aimed to  
61 exhaustively investigate the distribution over time of seven bioactive TTO components ( $\alpha$ -pinene,  $\beta$ -  
62 pinene, 1,8-cineole,  $\alpha$ -terpinene,  $\gamma$ -terpinene, 4-terpineol,  $\alpha$ -terpineol) in all “compartments” of the  
63 *in vitro* system (i.e., receiving phase, skin layers, residual amount in the formulation, amount released  
64 into the environment). The study focused on three main steps: 1) the application of a fully automated  
65 and solvent-free HS-SPME-GC-MS method to analyze and quantify TTO components in both the  
66 receiving phase and skin layers; 2) the evaluation of the distribution of bioactive components in the  
67 *stratum corneum*, epidermis, dermis and receiving phase when a 5% TTO model formulation is  
68 applied in a “finite dosing regimen” and in a non-occluded system mimicking the normal use of a  
69 topical formulation; and 3) the determination of the loss of TTO volatile bioactive components via  
70 spontaneous evaporation using an *ad hoc* modified static Franz cell to evaluate the “indicative”  
71 amount effectively undergoing the permeation process. The method was validated by measuring  
72 precision (repeatability and intermediate precision), linearity, LOD and LOQ values.

73

## 74 **2. Material and Methods**

### 75 **2.1. Chemicals and samples**

76 Tea tree essential oil (TTO) was supplied by Witt (Poirino, Turin, Italy). Its composition, reported in  
77 **Table 1**, complies with the ISO norms [14] and with European Pharmacopoeia [8].

78 The formulation was a 5% TTO oil/water emulsion, whose components were obtained from Merck-  
79 Sigma Aldrich (Milan, Italy) and are reported in **Table 2**.

80 Pure  $\alpha$ -pinene,  $\beta$ -pinene, 1,8-cineole,  $\alpha$ -terpinene,  $\gamma$ -terpinene, 4-terpineol,  $\alpha$ -terpineol, solvents  
81 (acetone, cyclohexane), phosphate saline buffer, sodium dodecyl sulfate and ammonium chloride  
82 were obtained from Merck-Sigma Aldrich.

83

## 84 **2.2. *In vitro* permeation tests**

85 TTO permeation tests were carried out in agreement with the SCCP guidelines [15] using: 1)  
86 conventional static glass Franz diffusion cells to monitor the permeation kinetics and 2) an *ad hoc*  
87 modified static glass Franz diffusion cell to monitor the loss of volatiles into the headspace during  
88 the permeation tests (Figure 1). Both cells were magnetically stirred. The study was carried out on  
89 pig-ear skin slices, belonging to different individuals, purchased from a local slaughterhouse and  
90 isolated with a dermatome (thickness 1 mm). The permeation tests were performed on 12 mg of the  
91 formulation (“finite dosing regimen”), at 32°C under constant stirring (1000 rpm). The donor  
92 compartment was kept open during the permeation test to mimic everyday use. A phosphate saline  
93 buffer 0.05M (pH 5.5) containing sodium dodecyl sulfate 0.1% was selected as the receiving phase,  
94 in accordance with previous studies [13, 16]. Generally, the receiving phase is used at physiological  
95 pH (7.4) to study transdermal formulations. In this case, a 5.5 pH coherent with the skin value was  
96 adopted.

97 Sampling was carried out at seven different times after the beginning of the experiment; 1, 2, 4, 8,  
98 10, 24, 27 hours. At each time point, the receiving phase (6 mL) was withdrawn and immediately  
99 replaced with the same volume of fresh buffer. The stability of the formulation was verified by  
100 analysis before and after the *in vitro* test along the whole working period. Each experiment was  
101 repeated three times.

102

## 103 **2.3. Skin layer separation**

104 The *stratum corneum* was completely removed using the tape stripping procedure (25 times) with an  
105 adhesive film (Scotch Booktape, 3M, USA).

106 Skin layers were separated using three different methods: 1) by immersing the skin in 1.8 mL of a  
107 0.22 M ammonium chloride solution (pH = 9.5) for 15 min at room temperature [modified from 17];  
108 2) via thermal-shock (i.e., immersion of the skin in 1.8 mL of water at 60°C for 30s, immediately

109 followed by cooling in ice at 0°C for 5 min) [17]; and 3) via mechanical separation with a cryostat  
110 (Cryostat Leica CM 1900, Nussloch GmbH) [17].

111

## 112 **2.4. SPME fibers**

113 Carboxen/divinylbenzene/PDMS (CAR/DVB/PDMS) SPME fibers were obtained from Supelco Co.  
114 (Bellafonte, PA, USA) and conditioned before use as recommended by the manufacturer. Three fibers  
115 were tested by analyzing a set of standard solutions, at different concentrations of the target TTO  
116 components, in the receiving phase. ANOVA was carried out to confirm the homogeneity of fiber  
117 performance and to discard those with different sampling behavior. The consistency of fiber  
118 performance was checked every 50 analyses using an in-fiber external standardization approach and  
119 a standard mixture of hydrocarbons (C<sub>9</sub>-C<sub>25</sub>) in cyclohexane (1 µL of a 0.1 mg/mL solution) [18,19].

120

## 121 **2.5. Sampling conditions**

122 *Receiving phase:* 1.8 mL of the receiving phase withdrawn at each time point and spiked with 3 µL  
123 of a 1.0 mg/mL tridecane (C<sub>13</sub>) solution in acetone, used as an internal standard, was sampled using  
124 HS-SPME with a CAR/DVB/PDMS fiber in a 20 mL headspace vial for 30 minutes at 35°C. The  
125 components below LOQ/LOD (i.e., α-pinene, β-pinene, α-terpinene and γ-terpinene) were quantified  
126 as the total amount involved in the permeation experiment by combining the total receiving phase  
127 withdrawn over 27 hours, and sampling 5.6 mL in a 20 mL sealed vial under the above conditions.

128 *Formulation and skin:* 5 mg of diluted formulation (1:50 with the same formulation without TTO)  
129 and suitable amounts of skin were sampled separately before and after permeation tests by HS-SPME  
130 in a 20 mL headspace vial under the above conditions.

131 *Headspace release:* seven *in vitro* permeation experiments were carried out separately in the *ad hoc*  
132 modified Franz cell. Each experiment was stopped at a different time point (i.e., 1, 2, 4, 8, 10, 24, 27  
133 hours). The TTO markers accumulated in the HS vial at the different times were sampled by HS-  
134 SPME and analyzed by GC-MS.

135 Each analysis was repeated three times. Blank runs did not show any carry-over effects.

136

## 137 **2.6. Analysis conditions**

138 Analyses were carried out using a MPS-2 multipurpose sampler (Gerstel, Mülheim a/d Ruhr,  
139 Germany) installed on a Shimadzu 2010 GC unit coupled to a Shimadzu QP2010 Mass spectrometer.

140 GC conditions: injector temperature: 280°C, injection mode: split; ratio: 1/20; carrier gas: helium;  
141 flow rate: 1 mL/min; fiber desorption time: 5 min; column: Mega SE52 (95% polydimethylsiloxane,  
142 5% phenyl) 25 m×0.25 mm dc×0.25 µm df, from MEGA (Milan, Italy). Temperature program: from  
143 50°C (1 min) to 125°C (0 min) at 3°C/min, then to 250°C (5 min) at 20°C/min.

144 MSD conditions: MS operated in EI mode (70 eV), scan range: 35-350 amu; dwell time: 40 ms, ion-  
145 source temperature: 230°C; quadrupole temperature: 150°C; transfer line temperature: 280°C. Marker  
146 compounds were identified by comparing their mass spectra and linear retention indices to those of  
147 authentic standards.

148

## 149 **2.7. Quantitation**

150 *Receiving phase quantitation:* a stock standard mixture of the markers to be quantified was prepared  
151 at 0.2 mg/mL of each pure standard in receiving phase. Suitably diluted solutions of the stock standard  
152 mixture were then prepared and renewed weekly. The resulting solutions (both stock and diluted)  
153 were stored at 4°C. Calibration curves were built up by analyzing, in triplicate, 1.8 mL of nine diluted  
154 mixtures in the 0.5-100 µg/mL concentration ranges using HS-SPME-GC-MS under the conditions  
155 reported above.

156 The calibration curves of  $\alpha$ -pinene,  $\beta$ -pinene,  $\alpha$ -terpinene and  $\gamma$ -terpinene were built up by analyzing,  
157 in triplicate, 5.6 mL of nine diluted mixtures in the 0.9-9 µg/mL concentration range using HS-SPME-  
158 GC-MS under the above conditions.

159 *Formulation and skin quantitation:* a stock standard mixture of each TTO marker was prepared in  
160 cyclohexane at a concentration of 10 mg/mL. Suitably diluted solutions of the stock standard mixture



161 were then prepared and renewed weekly and stored at 4°C. Calibration curves were built by analyzing,  
162 in triplicate, nine diluted mixtures in the 5 µg/mL-10 mg/mL concentration range using HS-SPME-  
163 GC-MS in multiple headspace extraction (MHE) mode under the above conditions.

164

## 165 **2.8. Method validation**

166 Method validation was run on a six-week protocol, over six-months; the following parameters were  
167 characterized: precision, linearity, Limit of Detection (LOD) and Limit of Quantitation (LOQ).  
168 Repeatability was evaluated via the HS-SPME-GC-MS analyses of one of the calibration levels in  
169 the receiving phase five times, over five consecutive days. Intermediate precision (inter-week  
170 precision) was measured on the internal standard contained in 1.8 mL of receiving phase at a  
171 concentration of 1.7 µg/mL once a week, over six months. Linearity was assessed using linear-  
172 regression analyses within the working range, over at least nine different concentration levels.

173 The Limit of Quantification (LOQ) was experimentally determined by analyzing decreasing  
174 concentrations of TTO in the receiving phase, and in the formulation, using HS-SPME-GC-MS. LOQ  
175 was the lowest concentration for which the instrumental response integration reported an RSD%  
176 below 20% across replicate analyses. The LOD of each analyte was calculated from the average “peak  
177 to peak” noise values sampled in its region of elution in the chromatogram, with a coverage factor of  
178 3. Each analysis was repeated three times.

179

## 180 **3. Results and discussion**

181 This study showed that in quantitative terms, TTO markers permeated in the receiving phase can be  
182 divided into two groups: i) those present above their LOD/LOQ (4-terpineol,  $\alpha$ -terpineol, 1,8-cineole)  
183 that were therefore quantified in each aliquot at each time, and ii) those present below their LOD/LOQ  
184 ( $\alpha$ -pinene,  $\beta$ -pinene,  $\alpha$ -terpinene,  $\gamma$ -terpinene) that were quantified as total amounts over the 27 hours  
185 as combined aliquots.

186 **Table 1** lists the TTO markers together with their partition coefficients (Log P), vapor pressures, the  
187 selected diagnostic ions used for their quantitation and their abundance (normalized relative % area)  
188 in the investigated essential oil.

189

### 190 **3.1. Set up of *in vitro* permeation tests**

191 Two different types of static Franz diffusion cells were used for the *in vitro* permeation tests (Figure  
192 1). Conventional static Franz cells (Figure 1A) were adopted to evaluate both the TTO components  
193 that permeated and distributed in ear pig skin layers (area surface 2.54 cm<sup>2</sup>); the donor compartment  
194 was kept open to mimic everyday use. Moreover, the static Franz cell was modified (Figure 1B) to  
195 measure the amounts of components vaporized during the permeation tests. The modified static Franz  
196 cell included a hermetically sealed glass vessel (75 mL) connected on-line to a donor compartment  
197 to collect the TTO components released by the formulation (i.e., the component(s) released into the  
198 environment during everyday use). The size of the glass vessel (75 mL) was selected to achieve the  
199 best compromise between a correct headspace/donor compartment ratio (data not shown) and a  
200 satisfactory analytical sensitivity.

201

### 202 **3.2. Optimization of skin-layer separation**

203 *Stratum corneum* was removed from the skin after each *in vitro* permeation study using the tape  
204 stripping approach. An average of 25 adhesive strips were used to ensure its complete elimination.  
205 The removed *stratum corneum* was then submitted to HS-SPME-GC-MS analysis to evaluate the  
206 presence of TTO markers. The *stratum corneum* headspace only contained  $\alpha$ -pinene at trace levels,  
207 although, in any case, below its LOQ.

208 Three approaches were investigated to split dermis from epidermis: the cryostat method was preferred  
209 as reference method because it provided well-separated skin layers ready for a direct HS sampling.

210 The resulting epidermis/dermis ratio was around 1:30, deriving from the average weight of epidermis  
211 (6.7±1.5 mg) and dermis (189±45.5 mg).

212 The thermal shock method provided quite satisfactory results with relatively low TTO-marker losses  
213 (i.e., below 15%) while the ammonium chloride method was abandoned because of the high solubility  
214 of 4-terpineol and  $\alpha$ -terpineol resulting in losses above 70 and 50 % respectively.

215

### 216 **3.3. *In vitro* TTO percutaneous permeation**

217 The amount of each TTO marker permeating into the receiving phase (expressed as  $\mu\text{g}/\text{cm}^2$ ) was  
218 quantitatively determined by HS-SPME-GC-MS with external standard calibration. The different  
219 calibration levels were directly prepared in an appropriate volume of receiving phase to overcome the  
220 matrix effect. **Table 3** reports the linearity range, the equations of the calibration curves and the  
221 correlation coefficients ( $r$ ) of each quantifiable marker.

222 The most permeated compounds were oxygenated monoterpenes: 4-terpineol,  $\alpha$ -terpineol and 1,8-  
223 cineole. **Figure 2** reports the *in vitro* kinetic permeation profiles obtained for 4-terpineol and  $\alpha$ -  
224 terpineol (Figure 2A), and 1,8-cineole (Figure 2B), by applying zero-order kinetics (i.e., cumulative  
225 amount per unit surface area ( $\mu\text{g}/\text{cm}^2$ ) plotted *versus* time). The reported data derive from twelve *in*  
226 *vitro* permeation tests carried out using skin samples belonging to different pig individuals. Kinetics  
227 are similar for all the oxygenated markers, although permeation rates differed quantitatively. The  
228 amounts over 27 hours were  $49.1 \mu\text{g}/\text{cm}^2$  for 4-terpineol,  $8.90 \mu\text{g}/\text{cm}^2$  for  $\alpha$ -terpineol and  $3.85 \mu\text{g}/\text{cm}^2$   
229 for 1,8-cineole. The kinetic permeation data of the other TTO markers (i.e.,  $\alpha$ -pinene,  $\beta$ -pinene,  $\alpha$ -  
230 terpinene,  $\gamma$ -terpinene) could not be measured because of the very low amounts permeated at each  
231 time. These amounts were below their LOD and LOQs, probably because of their high  
232 hydrophobicity. The total permeated amount of these components was measured over the entire *in*  
233 *vitro* permeation test, by quantifying them in an appropriate volume of the receiving phases collected  
234 over 27 hours combined (i.e., 5.6 mL). **Table 3** reports the calibration-curve equations and correlation  
235 coefficients ( $r$ ) for  $\alpha$ -pinene,  $\beta$ -pinene,  $\alpha$ -terpinene and  $\gamma$ -terpinene. The total amount of permeated

236 hydrocarbons was far lower than that of oxygenated monoterpenes, ranging from 0.0063  $\mu\text{g}/\text{cm}^2$  for  
237  $\beta$ -pinene to 0.017 for  $\alpha$ -pinene.

238 The higher permeation rate of the oxygenated compounds compared to that of the hydrocarbons is  
239 probably due to their higher relative abundance in the formulation (and in the TTO), and their better  
240 compatibility with the receiving phase (lower logP values). Conversely, the lower hydrocarbon  
241 permeation may also be due to their relatively high volatility responsible for a significant release from  
242 the formulation to the surrounding environment.

243

#### 244 **3.4. Skin retention of TTO marker compounds**

245 The amounts of TTO markers retained by the total skin, and by epidermis and dermis, were quantified  
246 by HS-SPME-GC-MS using the Multiple Headspace Extraction approach [20 and references cited  
247 therein], which affords to bypass the strong skin matrix effect. **Table 4** reports the regression  
248 equations of the calibration curves, the linearity range and the correlation coefficient for each  
249 quantified component.

250 **Table 5** reports the average total amount (expressed as  $\mu\text{g}$ ) of each TTO marker in whole skin and in  
251 the epidermis and dermis, separated using the cryostat method. In general, all TTO compounds were  
252 retained by the (whole) skin, although each one to a different extent; ranging from 0.031  $\mu\text{g}$  for  $\beta$ -  
253 pinene and 1.3  $\mu\text{g}$  for 4-terpineol. The two separated layers (epidermis and dermis) retained all TTO  
254 markers with the exception of  $\beta$ -pinene, which was exclusively found in the dermis (0.043  $\mu\text{g}$ ).  
255 Moreover, the absolute amounts of TTO components were found to be much higher in the dermis  
256 than in the epidermis, with a dermis/epidermis ratio corresponding to 7 for  $\alpha$ -pinene and 1,8-cineole,  
257 about 30 for  $\gamma$ -terpinene, 4-terpineol and  $\alpha$ -terpineol, and 170 for  $\alpha$ -terpinene. The greater  
258 accumulation in the dermis is probably due to its higher abundance compared to epidermis (about  
259 1:30 w/w). When the content of TTO markers is normalized to the weight of the skin-layer, the  
260 distribution between epidermis and dermis was comparable, in particular for  $\gamma$ -terpinene, 4-terpineol

261 and  $\alpha$ -terpineol dermis/epidermis ratio ranging from 0.8 to 1.3. Epidermis contained four-times more  
262  $\alpha$ -pinene and 1,8-cineole than dermis, while dermis accumulated more  $\alpha$ -terpinene than epidermis  
263 (dermis/epidermis ratio of about 5).

264

### 265 **3.5. Distribution of permeated TTO components**

266 **Figure 3** shows the marker % distribution in each compartment (i.e., receiving phase, epidermis,  
267 dermis and headspace/ambient, residual formulation after the permeation test) in relation to their  
268 lipophilicity and volatility.

269 TTO components were almost absent in the residual formulation after the permeation experiment,  
270 from, as expected, since it was applied at a finite dosing regimen in a non-occluded system.

271 TTO markers were retained in the skin layers in very low percentages, in general below 1%.

272 Hydrocarbons were poorly retained (always around 1%) and transferred to the receiving phases  
273 (always below 0.2% with the exception of  $\beta$ -pinene, 0.8%). Conversely, oxygenated compounds were  
274 transferred into the receiving phase at a high rate, ranging from about 12% for 1,8 cineole to about  
275 50% of the total content for 4-terpineol and  $\alpha$ -terpineol. The distribution of TTO markers was in good  
276 agreement with their physicochemical characteristics.

277

### 278 **3.6. TTO-component release into the headspace**

279 When working with volatiles, the evaluation of their release/loss into the surrounding environment  
280 during an *in vitro* test performed in a non-occluded system is a useful way to define the effective  
281 quantities involved in the permeation process. The headspace amount is conditioned by two  
282 simultaneous equilibria: i) the release of formulation components into the headspace; and ii) their  
283 permeation through the skin into the receiving phase.

284 TTO markers behaved differently depending on their polarity and volatility (vapor pressure).

285 The highest percentage of oxygenated compounds (i.e., 1,8-cineole, 4-terpineol,  $\alpha$ -terpineol) was  
286 released into the headspace within the first hour. These results confirmed the data observed in the *in*

287 *in vitro* permeation kinetic studies (**Table 5**). They are in agreement with those obtained with the mass  
288 balance, i.e., the summed amount of each analyte present in the skin layers, in the receiving phase  
289 and the residue in the formulation. **Figure 4** shows the percentage distribution of the oxygenated  
290 components in the three systems involved in the *in vitro* study: headspace, formulation and receiving  
291 phase. 4-Terpineol and  $\alpha$ -terpineol behave similarly as about 40-45% was released into the headspace.  
292 The value for 1,8-cineole was about 90% likely because of its relatively high volatility (1,8-cineole  
293 vapor pressure is about 30 times higher than that of 4-terpineol). After 2 hours, the release decreased,  
294 most probably because the equilibrium between formulation/ receiving phase became predominant.  
295 This equilibrium becomes prevalent in this closed system, possibly due to the relatively high water  
296 solubility of TTO oxygenated compounds inducing their redistribution accordingly.  
297 The opposite is true for hydrocarbons (i.e.,  $\alpha$ - and  $\beta$ -pinene,  $\alpha$ - and  $\gamma$ -terpinene), which are highly  
298 hydrophobic and whose percentages of release into the headspace were constant over the entire  
299 experiment (i.e. 27 hours), indicating a prevalent formulation/headspace equilibrium. The results  
300 show that about 80% of the hydrocarbons was already released in the headspace after the first hour,  
301 and the remaining 20% was in the formulation. The hydrocarbons were not quantifiable in the  
302 receiving phases at this time because below the LOD/LOQ of the method.

303

### 304 **3.7. Method validation**

305 The optimized HS-SPME-GC-MS method was validated by its precision (repeatability and  
306 intermediate precision), linearity, regression equation error, LOD and LOQ.

307 The method showed very good precision (repeatability), as the average RSD% for each TTO marker  
308 was always below 5.8%. The intermediate precision was equally satisfactory with the RSD%  
309 calculated on the internal standard (in the receiving phase for 143 analyses within a timeframe of six  
310 months) never exceeding 13.6%.

311 **Tables 3** and **4** report the LOD and LOQ values for all TTO markers, determined in HS-SPME-GC-  
312 MS and MHE-SPME-GC-MS. In the former case, the LOD values ranged from 0.1 ng/mL for  $\gamma$ -

313 terpinene to 16.4 ng/mL for  $\alpha$ -terpineol, and the LOQs varied from 0.33 ng/mL to 54.0 ng/mL  
314 respectively. In the latter case (MHE mode), the LOD values ranged from 0.2 ng for  $\gamma$ -terpinene to  
315 1.5 ng for  $\alpha$ -terpineol, while the LOQs ranged from 0.8 ng to 5.0 ng, respectively

316

#### 317 **4. Conclusion**

318 This results show that the *in vitro* permeation kinetics and distribution of TTO bioactive markers in  
319 a model formulation and at a finite dosing regimen can be monitored with a fully automated solvent-  
320 free method.

321 To the best of the authors' knowledge, this is the first time that: 1) a solvent-free method has been  
322 applied to monitor quantitatively the distribution of TTO components in skin layers, and their residual  
323 amounts in the formulation in an *in vitro* permeation test; 2) a modified static Franz cell has been  
324 used to evaluate the evaporation of TTO components from the formulation during the permeation  
325 process. The method has shown itself to be highly reliable and sensitive for permeation experiments,  
326 demonstrating high repeatability and intermediate precision.

327 The permeation results indicated that TTO component behavior changed as a function of chemical  
328 structure; hydrocarbons did not pass the skin barrier (or only as traces), while oxygenated compounds  
329 permeated at percentages ranging from 12% (1,8-cineole) to 53.3% ( $\alpha$ -terpineol).

330 This model study allows to evaluate for the first time the effect of the skin barrier on the permeation  
331 of volatile bioactive compounds of differing nature in a topical formulation, while also determining  
332 the true amount(s) of each that: 1) overcome the natural protective barrier (whole skin, its separated  
333 layers and pH) and; 2) can potentially be absorbed, and/or lost because of evaporation.

334 More generally, these model studies provide data useful to design topical formulations containing  
335 volatile bioactive compounds in amounts not only sufficient for their biological activity, but also  
336 within the limits fixed by regulatory authorities.

337

#### 338 **Acknowledgment**

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342

#### 343 **Declaration of interest**

344 None

345

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### Caption to Figures

403 **Figure 1.** Scheme of the two different types of static Franz diffusion cells used for the *in vitro*  
404 permeation tests: conventional static Franz cells (Figure 1A) and; modified static Franz cell (Figure  
405 1B)

406

407 **Figure 2.** *In vitro* kinetic permeation profiles for 4-terpineol and  $\alpha$ -terpineol (Figure 2A) and 1,8-  
408 cineole (Figure 2B), obtained by applying a zero-order kinetics (i.e., cumulative amount per unit  
409 surface area ( $\mu\text{g}/\text{cm}^2$ ) plotted *versus* time).

410

411 **Figure 3.** Marker % distribution in each compartment (i.e., receiving phase, epidermis, dermis and  
412 headspace/ambient, residual formulation after permeation test) related to lipophilicity and volatility.

413

414 **Figure 4.** Percentage distribution of oxygenated components in the three systems in the *in vitro* study  
415 (i.e., headspace, formulation, receiving phase).

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418 Figure 1

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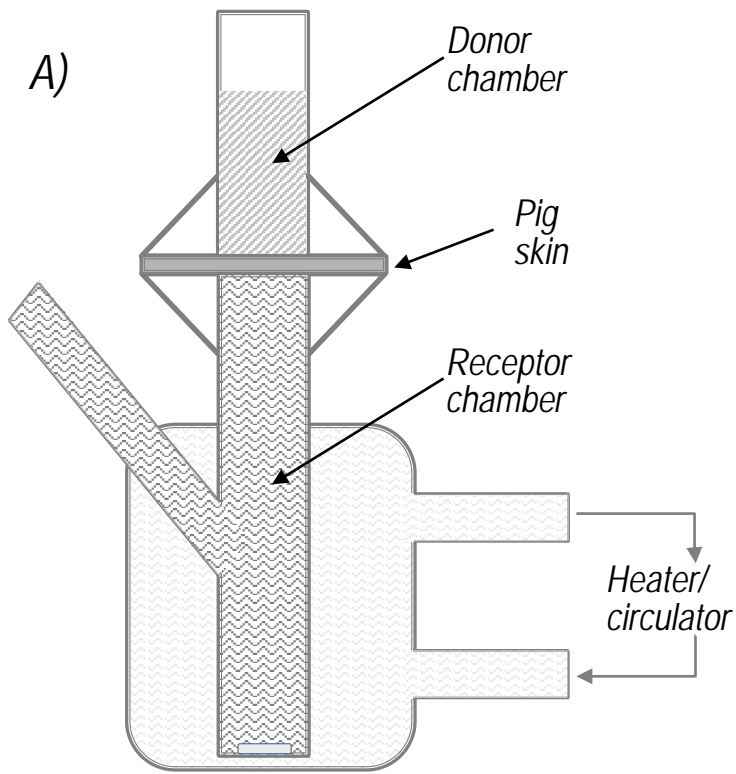
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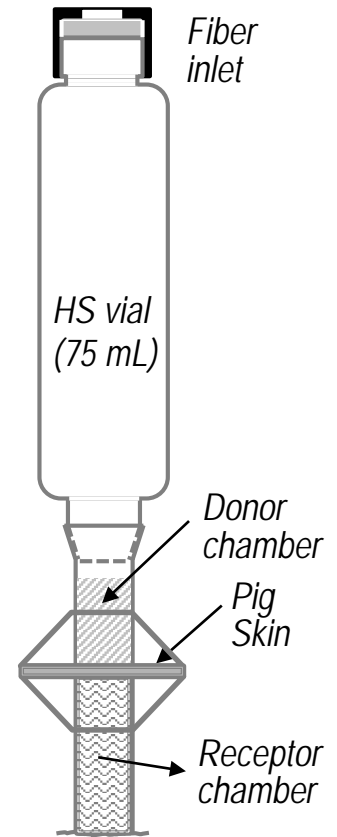
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432 Figure 2

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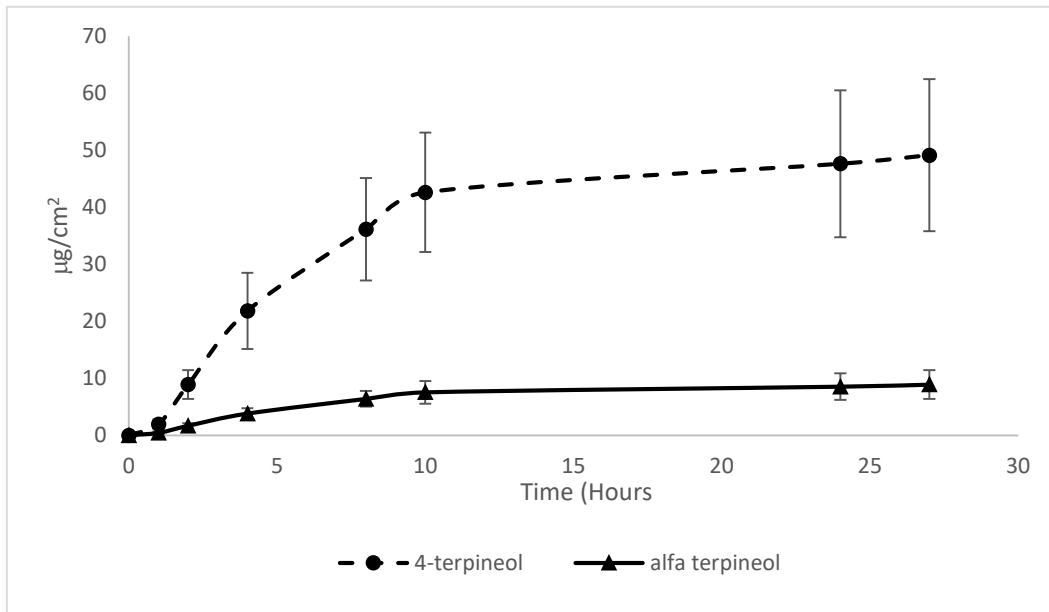
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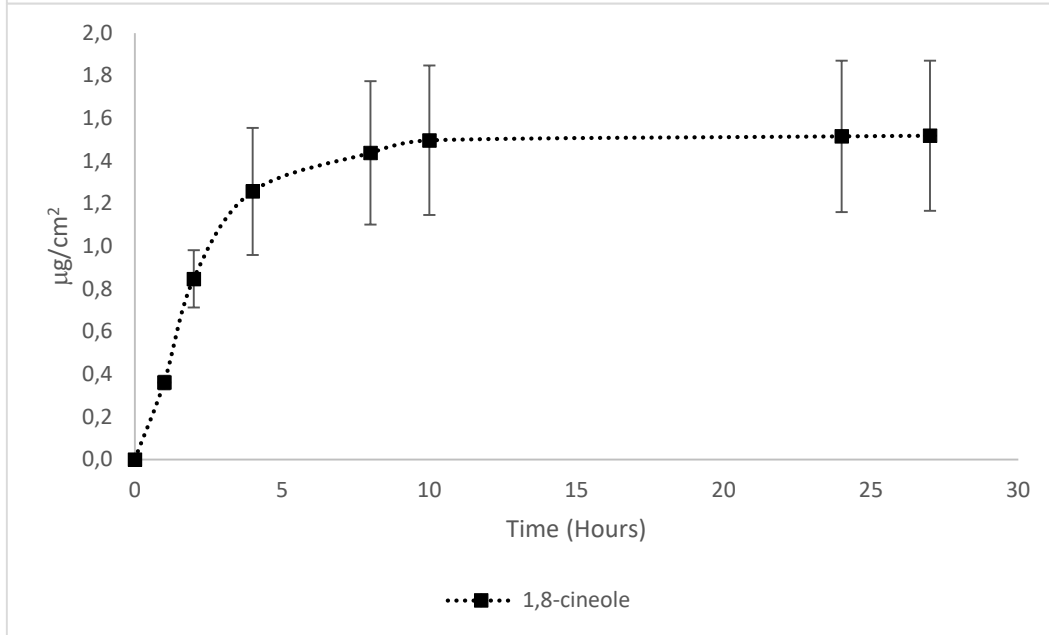
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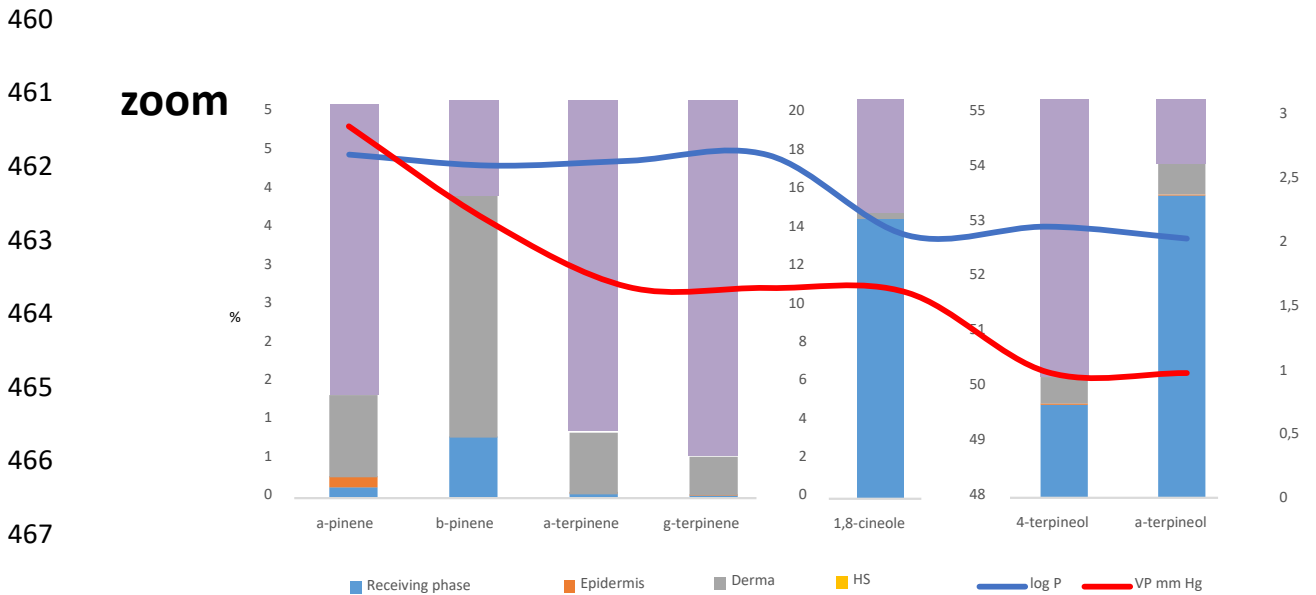
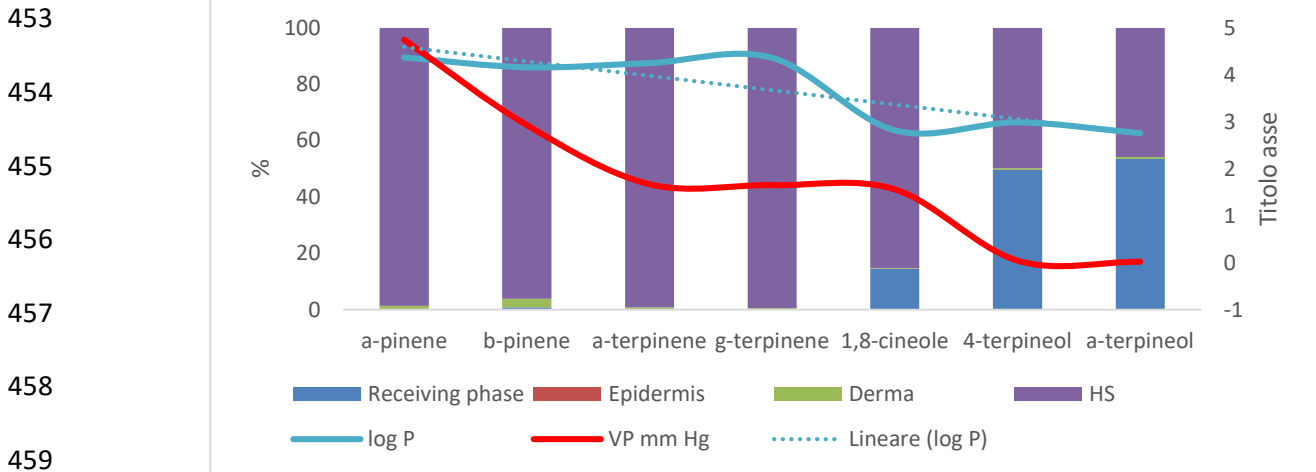
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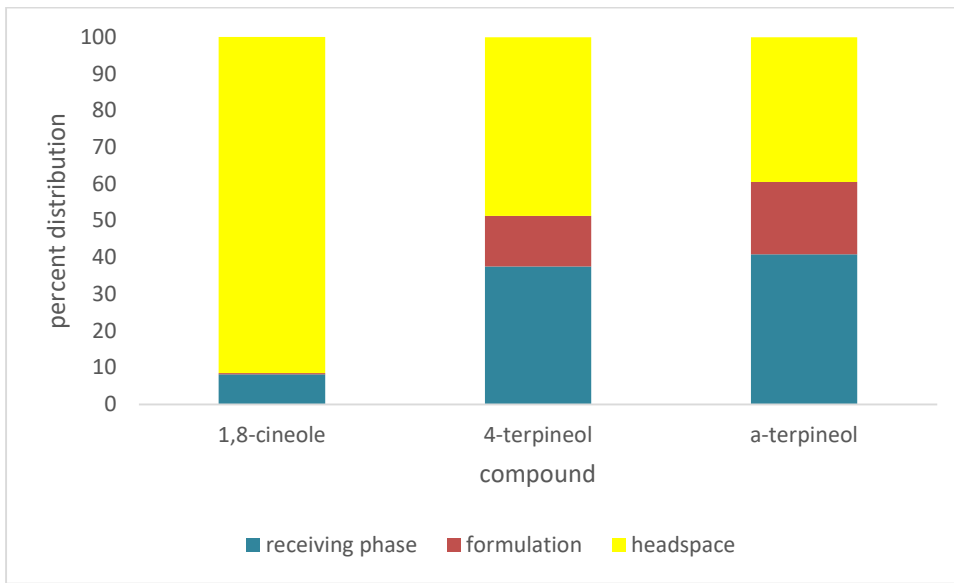
452 Figure 3



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470 Figure 4



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474 **Table 1.** List of the TTO markers together with their partition coefficients (Log P) and vapour pressure  
 475 values, the selected diagnostic ions used for their quantitation and their abundance in the investigated  
 476 essential oil (expressed as relative % area).

Compounds	m/z	Log P*	Vapour pressure (mm Hg)*	Area%
$\alpha$ -pinene	93	4.37	4.75	49.9
$\beta$ -pinene	93	4.16	2.93	0.3
1,8-cineole	43	2.82	1.56	5.2
$\alpha$ -terpinene	93	4.25	1.67	7.1
$\gamma$ -terpinene	93	4.36	1.65	16.4
4-terpineol	71	2.99	0.0427	41.9
$\alpha$ -terpineol	59	2.79	0.0196	7.0

483 \* Episuite database

484



485 **Table 2.** Linearity range, equations of the calibration curves and correlation coefficients (r) for 1,8-cineole,  
 486 4-terpineol and  $\alpha$ -terpineol.

487

Compound	linearity range ( $\mu\text{g/mL}$ )	slope	intercept	r	LOQ ( $\mu\text{g/mL}$ )	LOD ( $\mu\text{g/mL}$ )
1,8 cineole	0.1-1.0	1.28E-01	7.10E-03	0.9918	0.0034	0.0010
	2.5-75.0	4.19E-02	1.05E-01	0.9993		
4-terpineol	0.1-1.0	5.39E-02	-8.00E-04	0.9996	0.0174	0.0053
	2.5-75.0	3.58E-02	4.35E-02	0.9996		
$\alpha$ -terpineol	0.3-1.5	1.45E-02	9.00E-04	0.9990	0.0540	0.0164
	3.1-46.3	1.75E-02	-1.59E-02	0.9988		
Compound	linearity range ( $\text{ng/mL}$ )	slope	intercept	r	LOQ ( $\text{ng/mL}$ )	LOD ( $\text{ng/mL}$ )
$\alpha$ -pinene	0.9-9	8.98E+04	-4.39E+03	0.9932	0.45	0.14
$\beta$ -pinene	0.9-9	8.19E+04	-1.11E+03	0.9952	0.48	0.15
$\alpha$ -terpinene	0.9-9	3.86E+04	-9.51E+03	0.9948	0.65	0.20
$\gamma$ -terpinene	0.9-9	8.49E+04	-2.02E+03	0.9969	0.33	0.10

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489

490 **Table 3.** Regression equations of the calibration curves, linearity range, correlation coefficient for each  
 491 quantified component obtained by HS-SPME-GC-MS with the Multiple Headspace Extraction approach.

492

Compound	linearity range (µg)	slope	intercept	r	LOQ (µg)	LOD (µg)
α-pinene	10 - 1.0	1.00E+08	-8.00E+07	0.993	0.0025	0.0008
	0.50 - 0.05	3.00E+07	-7.99E+05	0.999		
	0.05 - 0.005	8.00E+06	7.18E+04	0.997		
β-pinene	5.0 - 0.5	3.00E+07	-5.00E+06	1.000	0.0020	0.0006
	0.50 - 0.05	2.00E+07	-4.65E+05	0.998		
	0.05 - 0.005	5.00E+06	65456	0.9963		
α-terpinene	10.0 - 1.0	8.00E+06	2.00E+06	0.991	0.0012	0.0004
	0.50 - 0.05	6.00E+06	-5.18E+04	0.987		
	0.05 - 0.005	2.00E+06	2.15E+04	0.997		
1,8-cineole	5.0 - 0.05	2.00E+07	-5.84E+05	1.000	0.0010	0.0003
	0.01 - 0.001	9.00E+06	7.61E+04	0.984		
γ-terpinene	5.0 - 0.1	2.00E+07	7.16E+05	0.999	0.0008	0.0002
	0.01 - 0.001	1.00E+07	1.76E+04	0.999		
4-terpineol	5.0 - 0.5	2.00E+07	1.00E+06	1.000	0.0016	0.0005
	0.5 - 0.05	3.00E+07	-3.18E+05	1.000		
	0.05 - 0.005	2.00E+07	7.00E+00	1.000		
α-terpineol	3.0 - 0.01	2.00E+07	-2.87E+05	1.000	0.0050	0.0015

493

494 **Table 4.** Average total amount (expressed as  $\mu\text{g}$ ) of each TTO marker in whole skin and in epidermis and dermis separated with the cryostat method,  
 495 in receiving phase, in the remaining formulation after the *in vitro* permeation test and in the surrounding environment.

Total amount in formulation ( $\mu\text{g}$ )	$\alpha$ -pinene			$\beta$ -pinene			$\alpha$ -terpinene			1,8-cineole			$\gamma$ -terpinene			4-terpineol			$\alpha$ -terpineol		
	$\mu\text{g}$	$\sigma$	% of the total	$\mu\text{g}$	$\sigma$	% of the total	$\mu\text{g}$	$\sigma$	% of the total	$\mu\text{g}$	$\sigma$	% of the total	$\mu\text{g}$	$\sigma$	% of the total	$\mu\text{g}$	$\sigma$	% of the total	$\mu\text{g}$	$\sigma$	% of the total
	29.1			2.0			42.3			31.1			98.5			251			42.2		
Epidermis	0.042	0.007	<b>0.14</b>	< LOD		-	< LOD			0.013	0.007	<b>0.04</b>	0.016	0.002	<b>0.01</b>	0.035	0.003	<b>0.01</b>	0.012	0.002	<b>0.03</b>
Dermis	0.311	0.122	<b>1.07</b>	0.031	0.008	<b>1.55</b>	0.344	0.072	<b>0.80</b>	0.089	0.068	<b>0.29</b>	0.501	0.085	<b>0.51</b>	1.260	0.452	<b>0.50</b>	0.263	0.053	<b>0.62</b>
Total skin*	0.353	0.129	<b>1.21</b>	0.031	0.008	<b>1.55</b>	0.346	0.075	<b>0.82</b>	0.102	0.075	<b>0.33</b>	0.517	0.087	<b>0.52</b>	1.295	0.455	<b>0.51</b>	0.275	0.055	0.65
Receiving phase	0.042	0.008	<b>0.14</b>	0.016	0.005	<b>0.80</b>	0.027	0.004	<b>0.06</b>	3.85	0.89	<b>12.39</b>	0.031	0.005	<b>0.03</b>	124.80	33.83	<b>49.68</b>	22.61	6.45	<b>53.50</b>
Remaining amount in formulation	0.200	0.031	<b>0.69</b>	0.016	0.001	<b>0.80</b>	0.061	0.006	<b>0.14</b>	0.000	0.000	<b>0.00</b>	0.072	0.013	<b>0.07</b>	0.034	0.015	<b>0.01</b>	0.016	0.005	<b>0.04</b>

496

497 \* Sum of epidermis and dermis amount

498 Table 5. Composition of the topic O/W formulation used for *in vitro* permeation studies.

Component	% in the formulation
Deionized water	84.5
Glycerin	4.6
PEG 400	0.6
Disodium EDTA	0.1
Carbomer 341	0.4
Mineral oil	3.6
Cetyl alcohol	0.2
Triethylamine	0.3
Dimethicone	0.5
Methyl paraben	0.2
Tea Tree ( <i>Melaleuca alternifolia</i> ) essential oil	5.0

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