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

(Article begins on next page)

07 January 2023

1 **Tea tree (*Melaleuca alternifolia*) essential oil: evaluation of skin permeation and distribution**

2 **from topical formulations with a solvent-free analytical method.**

3 Francesca Capetti[a], Barbara Sgorbini[a]*, Cecilia Cagliero[a], Monica Argenziano[a], Roberta Cavalli[a],

4 Luisella Milano[b], Carlo Bicchi[a], Patrizia Rubiolo[a]

5 [a]Dipartimento di Scienza e Tecnologia del Farmaco, Università degli Studi di Torino, Via P. Giuria

6 9, I-10125, Turin, Italy

7 [b]Dipartimento di Neuroscienze Rita Levi-Montalcini, Università degli Studi di Torino, Corso

8 Raffaello 30, I-10125, Turin, Italy

9 *Corresponding author: barbara.sgorbini@unito.it, +39 011 6707135

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., α-pinene,

18   β-pinene, α-terpinene, 1,8-cineole, γ-terpinene, 4-terpineol, α-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

1. Introduction

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

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

To the best of the authors' knowledge, only a few studies are available on TTO permeation [10-12], all of them investigating markers accumulated in the skin by solvent extraction. In 2006, Reichling *et al.* [5], compared several formulations, and studied the permeation of the major TTO component, i.e., 4-terpineol, without measuring its distribution in the skin layers. In 2008, Cross *et al.* [10] studied the epidermal retention of neat TTO and of a 20% TTO solution in ethanol by submitting skin layers to extraction with acetonitrile. In 2016, Sgorbini *et al.* studied the influence of both the formulation 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 (α-pinene, β-

62 pinene, 1,8-cineole, α-terpinene, γ-terpinene, 4-terpineol, α-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 α-pinene, β-pinene, 1,8-cineole, α-terpinene, γ-terpinene, 4-terpineol, α-terpineol, solvents

81 (acetone, cyclohexane), phosphate saline buffer, sodium dodecyl sulfate and ammonium chloride

82 were obtained from Merck-Sigma Aldrich.

83

2.2. *In vitro* permeation tests

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

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

2.3. Skin layer separation

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

Skin layers were separated using three different methods: 1) by immersing the skin in 1.8 mL of a 0.22 M ammonium chloride solution (pH = 9.5) for 15 min at room temperature [modified from 17]; 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 (C9-C25) 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_{13}) 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

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

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 α-pinene, β-pinene, α-terpinene and γ-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

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

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, α-terpineol, 1,8-cineole)

183 that were therefore quantified in each aliquot at each time, and ii) those present below their LOD/LOQ

184 (α-pinene, β-pinene, α-terpinene, γ-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^2); 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 α-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 α-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 $\mu g/cm^2$) 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, α-terpineol and 1,8-

223 cineole. **Figure 2** reports the *in vitro* kinetic permeation profiles obtained for 4-terpineol and α-

224 terpineol (Figure 2A), and 1,8-cineole (Figure 2B), by applying zero-order kinetics (i.e., cumulative

225 amount per unit surface area ($\mu g/cm^2$) 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 $\mu g/cm^2$ for 4-terpineol, 8.90 $\mu g/cm^2$ for α-terpineol and 3.85 $\mu g/cm^2$

229 for 1,8-cineole. The kinetic permeation data of the other TTO markers (i.e., α-pinene, β-pinene, α-

230 terpinene, γ-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 α-pinene, β-pinene, α-terpinene and γ-terpinene. The total amount of permeated

236   hydrocarbons was far lower than that of oxygenated monoterpenes, ranging from 0.0063 $\mu g/cm^2$ for

237   β-pinene to 0.017 for α-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 μg) 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 μg for β-

253   pinene and 1.3 μg for 4-terpineol. The two separated layers (epidermis and dermis) retained all TTO

254   markers with the exception of β-pinene, which was exclusively found in the dermis (0.043 μg).

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 α-pinene and 1,8-cineole,

257   about 30 for γ-terpinene, 4-terpineol and α-terpineol, and 170 for α-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 γ-terpinene, 4-terpineol

261   and α-terpineol dermis/epidermis ratio ranging from 0.8 to 1.3. Epidermis contained four-times more

262   α-pinene and 1,8-cineole than dermis, while dermis accumulated more α-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 β-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 α-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, α-terpineol) was

286   released into the headspace within the first hour These results confirmed the data observed in the *in*

287   *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 α-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., α- and β-pinene, α- and γ-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 γ-

terpinene to 16.4 ng/mL for α-terpineol, and the LOQs varied from 0.33 ng/mL to 54.0 ng/mL

respectively. In the latter case (MHE mode), the LOD values ranged from 0.2 ng for γ-terpinene to

1.5 ng for α-terpineol, while the LOQs ranged from 0.8 ng to 5.0 ng, respectively

4. Conclusion

This results show that the *in vitro* permeation kinetics and distribution of TTO bioactive markers in

a model formulation and at a finite dosing regimen can be monitored with a fully automated solvent-

free method.

To the best of the authors' knowledge, this is the first time that: 1) a solvent-free method has been

applied to monitor quantitatively the distribution of TTO components in skin layers, and their residual

amounts in the formulation in an *in vitro* permeation test; 2) a modified static Franz cell has been

used to evaluate the evaporation of TTO components from the formulation during the permeation

process. The method has shown itself to be highly reliable and sensitive for permeation experiments,

demonstrating high repeatability and intermediate precision.

The permeation results indicated that TTO component behavior changed as a function of chemical

structure; hydrocarbons did not pass the skin barrier (or only as traces), while oxygenated compounds

permeated at percentages ranging from 12% (1,8-cineole) to 53.3% (α-terpineol).

This model study allows to evaluate for the first time the effect of the skin barrier on the permeation

of volatile bioactive compounds of differing nature in a topical formulation, while also determining

the true amount(s) of each that: 1) overcome the natural protective barrier (whole skin, its separated

layers and pH) and; 2) can potentially be absorbed, and/or lost because of evaporation.

More generally, these model studies provide data useful to design topical formulations containing

volatile bioactive compounds in amounts not only sufficient for their biological activity, but also

within the limits fixed by regulatory authorities.

343   **Declaration of interest**

344   None

345

346   **References**

347   [1] Directive 2003/15/Ec of The European Parliament and of the Council of 27 February 2003

348   [2] Scientific Committee on Consumer Safety. Opinion on Fragrance allergens in cosmetic products.

349   2011 [May 1st, 2016]

350   [3] C.F. Carson, K.A. Hammer, T.V. Rileya, *Melaleuca alternifolia* (tea tree) oil: a review of

351   antimicrobial and other medicinal properties, Clin. Microbiol. Rev. 19 (2006) 50-62.

352   https://doi.org/10.1128/CMR.19.1.50–62.2006.

353   [4] K.A. Hammer, C.F. Carson, T.V. Rileya, Effect of *Melaleuca alternifolia* (tea tree) essential oil

354   and the major component terpinen-4-ol on the development of single – and multistep antibiotic

355   resistance and antimicrobial susceptibility, Antimicrob. Agents Chemother. 56 (2012) 909-915.

356   https://doi.org/10.1128/AAC.05741-11.

357   [5] J. Reichling, U. Landvatter, H. Wagner, K.H. Kostka, U.F. Schaefer, *In vitro* studies on release

358   and human skin permeation of Australian tea tree oil (TTO) from topical formulations, Eur. J. Pharm.

359   Biopharm. 64 (2006) 222-228. https://doi.org/10.1016/j.ejpb.2006.05.006

360   [6] N. Pazyar, R. Yaghoobi, N. Bagherani, A. Kazerouni, A review of applications of tea tree oil in

361   dermatology, Int. J. Dermatol. 52 (2013) 784-790. https://doi.org/10.1111/j.1365-

362   4632.2012.05654.x.

363 [7] J. Thomas, C.F. Carson, G.M. Peterson, S.F. Walton, K.A. Hammer, M. Naunton, R.C. Davey, T.

364 Spelman, P. Dettwiller, G. Kyle, G.M. Cooper, K.E. Baby, Therapeutic potential of Tea Tree Oil for

365 scabies, Am. J. Trop. Med. Hyg. 94 (2016) 258-266. https://doi.org/10.4269/ajtmh.14-0515.

366 [8] European Pharmacopoeia, 8th Edition, 2016

367 [9] SCCP Opinion on Tea Tree Oil, 2008, European Commission – Health and Consumer Protection

368 Directorate General, SCCP/1155/08.

369 [10] S.E. Cross, M. Russell, I. Southwell, M.S. Roberts, Human skin penetration of the major

370 components of Australian tea tree oil applied in its pure form and as a 20% solution in vitro, Eur. J.

371 Pharm. Biopharm. 69 (2008) 214-222. https://doi.org/10.1016/j.ejpb.2007.10.002.

372 [11] P. Sinha, S. Srivastava, N. Mishra, D. Kumar Singh, S. Luqman, D. Chanda, N. Prasad

373 YadavaSinh, Development, optimization, and characterization of a novel tea tree oil nanogel using

374 response surface methodology, Ind. Pharm. 42 (2016) 1434-1445.

375 https://doi.org/10.3109/03639045.2016.1141931.

376 [12] L. Dong, C. Liu, D. Cun, L. Fang, The effect of rheological behavior and microstructure of the

377 emulgels on the release and permeation profiles of Terpinen-4-ol, Eur. J. Pharm. Biopharm. 78 (2015)

378 140-150. https://doi.org/10.1016/j.ejps.2015.07.003.

379 [13] B. Sgorbini, C. Cagliero, M. Argenziano, R. Cavalli, C. Bicchi, P. Rubiolo, In vitro release and

380 permeation kinetics of *Melaleuca alternifolia* (tea tree) essential oil bioactive compounds from

381 topical formulations, Flav. Fragr. J. 32 (2017) 354-361. https://doi.org/10.1002/ffj.3403

382 [14] ISO norm 4730:2017, Essential oil of melaleuca, terpinen-4-ol type (tea tree oil)

383 [15] Basic criteria for the in vitro assessment of dermal absorption of cosmetic ingredients - updated

384 March 2006, Scientific Committee On Consumer Products

385 [16] M. Argenziano, A. Haimhoffer, C. Bastiancich, L. Jicsinszky, F. Caldera, Trotta F, 2019. *In vitro*

386 enhanced skin permeation and retention of imiquimod loaded in β-cyclodextrin nanosponge hydrogel,

387 Pharmaceutics, 11, 138. https://doi.org/10.3390/pharmaceutics11030138.

388 [17] D.I. Wilkinson, J.T. Walsh, Effect of Various Methods of Epidermal-Dermal Separation on the

389 Distribution of 14C-Acetate-Labeled Polyunsaturated Fatty Acids in Skin Compartments, J. Investig.

390 Dermatol., 62 (1974) 517–521. https://doi.org/10.1111/1523-1747.ep12681061.

391 [18] Y. Wang, J. O'Reilly, Y. Chen, J. Pawliszyn, Equilibrium in-fibre standardisation technique for

392 solid-phase   microextraction,   J.   Chromatogr.   A   1072   (2005)   13-17.

393 https://doi.org/10.1016/j.chroma.2004.12.084

394 [19] C. Bicchi, C. Cordero, E. Liberto, B. Sgorbini, P. Rubiolo, Reliability of fibres in Solid Phase

395 Microextraction for routine analysis of the headspace of aromatic and medicinal plants, J.

396 Chromatogr. A 1152 (2007) 138-149. https://doi.org/10.1016/j.chroma.2007.02.011.

397 [20] B. Sgorbini, C. Bicchi, C. Cagliero, C. Cordero, E. Liberto, P. Rubiolo, Herbs and spices:

398 Characterization and quantitation of biologically-active markers for routine quality control by

399 multiple headspace solid-phase microextraction combined with separative or non-separative analysis,

400 J. Chromatogr. A 1376 (2015) 9-17. https://doi.org/10.1016/j.chroma.2014.12.007.

401

Caption to Figures

402

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 α-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 (μg/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).
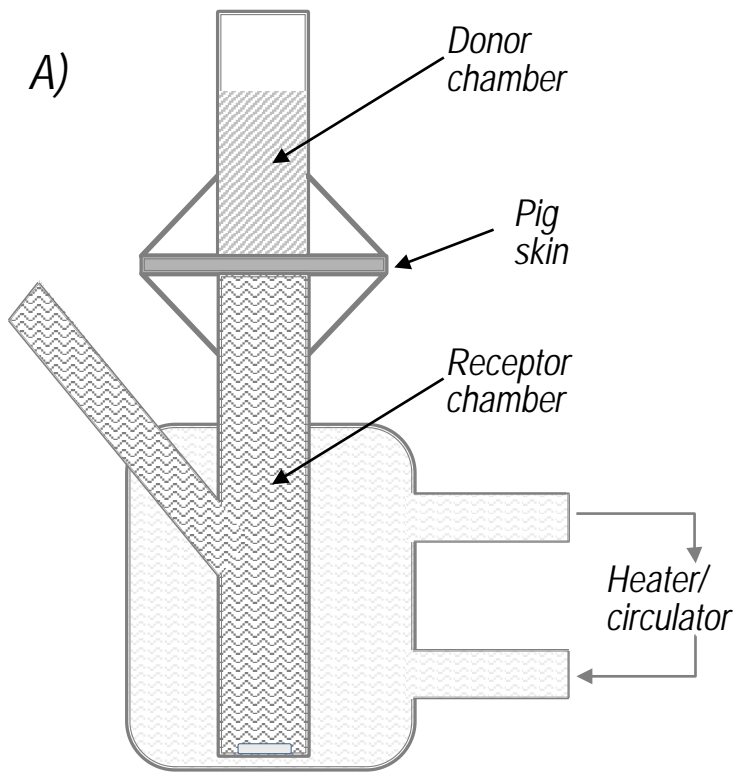
416

417

418 Figure 1

419

420 *A)*   Donor
chamber
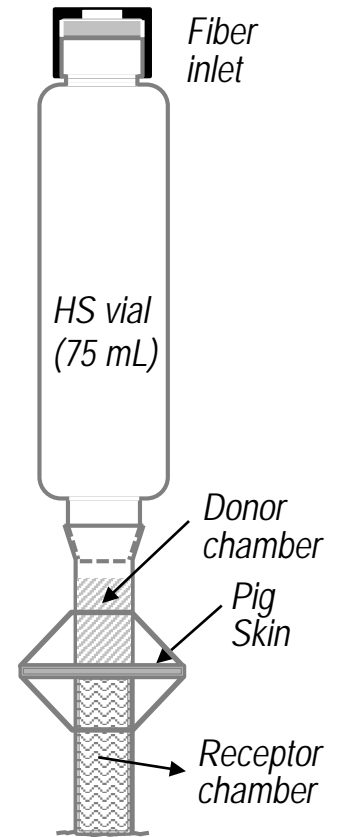
421

422   Pig
skin

423

424   Receptor
chamber

425

426

427   Heater/
circulator

428

429

430

431

B)   Fiber
inlet

HS vial
(75 mL)

Donor
chamber

Pig
Skin

Receptor
chamber

432   Figure 2

452   Figure 3

453

zoom
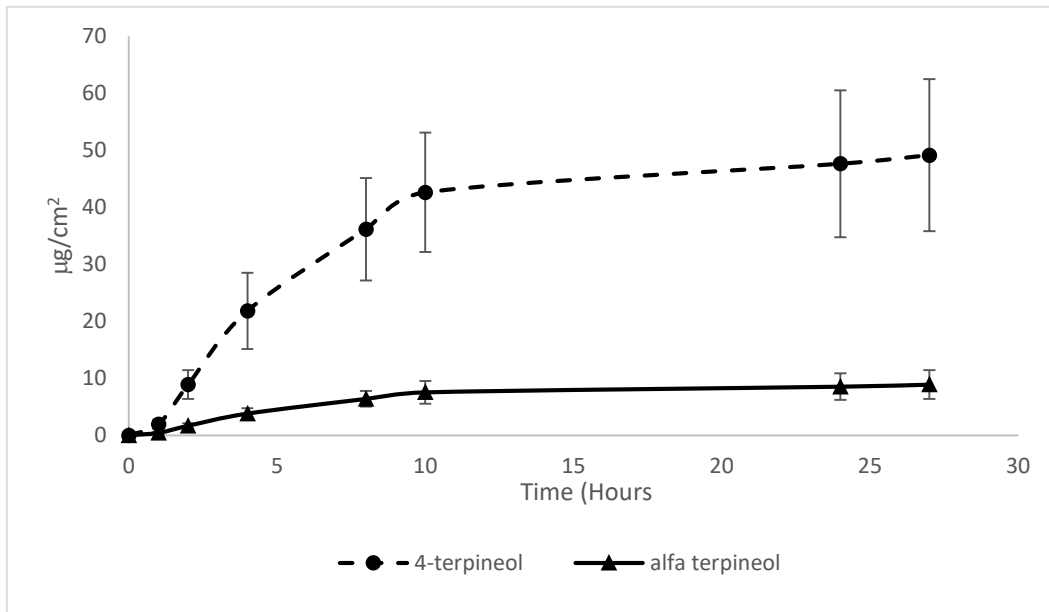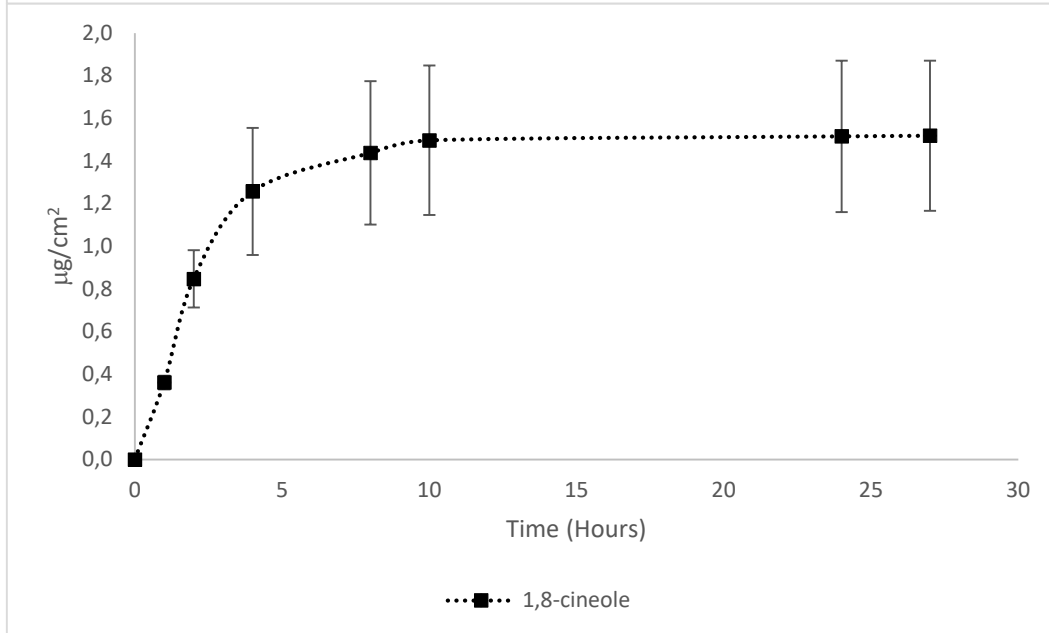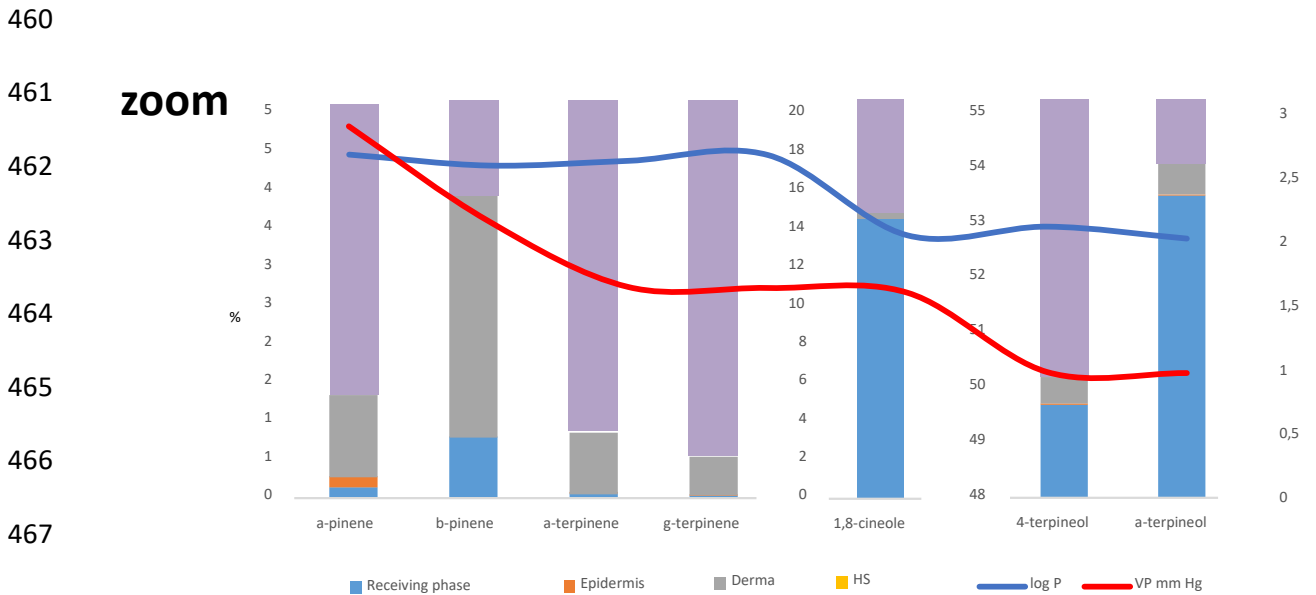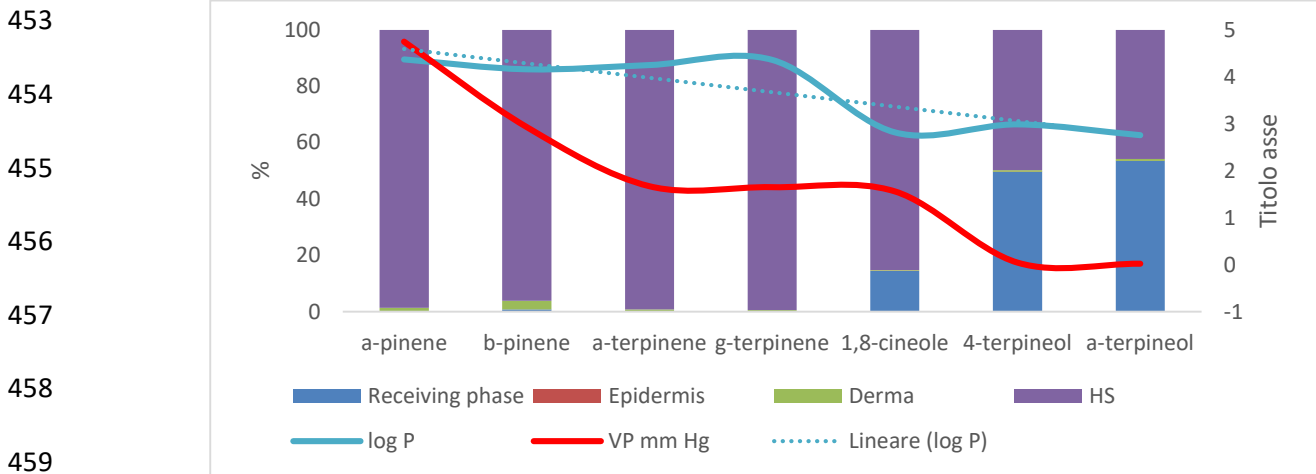
470   Figure 4
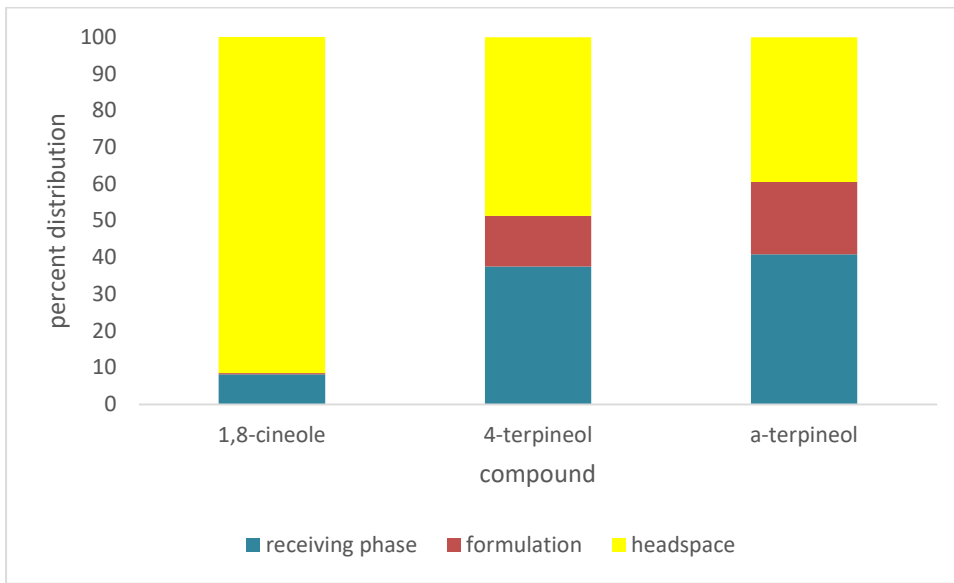
471

472

473

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 %
α-pinene	93	4.37	4.75	44.9
β-pinene	93	4.16	2.93	0.3
1,8-cineole	43	2.82	1.56	5.2
α-terpinene	93	4.25	1.67	7.1
γ-terpinene	93	4.36	1.65	16.4
4-terpineol	71	2.99	0.0427	41.9
α-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 α-terpineol.

487

Compound	linearity range (µg/mL)	slope	intercept	r	LOQ (µg/mL)	LOD (µg/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		
α-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 (ng/mL)	slope	intercept	r	LOQ (ng/mL)	LOD (ng/mL)
α-pinene	0.9-9	8.98E+04	-4.39E+03	0.9932	0.45	0.14
β-pinene	0.9-9	8.19E+04	-1.11E+03	0.9952	0.48	0.15
α-terpinene	0.9-9	3.86E+04	-9.51E+03	0.9948	0.65	0.20
γ-terpinene	0.9-9	8.49E+04	-2.02E+03	0.9969	0.33	0.10

488

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 µg) 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.

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

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 (*Melaleuca alternifolia*) essential oil	5.0

499

500

501

502

503

504

505

506

507

508

509

510