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

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

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Abstract

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

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

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

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

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

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

2. Material and Methods

2.1. Chemicals and samples

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

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

Pure α-pinene, β-pinene, 1,8-cineole, α-terpinene, γ-terpinene, 4-terpineol, α-terpineol, solvents (acetone, cyclohexane), phosphate saline buffer, sodium dodecyl sulfate and ammonium chloride were obtained from Merck-Sigma Aldrich.

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

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

2.4. SPME fibers

Carboxen/divinylbenzene/PDMS (CAR/DVB/PDMS) SPME fibers were obtained from Supelco Co. (Bellafonte, PA, USA) and conditioned before use as recommended by the manufacturer. Three fibers were tested by analyzing a set of standard solutions, at different concentrations of the target TTO components, in the receiving phase. ANOVA was carried out to confirm the homogeneity of fiber performance and to discard those with different sampling behavior. The consistency of fiber performance was checked every 50 analyses using an in-fiber external standardization approach and a standard mixture of hydrocarbons (C9-C25) in cyclohexane (1 μL of a 0.1 mg/mL solution) [18,19].

2.5. Sampling conditions

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

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

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

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

2.6. Analysis conditions

Analyses were carried out using a MPS-2 multipurpose sampler (Gerstel, Mülheim a/d Ruhr, Germany) installed on a Shimadzu 2010 GC unit coupled to a Shimadzu QP2010 Mass spectrometer. GC conditions: injector temperature: 280°C, injection mode: split; ratio: 1/20; carrier gas: helium; flow rate: 1 mL/min; fiber desorption time: 5 min; column: Mega SE52 (95% polydimethylsiloxane, 5% phenyl) 25 m×0.25 mm dc×0.25 μm df, from MEGA (Milan, Italy). Temperature program: from 50°C (1 min) to 125°C (0 min) at 3°C/min, then to 250°C (5 min) at 20°C/min.

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

2.7. Quantitation

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

The calibration curves of α-pinene, β-pinene, α-terpinene and γ-terpinene were built up by analyzing, in triplicate, 5.6 mL of nine diluted mixtures in the 0.9-9 μg/mL concentration range using HS-SPME-GC-MS under the above conditions.

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

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

2.8. Method validation

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

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

3. Results and discussion

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

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

3.1. Set up of *in vitro* permeation tests

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

3.2. Optimization of skin-layer separation

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

Three approaches were investigated to split dermis from epidermis: the cryostat method was preferred as reference method because it provided well-separated skin layers ready for a direct HS sampling. The resulting epidermis/dermis ratio was around 1:30, deriving from the average weight of epidermis (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 μg/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 (μg/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 μg/cm^2 for 4-terpineol, 8.90 μg/cm^2 for α-terpineol and 3.85 μg/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

hydrocarbons was far lower than that of oxygenated monoterpenes, ranging from 0.0063 μg/cm^2 for β-pinene to 0.017 for α-pinene.

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

3.4. Skin retention of TTO marker compounds

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

Table 5 reports the average total amount (expressed as μg) of each TTO marker in whole skin and in the epidermis and dermis, separated using the cryostat method. In general, all TTO compounds were retained by the (whole) skin, although each one to a different extent; ranging from 0.031 μg for β-pinene and 1.3 μg for 4-terpineol. The two separated layers (epidermis and dermis) retained all TTO markers with the exception of β-pinene, which was exclusively found in the dermis (0.043 μg). Moreover, the absolute amounts of TTO components were found to be much higher in the dermis than in the epidermis, with a dermis/epidermis ratio corresponding to 7 for α-pinene and 1,8-cineole, about 30 for γ-terpinene, 4-terpineol and α-terpineol, and 170 for α-terpinene. The greater accumulation in the dermis is probably due to its higher abundance compared to epidermis (about 1:30 w/w). When the content of TTO markers is normalized to the weight of the skin-layer, the distribution between epidermis and dermis was comparable, in particular for γ-terpinene, 4-terpineol

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

3.5. Distribution of permeated TTO components

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

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

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

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

3.6. TTO-component release into the headspace

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

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

The highest percentage of oxygenated compounds (i.e., 1,8-cineole, 4-terpineol, α-terpineol) was 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.

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Declaration of interest

None

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401

Caption to Figures

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

Figure 2. *In vitro* kinetic permeation profiles for 4-terpineol and α-terpineol (Figure 2A) and 1,8-cineole (Figure 2B), obtained by applying a zero-order kinetics (i.e., cumulative amount per unit surface area (μg/cm^2) plotted *versus* time).

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

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

Figure 1

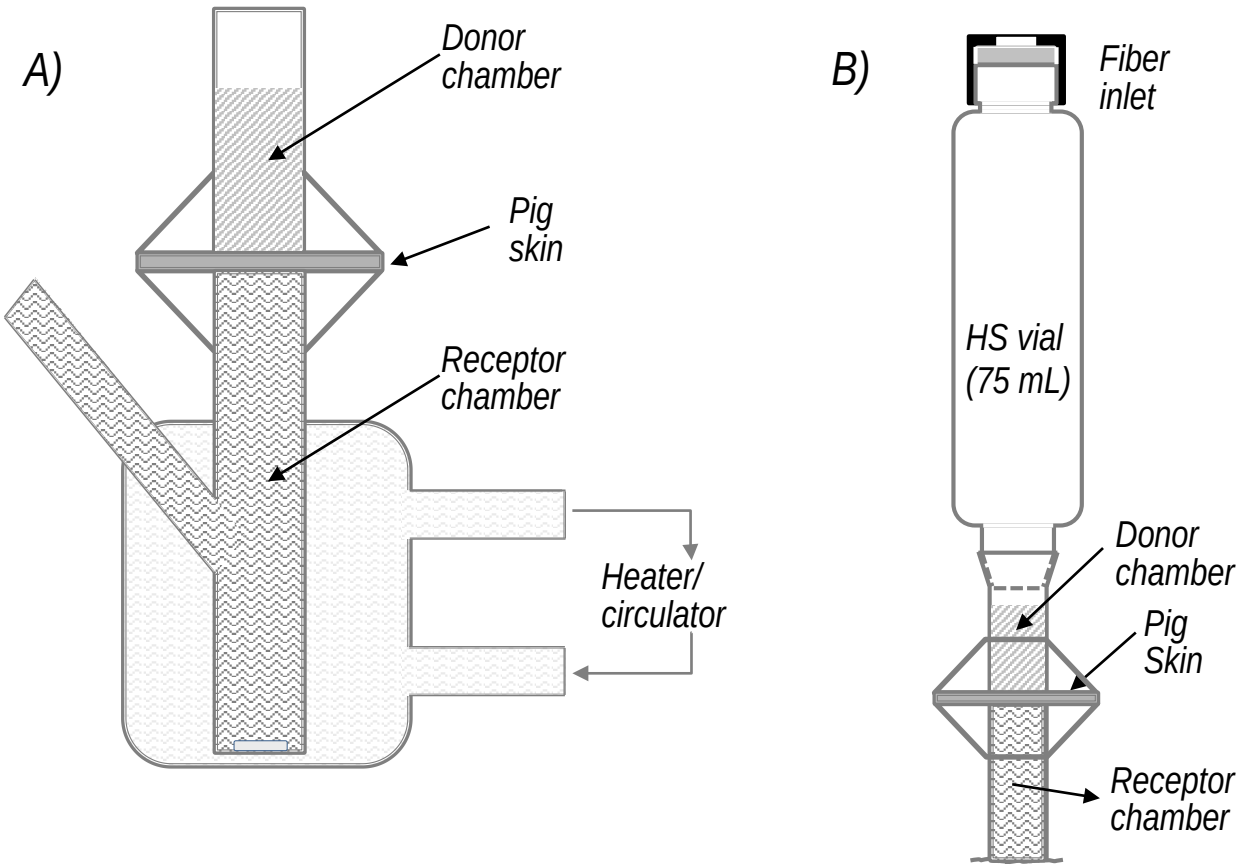

A)
Donor chamber
Pig skin
Receptor chamber
Heater/ circulator
B)
Fiber inlet
HS vial (75 mL)
Donor chamber
Pig Skin
Receptor chamber

Figure 2

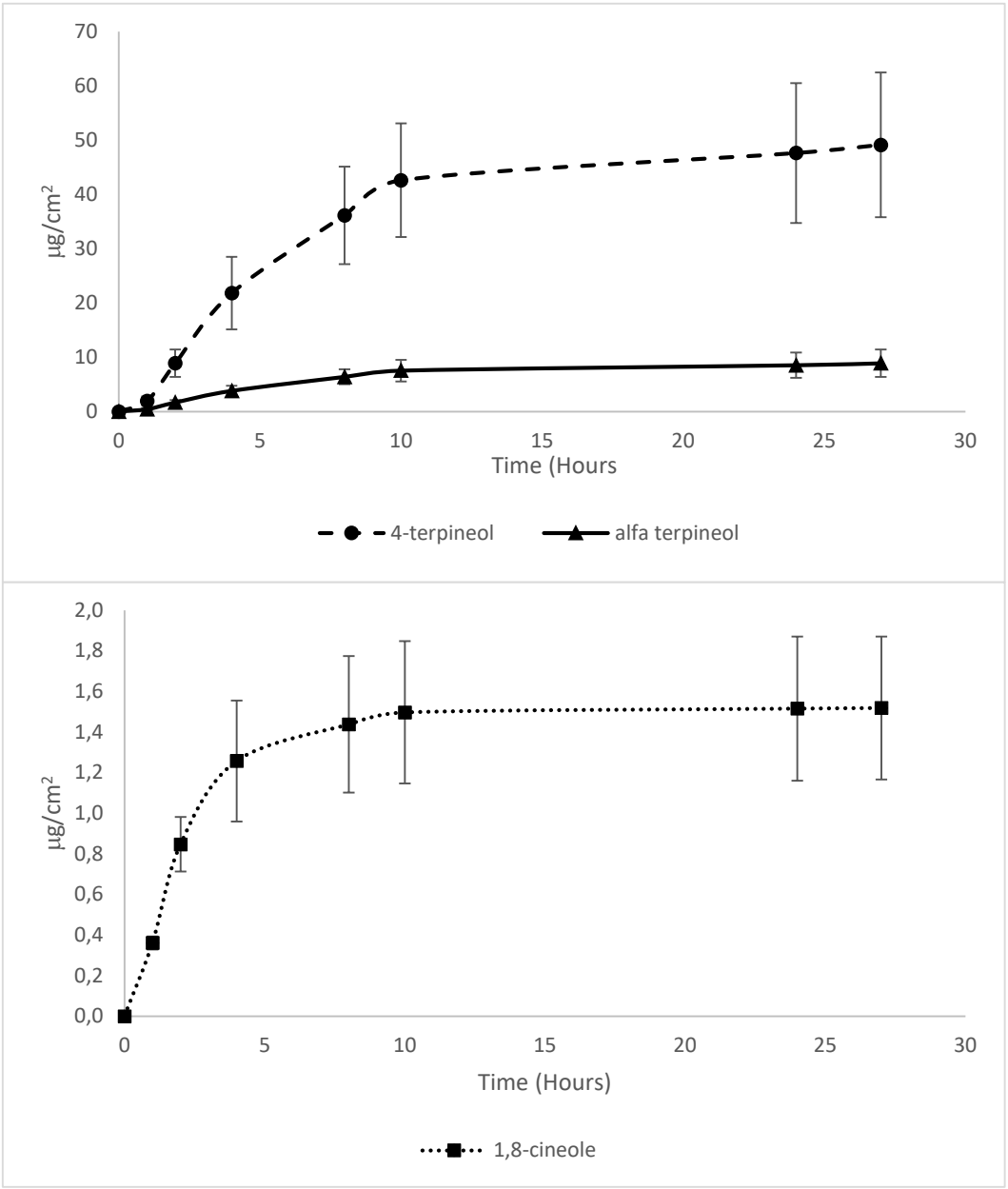
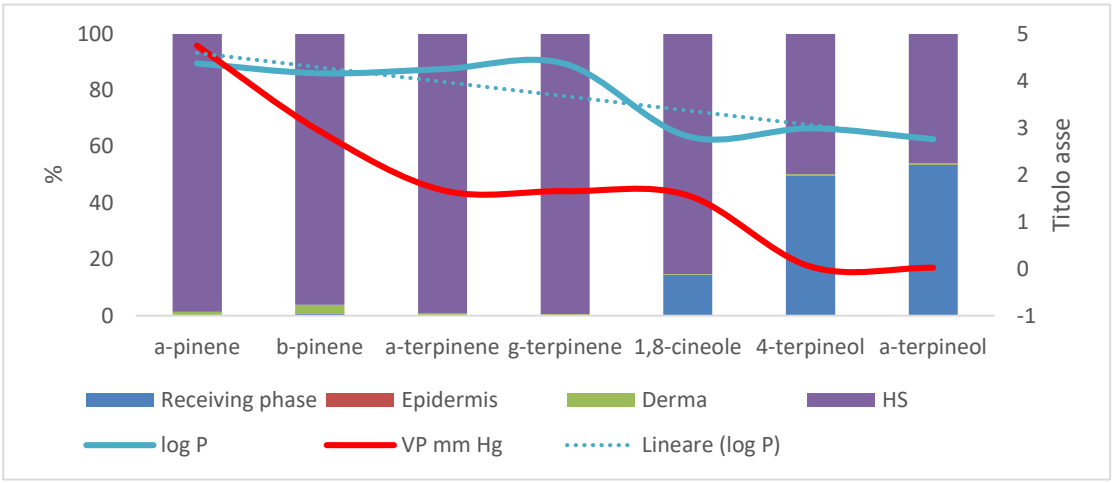
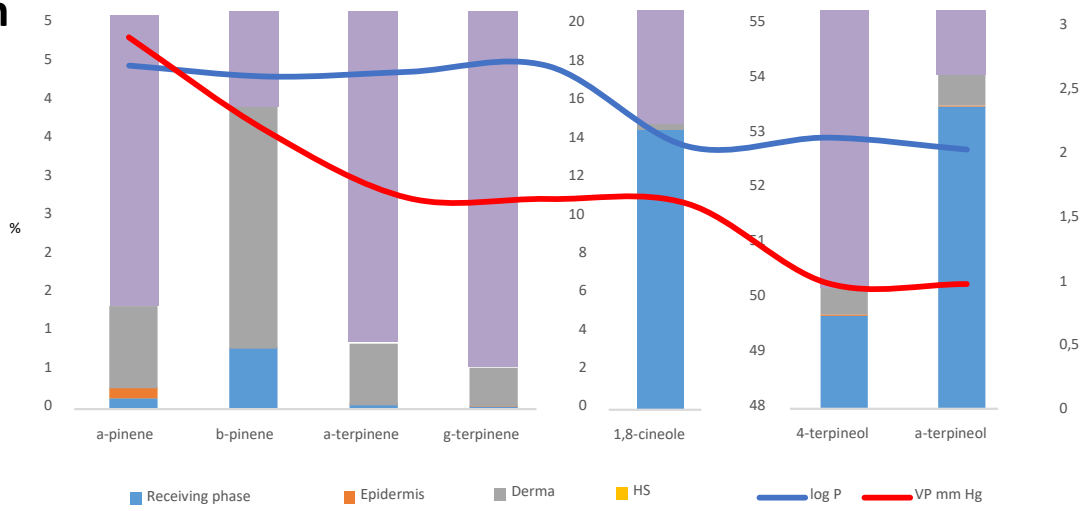

70
60
50
40
30
20
10
0
µg/cm²
0
5
10
15
20
25
30
Time (Hours
4-terpineol
alfa terpineol
2,0
1,8
1,6
1,4
1,2
1,0
0,8
0,6
0,4
0,2
0,0
µg/cm²
0
5
10
15
20
25
30
Time (Hours)
1,8-cineole

Figure 3



zoom



470 Figure 4

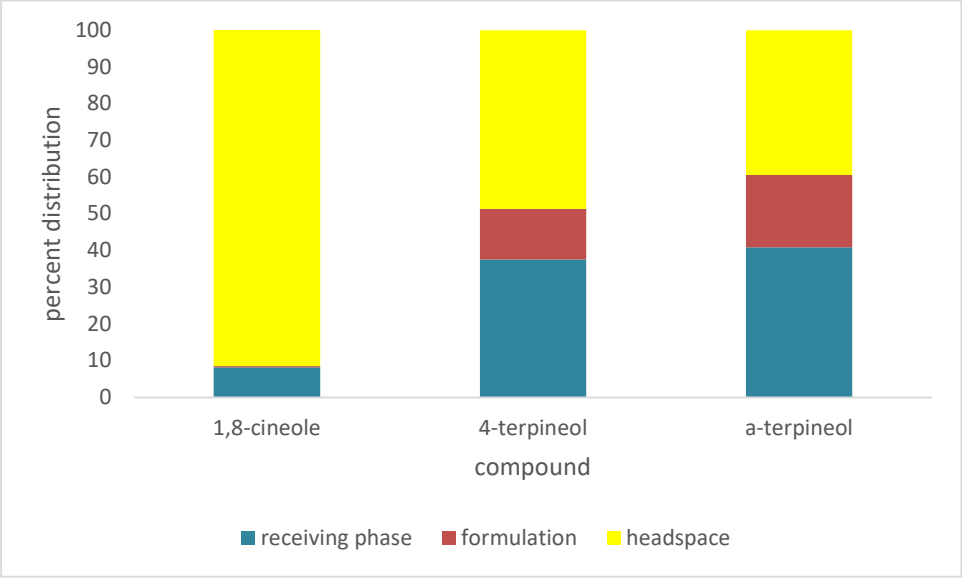


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Table 1. List of the TTO markers together with their partition coefficients (Log P) and vapour pressure values, the selected diagnostic ions used for their quantitation and their abundance in the investigated essential oil (expressed as relative %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

Compounds	m/z	Log P*	Vapour pressure (mm Hg)*	Area %
α-pinene	93	4.37	4.75	4.7
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

* Episuite database

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

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