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In vitro evaluation of the permeation through reconstructed human epidermis of essentials oils from cosmetic formulations

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ABSTRACT

The permeation of essential oils through SkinEthic® reconstructed human epidermis, (RHE), was studied *in vitro* to establish a convenient tool to monitor the kinetics of release of active principles from cosmetic formulations. Twelve days old human epidermis held on polycarbonate disks was revitalized by addition of growth medium and incubated at 37 °C in 5% CO_2 atmosphere for five days prior to investigation. A system of six custom designed glass Franz-type diffusion cells were used for the permeation studies at 34 °C. The diffusion kinetic for 8 selected terpenes (camphor, carvone, 1,8-cineole, linalool, menthol, α-thujone, menthone, *t*-anethole), chosen as analytical markers of a mixture of plant essential oils contained in a cosmetic formulation, was probed by HS/SPME–GC–MS analysis and elaborated according to Fick's first law to obtain skin permeability coefficients (P_S = 1.51, 1.47, 1.36, 0.80, 0.62, 0.40 and 0.14 × 10^{-3} cm/h, respectively). The method proved to be sensitive, simple and reproducible, and RHE represents a convenient model for safety/quality assessment of cosmetic formulations.

1. Introduction

Essential oils are commonly employed in cosmetic formulations, particularly in perfumes and in massage oils. They are known to penetrate human skin [1–3], mostly by passive diffusion [4], and to influence the skin permeability to other bio-active ingredients delivered from topical formulations [5–7]. As a consequence evaluating their percutaneous release from cosmetic formulations would be highly relevant for their quality and safety assessment, however studies on their skin absorption are rare. While *in vivo* studies in humans would provide the most valuable information they would also raise major ethical issues. On the other hand current EU regulations prohibit animal testing for cosmetic formulations [8]. Several *in vitro* protocols based of Franz-type diffusion cells have been described and OECD guidelines have become available for the development and validation of such protocols [9,10]. These are commonly based on the use of animal skin (particularly mouse and pig) [7,11–13], or human skin from reductive mastoplastic, from other reductive surgery or from cadaver [12,14,15]. While the use of animal skin for cosmetics testing would somewhat conflict with the ethical principles that inspired EU regulations, human skin from surgery or cadaver poses major problems of availability. Furthermore these approaches require skilled histological manipulation to remove hair, derma and subcutaneous tissues, which may compromise the integrity of epidermis. Biological variability also suggests that a large number of measurements should be performed to obtain representative data. Recently reconstructed human epidermis (RHE) from cell culture has become available on the market. This is usually delivered in disks of different size, developed for different uses, including permeation studies [16]. The use of RHE for *in vitro* testing is considered in OECD guidelines [9] and is strongly encouraged by COLIPA.

Previous studies have shown that RHE bears reasonable similitude to native human epidermis both in terms of morphology and lipid composition [16,17]. Although it might be less selective than native epidermis to the permeation of some drugs [16,18], recent investigation suggests it is a valid substitute for *in vitro* testing of topical formulations [19,20].

Aiming to develop a convenient analytical tool for *in vitro* testing of the percutaneous release of essential oils from cosmetic formulation, we have designed a system of thermostatted Franz-type diffusion cells whose geometry has been optimized for the use of SkinEthic® RHE disks, and we have set up a procedure for the analysis of major terpene components of essential oils released in the receptor medium. This is based on a head-space solid phase micro extraction (HS/SPME) of selected terpene markers followed by GC–MS analysis. With these settings we have investigated the diffusion kinetics of terpenes from essential oils

Abbreviations: RHE, reconstructed human epidermis; SC, stratum corneum; egf, epidermis growth factor; EO, essential oil; HS, head space; SPME, solid phase micro extraction; IS, internal standard; TIC, total ion current; SIM, single ion monitoring; LOD, limit of detection; LOQ, limit of quantitation.

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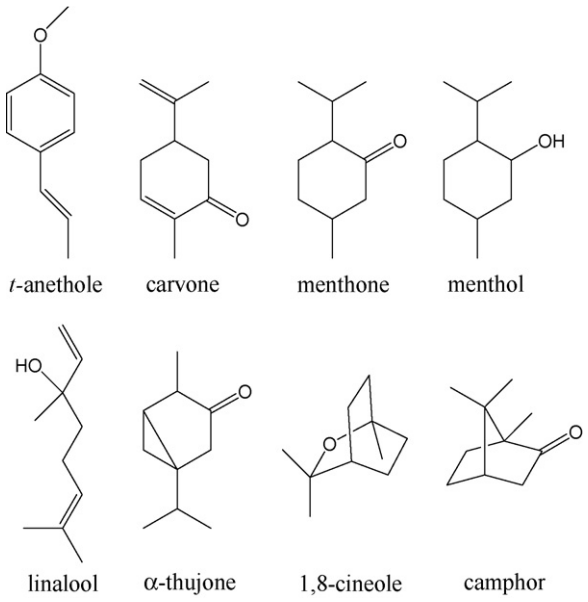

Chart 1. Terpenes investigated in this study.

contained in a typical cosmetic formulation (massage oil) through RHE (Chart 1).

2. Materials and methods

2.1. Materials

(−)-Myrtenal (>99%), eucalyptol (1,8-cineole ≥99.0%), (−)-linalool (≥98.5% sum of enantiomers), (−)-α-thujone (ca. 99%), (−)-camphor (≥99.0%), (−)-menthone (≥99.0% sum of enantiomers), (−)-menthol (≥99.0% sum of enantiomers), (−)-carvone (≥99.0% sum of enantiomers), *trans*-anethole (99%) were purchased from Fluka–Sigma–Aldrich. Essential oils of sage, caraway and coriander were purchased from Maraschi & Quirici s.p.a. (Riva Presso Chieri, TO, Italy), essential oils of eucalyptus and star anise were purchased from Muller & Koster s.p.a. (Milano, MI, Italy), essential oil of peppermint was purchased from Cydea s.r.l. (Almese, TO, Italy), essential oil of camphor was purchased from Agrar s.r.l. (Roma, RM, Italy), Reconstituted Human Epidermis 12-day-old, tissue surface 4.0 cm^2 and SkinEthic Growth Medium (1.5 mM calcium chloride, 25 mg/mL gentamycin, 5 mg/mL insulin, 1 ng/mL egf) were purchased from SkinEthic Laboratories (Nice, France). Sulphuric acid standard solution 1 M, ethyl ether, methanol (≥99.8%), sodium hydroxide (≥98%), sodium chloride (≥99.5%), isopropanol (≥99.8%), potassium chloride (≥99.5%), sodium phosphate dibasic anhydrous (≥99%) and crystal violet solution indicator were purchased from Sigma Chemical (St. Louis, MO, U.S.A.). Potassium di-hydrogen phosphate (≥99.5%) was purchased from Merck KgaA (Darmstadt, Germany). Sweet almond oil and jojoba oil cosmetic-grade were purchased from Agrar s.r.l. (Rome, RM, Italy). Dibutyl adipate (Cetiol B) and polisorbate 20 (Eumulgin SML 20) cosmetic-grade, were purchased from Cognis s.p.a. (Fino Mornasco, Co., Italy).

2.2. Apparatus and chromatographic conditions

2.2.1. Franz-type diffusion cells

Franz-type diffusion cells were designed in our labs to optimize the use of SkinEthic® RHE disks (surface 4.0 cm^2), and were manufactured by FAVS S.n.c. (Bologna, Italy). The donor compartment was 10 mL of internal volume and was closed from the outside by a screw cap with PTFE seal to avoid dispersion of volatile components. The receptor compartment was 14.8 ± 0.1 mL, and the exact receptor volume was determined for each cell and used in calculations. The useful diffusion surface was 1.54 cm^2 (diameter = 1.40 cm). Thermostatting was accomplished by circulating water for the entire extension of the receptor compartment, up to the membrane layer, to ensure homogenous temperature. The cells could be used either isolated with discontinuous sampling of the receptor from the sampling port, or in serial connection with continuous flow of the receptor through a flow cell for continuous reading. When the cells were used isolated with discontinuous sampling, as in our current measurements, the continuous flow ports were sealed with PTFE plungers. A picture of the diffusion cells is shown in Fig. 2. Each cell was equipped with a 10 mm × 2 mm PTFE coated stirring bar and a battery of 6 cells were mounted on a Magnetic 6 Stirrer (VELP scientifica s.r.l., Milan, Italy) and connected to a water bath MP BASIS (Julabo labortechnik Gmbh, Seelbach, Germany). Homogenous concentration of the analytes within the receptor compartment under our experimental conditions was ensured during preliminary tests by monitoring the diffusion of blue-colored crystal violet solutions.

2.2.2. GC–MS analysis

GC–MS analysis was carried out on a Gas-Chromatograph Star 3400 CX (Varian) equipped with a Ion-trap Mass Spectrometer detector Saturn 2000 (Varian), mounting 2 split/splitless 1078 Universal Capillary Injectors (Varian) one of which equipped with SPME splitless inlet liner 54 mm × 5 mm × 0.8 mm (Supelco, Bellefonte, PA, U.S.A.). Analysis was performed on capillary columns (ZB-5, 5% phenyl-95% dimethyl-polysiloxane, 30 m × 0.25 mm × 0.25 μm) purchased from Phenomenex (Torrance, CA, U.S.A.). The helium carrier head column pressure was 14 PSI (1.0 mL/min). Temperature programming was from 50 to 125 °C at 3 °C/min and that of the transfer line and ion trap were was 180 °C. All MS analyses were made in the electron impact (EI+) mode at 70 eV, the mass range was from 40 to 650 m/z and the chromatogram acquired in total ion current (TIC); single ion monitoring (SIM) chromatograms, for the quantitative analysis, were reconstructed at the ions indicated in Table 1, corresponding to the respective base peak except for linalool (base peak m/z 71), which was quantified at m/z 43 together with 1,8-cineole. The content of terpene markers (major constituents) in each essential oil and in the essential oil mixture was obtained from GC–MS analysis following 1 μL injection of standard solution in ethyl ether containing myrtenal as internal standard (IS). A five levels calibration for each terpene was obtained with authentic standards by 1 μL injection of ethyl ether solutions containing the IS.

2.2.3. SPME procedure

Samples of receptor medium and analytical standard solutions for calibration were subjected to head-space solid phase micro extraction. A volume of 100 μL of sample or standard solution, containing myrtenal (IS) was transferred in a 2 mL clear glass vial (Chromacol Ltd., Herts, United Kingdom) with silicone/PTFE screw cap. Polydimethylsiloxane 100 μm fibre was mounted on a SPME manual holder (Supelco, Bellefonte, PA, U.S.A.) and exposed in head space for 10 min at 25 °C. After exposition the fibre was retracted into holder and, exposed for 30 s at injector temperature (250 °C), then cleaned for 2 min in a baking unit. A GC–MS chromatogram was collected and the markers peaks identified and integrated.

2.3. Assay procedure

2.3.1. Calibration for HS–SPME–GC–MS analysis

For each terpene a 13 levels calibration curve was built by adding known volumes (0.2–20 μL) of a concentrated (10% w/v) standard solution in methanol and 1.0 μL of myrtenal (IS) methanol solu-

Table 1
Terpenes 13 levels calibration parameters expressed as [area] *vs.* [amount in receptor volume (14.8 mL)], linearity and LOD/LOQ values for HS/SPME–GC–MS analysis.

Terpene marker	Quantitation ion	LOD/LOQ [μg/mL]	Amount range [μg]	Slope (±S.D.)	*y*-Intercept (±S.D.)	r^2
1,8-Cineole	43	0.0699/0.0218	0.1–590	9,816.6 (±103.2)	209,467.8 (±26,943.9)	0.9987
Linalool	43[a]	0.0363/0.110	1.1–568	2,176.7 (±37.2)	17,886.5 (±9,340.5)	0.9968
α-Thujone	67	0.0049/0.0149	0.2–302	12,140.0 (±243.8)	77,730.6 (±32,581.4)	0.9956
Camphor	95	0.0389/0.118	0.5–1159	7,829.2 (±123.8)	116,363.7 (±63,440.4)	0.9973
Menthone	112	0.0139/0.0423	0.3–241	9,573.2 (±166.5)	24,409.4 (±17,771.7)	0.9967
Menthol	123	0.0788/0.238	1.8–456	1,720.9 (±27.4)	12,753.7 (±5,533.7)	0.9972
Carvone	82	0.0729/0.221	1.4–477	2,690.9 (±47.1)	11,924.9 (±9,944.6)	0.9966
t-Anethole	148	0.0482/0.146	0.8–533	4,710.1 (±65.3)	11,466.1 (±15,400.3)	0.9979

[a] Base peak: *m*/*z* 71.

Table 2
Composition of essentials oils (EO) in mixture and physical–chemical properties of selected terpene markers.

EO	EO in mixture [%, w/w]	Terpene marker	Terpene in EO [%, w/w]	$D^{20\,°C}$ [g/cm^3]	Solubil. $H_2O^{25\,°C}$ [mg/L]	log *P*	Vapor pressure$^{25\,°C}$ [mmHg]
Sage	16.0	α-Thujone	32.1	0.914	408	2.65	0.20
		Camphor	22.2	0.992	1600	2.38	0.65
		1,8-Cineole	9.0	0.924	3500	2.74	1.90
Coriander	16.0	Linalool	60.4	0.863	1590	2.97	0.16
Caraway	16.0	Carvone	51.0	0.960	1300	3.07	
Peppermint	16.0	Menthol	50.7	–	490	3.40	–
		Menthone	26.5	–	688	2.87	0.278
Camphor	16.0	Camphor	100.0	0.992	1600	2.38	0.65
Star anise	10.0	*t*-Anethole	89.2	0.988	111	3.39	0.09
Eucalyptus	10.0	1,8-Cineole	85.4	0.924	3500	2.74	1.90
Internal std.	0.00	Myrtenal	–	0.988	–	2.98	–

tion to the receptor medium, up to a final volume of 14.8 mL, in a 20 mL head space clear glass vial with silicone/ptfe crimp cap (Supelco, Bellefonte, PA, U.S.A.) well stirred over magnetic stirrer; 100 μL of solution was sampled and transferred in a 2 mL clear glass vial (Chromacol Ltd., Herts, United Kingdom) with silicone/PTFE screw cap. Then it was subjected to head-space SPME procedure. The GC–MS chromatogram was collected and the markers peaks integrated. Calibration was obtained as (peak area)/(IS peak area) *vs.* [concentration]/[IS concentration] for each terpene and results are collected in Table 1. LOD and LOQ values were obtained from linear regression of the 5 data point at lower concentration as LOD = $3.3\sigma_a/b$ and LOQ = $10\sigma_a/b$, where σ_a is the standard error on the intercept and *b* is the slope of the regression curve. LOD/LOQ values are expressed in Table 1 as concentration (μg/mL) in the receptor fluid, the corresponding absolute amounts of terpenes (in μg) can be obtained by multiplying by the average receptor volume of 14.8 mL.

2.3.2. Preparation of the essential oils mixture and donor composition

A mixture of essential oils (EO) was prepared according to the composition reported in Table 2. This EO mixture was used both for the validation of the analytical procedure and for the formulation of the massage oil (donor). The donor formulation used for kinetic measurements consisted of an *oleolite* [21] solution containing 5% (w/w) of EO mixture, dibutyl adipate (90%, w/w), sweet almond oil (2.5%, w/w) and jojoba oil (2.5%, w/w). The final oleolite density was 0.9838 g/mL.

2.3.3. Validation of the analytical procedure

The EO mixture (sage, caraway, coriander, eucalyptus, star anise, peppermint and camphor), used for the preparation of the oleolite, was added in known amounts (0.2, 0.5, 1.0 μL) to 14.8 mL of receptor medium with 1.0 μL of myrtenal methanol solution as internal standard in a 20 mL head space clear glass vial with silicone/PTFE

Table 3
Content of terpenes in the essential oil mixture determined by GC–MS analysis and validation of the HS/SPME procedure for their analysis in the receptor medium.

Terpene marker	Conc. in EO mixture mean ± S.D. (*n* = 5) [mg/mL][a]	Level 1[b]		Level 2[b]		Level 3[b]	
		Added in receptor [μg]/%recovery	Intra-day (*n* = 5)/inter-day (*n* = 15) precision R.S.D.%	Added in receptor [μg]/%recovery	Intra-day (*n* = 5)/inter-day (*n* = 15) precision R.S.D.%	Added in receptor [μg]/%recovery	Intra-day (*n* = 5)/inter-day (*n* = 15) precision R.S.D.%
α-Thujone	50.36 ± 1.30	10/90.0%	9.3/13.4	25/100%	3.4/3.9	50/102.0%	4.5/5.1
Camphor	193.25 ± 1.16	39/94.9%	7.9/8.6	97/101.0%	2.0/3.5	193/99.0%	2.2/4.1
1,8-Cineole	98.47 ± 0.72	20/95.0%	6.3/8.1	49/95.9%	7.2/8.3	98/96.9%	6.1/7.4
Linalool	94.72 ± 1.10	19/89.5%	7.8/10.2	47/102.1%	3.6/5.1	95/97.9%	4.6/7.0
Carvone	79.49 ± 1.12	16/93.8%	9.8/12.6	40/97.5%	5.5/5.9	79/101.2%	3.5/4.2
Menthol	75.96 ± 0.65	15/93.3%	7.5/9.9	38/102.6%	3.7/4.4	76/100%	2.8/3.5
Menthone	40.26 ± 0.52	8/112.5%	6.6/10.4	20/95.0%	6.0/8.1	40/97.5%	4.7/5.8
t-Anethole	88.85 ± 1.04	18/105.6%	7.2/11.8	44/97.7%	4.2/5.6	89/101.1%	2.6/3.1

[a] From 1 μL injection and GC–MS analysis (see Section 2.2.2).
[b] From HS/SPME–GC–MS analysis (see Section 2.3.3).

crimp cap. HS/SPME was performed in a 2 mL vial on 100 μL of sampled solution, followed by GC–MS analysis. Results are collected in Table 3.

2.3.4. Preparation of reconstructed human epidermis

Twelve days old reconstructed human epidermis (SkinEthic, Nice, Fr.) held on polycarbonate disks were revitalized by addition of 4 mL of growth medium for skin for day (1.5 mM calcium chloride, 25 mg/mL gentamycin, 5 mg/mL insulin, 1 ng/mL egf) in sterile Petri boxes under laminar flux cabinet mod. 1200 FLV (Asal s.r.l. Cernusco S.N., MI, Italy) and incubated at 37 °C in 5% CO_2 atmosphere in a Infrabator CO_2 incubator (F.lli Galli G.&P., Milan, Italy) for five days prior to investigation.

2.3.5. In vitro percutaneous absorption

Glass Franz-type diffusion cells were mounted on a Magnetic 6 Stirrer (VELP scientifica s.r.l., Milan, Italy) and connected to a water bath MP BASIS (Julabo labortechnik Gmbh, Seelbach, Germany). The stirring rate and temperature were maintained at 600 rpm and 34 °C, respectively. The receptor medium was phosphate saline buffer pH 7.4 (8 g/L NaCl, 0.2 g/L KCl, 0.2 g/L KH_2PO_4 and 1.15 g/L $NaHPO_4$ in bi-distilled water), 3% (w/w) of polysorbate-20 and 0.25% (w/w) of isopropanol. In each cell was introduced a 10 mm × 2 mm magnetic stirring bar, the receiver compartment was filled with receptor medium and the skin membranes were cut from plastic support and mounted with their polycarbonate filter on the receptor compartment with the epidermal side facing upward into the donor compartment. Donor and receptor chambers were watertight closed with a metallic clamp, water circulation and magnetic stirring were started and a dose of 1.0 mL of oleolite (donor) was applied to the surface of the epidermal side of the mounted skin. Donor compartment was sealed with a screw cap to prevent evaporation of the essential oils. At time intervals of 30 min within 12 h from application, a 100 μL aliquot of the receptor medium was withdrawn and immediately replaced with an equal volume of fresh buffer [13]. An additional sampling was made after 24 h. The amount of marker terpenes released in the receptor medium was determined using the HS/SPME–GC–MS method. Samples that could not be analyzed immediately upon withdrawal were sealed in 2 mL vials with silicone/PTFE screw cap and stored at +4 °C.

2.3.6. Data analysis

The experimental data were elaborated according to Fick's first law (Eq. (1)) to obtain the flux and skin permeability coefficients of terpenes. The cumulative amount of substance through a unit of surface of skin (Q; μg/cm^2) was obtained by HS/SPME–GC–MS of the receptor medium, corrected for the amount of analytes subtracted at every sampling [13].

2.3.7. Statistical analysis

All data presented are the mean ± S.D. or S.E. The equivalence of variance was tested with the one-way ANOVA. Statistical comparisons were made using the unpaired t-test. p-Values of less than 0.05 were considered significant.

3. Results and discussion

3.1. Method of analysis

The chromatographic conditions (temperature programming) were optimized to achieve sufficient resolution of the analytes while maintaining the run-time as short as possible (25 min), in order to keep the pace with sampling from the receptor compartment in case a single diffusion cell is used at a time (*vide infra*). Partial overlapping of 1,8-cineole with limonene, which is almost ubiquitous in essential oils, was resolved in SIM reconstructed chromatograms, by selecting a convenient quantitation ion (Fig. 1 and Table 1).

Due to the apolar nature of terpenes, the barrier offered to their diffusion by RHE, and the aqueous composition of the receptor medium (*vide infra*), our study had to include the setting of a practical method to extract the analytes at very low concentration from the receptor medium. A manual SPME procedure with polydimethylsiloxane fibre exposed in the head space at 25 °C offered a convenient approach with satisfying linearity in a concentration range suitable to the kinetic investigation.

The limit of detection (LOD) and the limit of quantitation (LOQ) were determined for each terpene from regression of the 5 data points at lowest concentration, as the concentrations giving a signal to noise ratio (S/N) of 3.3 and 10, respectively, in the SIM reconstructed chromatograms. LOD values (expressed in Table 1) correspond to 0.07–1.1 μg range when multiplied by the total volume (14.8 mL) of receptor solution and are well suited to the sensitivity needed for such kinetic investigations. The calibration parameters and linearity data for HS/SPME–GC–MS analysis of terpene markers in the receptor medium are collected in Table 1 together with LOD/LOQ values.

The selection of representative markers for each essential oil (EO) was necessary due to the complex composition of many of them and the impossibility to quantitatively monitor the diffusion kinetics for each individual component. Preliminary experiments suggested that release of highly lipophylic volatiles from an oleolite into an aqueous receptor medium can be erratic for compounds having at the same time high vapour pressure and very high O/W partition coefficient ($\log P > 4$). Therefore main components having $\log P < 4$ were selected for each essential oil and monitored in the kinetic investigation. The content of such terpenes in the investigated essential oils, the amount of each essential oil used to prepare the EO mixture, together with some relevant physical-chemical properties of the selected terpenes are summarized in Table 2.

The actual content of each terpene marker in the EO mixture was determined by GC–MS analysis using authentic standards for calibration and myrtenal as IS. Results are displayed in Table 3 (column 2).

In order to validate our analytical method we tested its accuracy and precision by adding 0.2, 0.5 or 1.0 μL of the EO mixture to a volume of receptor solution corresponding to the receptor volume of diffusion cells. Such 3 levels validation covered the concentration range used in subsequent kinetic measurements. The content of each terpene in the receptor was then analyzed by our HS/SPME–GC–MS procedure for three consecutive days (15 replicate analyses). For each level, accuracy and precision were evaluated from mean percent recovery and %Relative S.D. as detailed in Table 3. Overall the performance of the analytical method was judged adequate to the subsequent kinetic investigation. A typical chromatogram is shown in Fig. 1.

3.2. Percutaneous absorption kinetics

The same EO mixture employed for the analytical validation was used (5%, w/w) to prepare the massage oil, which represented the donor medium in our investigation (see Section 2). A diffusion cell designed for this work, whose geometry was optimized to accommodate SkinEthic® RHE 4.0 cm^2 disks, is depicted in Fig. 2. The receptor medium composition was chosen to mimic physiological extracellular fluids; polysorbate-20 (3%, w/w) and isopropanol (0.25%, w/w) were added to increase the solubility of apolar analytes, in line with other *in vitro* studies [12,13] and with OECD recommendations. The concentration of such solubilizers was however kept as low as possible to avoid toxicity in RHE.

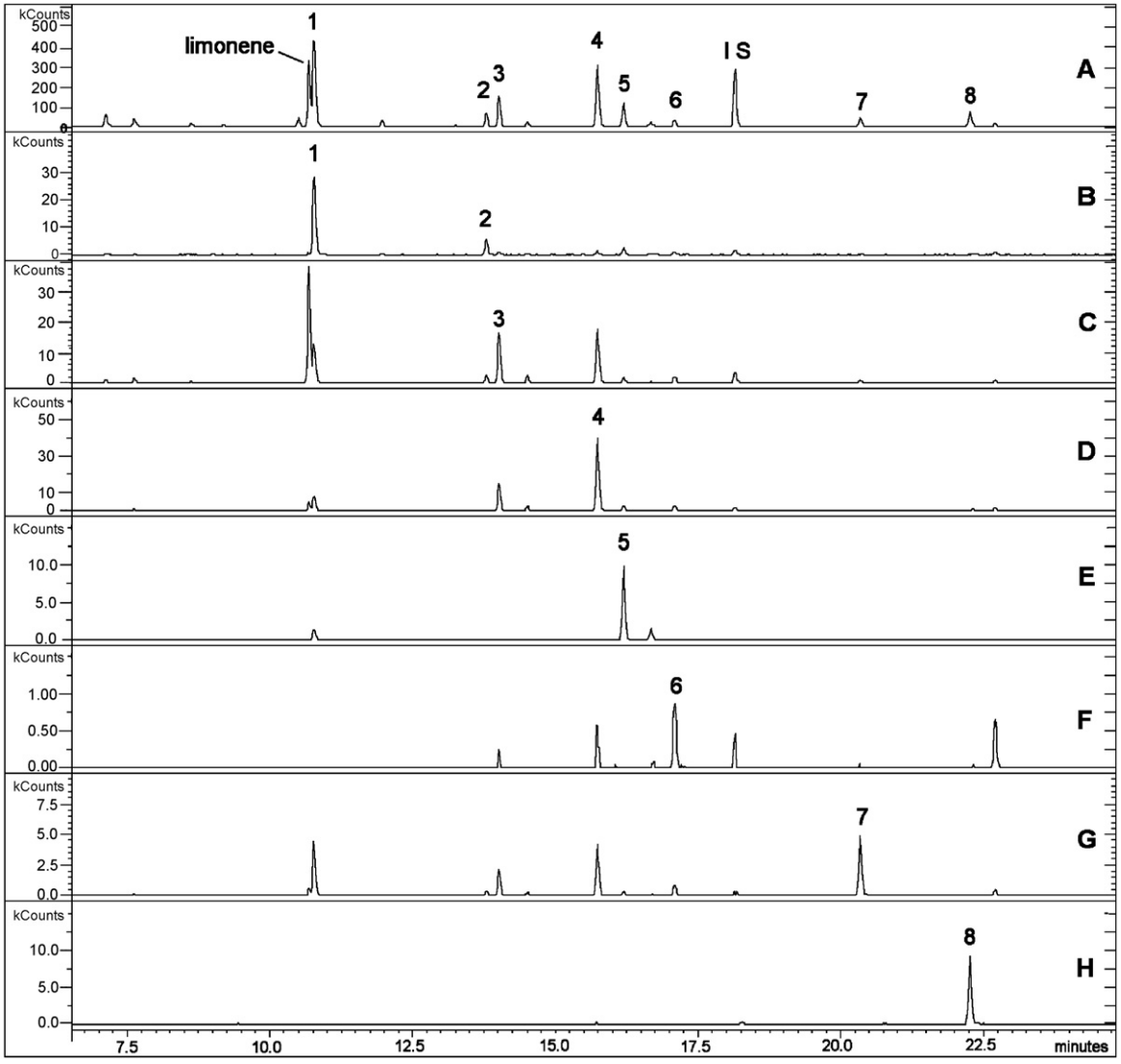

Fig. 1. TIC chromatogram (40–650 *m*/*z*) obtained from GC–MS analysis (ZB5 column, He 1 mL/min, 50–125 °C at 3 °C/min) of HS/SPME of the receptor medium (14.8 mL) addition of 1 μL of EO mixture (A); reconstructed SIM chromatograms at *m*/*z* 43 (B); *m*/*z* 67 (C); *m*/*z* 95 (D); *m*/*z* 112 (E); *m*/*z* 123 (F); *m*/*z* 82 (G); and *m*/*z* 148 (H). Peak numbers correspond to: 1 = 1,8-cineole; 2 = linalool; 3 = α-thujone; 4 = camphor; 5 = menthone; 6 = menthol; 7 = carvone; 8 = *t*-anethole.

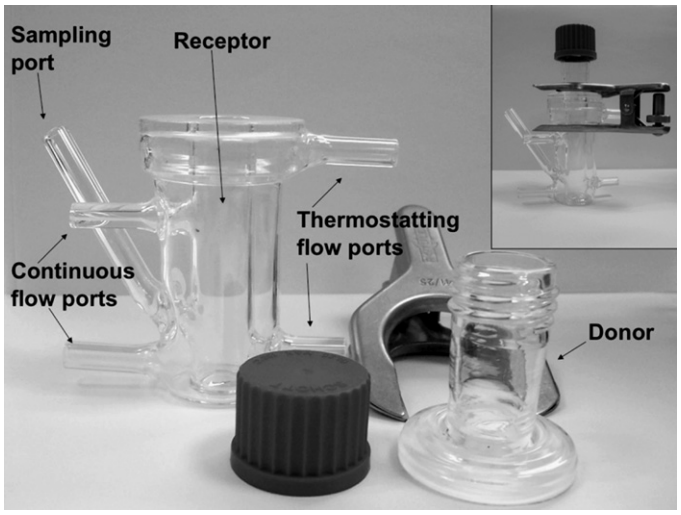

Fig. 2. Franz-type diffusion cells developed for this study.

At the temperature of 34 ± 1 °C the donor compartment was loaded with 1.0 mL of oleolite so to operate under "infinite dose" conditions and avoid significant decrease of analytes' concentration in the donor during the diffusion experiment. The percutaneous absorption profile was determined by HS/SPME–GC–MS analysis of the receptor medium, sampling from each diffusion cell at regular intervals. Results are shown in Fig. 3. Under the steady-state approximation, which is valid when the concentration gradient through the skin membrane is constant, the diffusion process occurs in accordance with Fick's first law (Eq. (1)).

$$\frac{dQ}{dt} = P_S(C_D - C_R) \quad (1)$$

Here P_S (cm/h) is the skin permeability coefficient and C_D and C_R (μg/cm^3) are the drug concentrations in the donor and receptor chambers, Q is the cumulative amount of substance diffused through a unit of skin surface (μg/cm^2), obtained by HS/SPME–GC–MS corrected for the amount of analyte subtracted at every sampling. Integration of equation 1 gives:

$$Q = P_S(C_D - C_R)t \quad (2)$$

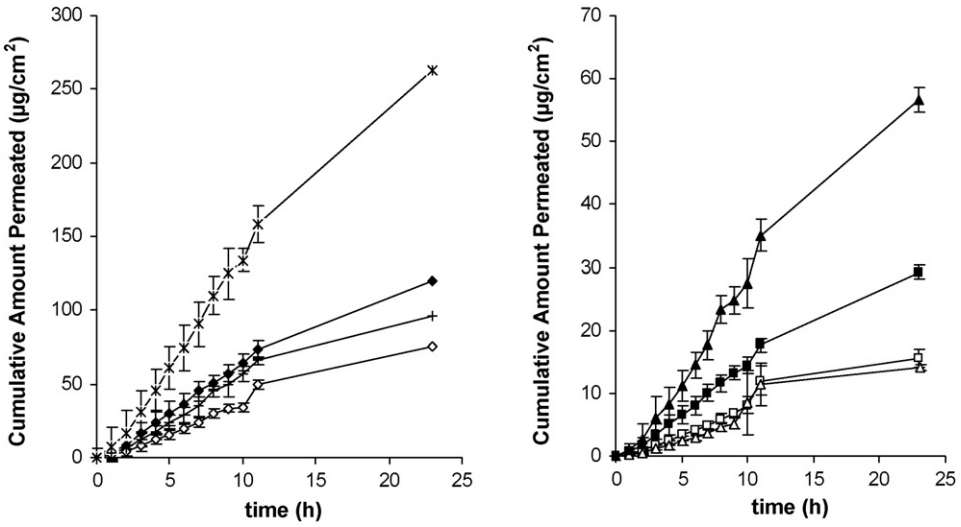

Fig. 3. Time course of mean cumulative amounts of terpenes permeated through 1.54 cm^2 of 12-day-old reconstituted human epidermis membrane (incubated for five days) released from the oleolite. *Key*: (✳) Camphor, (♦) 1,8-cineole, (+) carvone, (◊) linalool, (▲) menthol, (■) α-thujone, (□) menthone, (△) *t*-anethole. Each point represents mean ± S.D. of 6 diffusion experiments performed in two non-consecutive days. The rather large error bars are due to variability in the lag-time (see text).

Fig. 3 shows that the quantity Q varied linearly with time, during the first 12 h, in agreement with Eq. (2) assuming that $C_D \gg C_R$ (infinite dose condition). Deviation form linear behaviour was instead observed at 24 h, when lower that expected values of Q were recorded for all terpenes. This can be due to a combination of factors, among which the evaporation of terpenes from the sampling port (sealed only with a rubber septum) and loss of cell functionality in the membrane, resulting in a conformational change of cell membranes and variation of permeability. Similar findings were previously reported with SkinEthic® RHE [12].

The flux J (μg/cm^2/h) was calculated from the slope of cumulative uptake of substance through a unit of skin surface area (Q in μg/cm^2) as a function of time during the first 8 h, when excellent linear behaviour was recorded and no loss of cell viability is expected in the membrane. We obtained the skin permeability coefficient according to Eq. (3).

$$P_S = \frac{J}{C_D} \tag{3}$$

The lag-time L_t (h) is the time employed by the drug to start its diffusion through the skin in the receptor medium: it was graphically estimated by extrapolation of steady state portion of curve Q *vs.* t.

Data of percutaneous penetration and physiochemical parameters (flux, permeability coefficient, lag-time), averaged over six experiments in two non-consecutive days, are collected in Table 4.

Table 4
Donor concentration (C_D), lag-time (L_t), flux (J) and permeability coefficient (P_S) through RHE of terpenes released from a mixture of essential oils (sage, carvi, coriander, mint, camphor, eucalyptus, star anise) in oleolite.[a,b].

Donor	C_D [mg/mL]	L_t [h]	$J \times 10^3$ [mg/cm^2/h]	$P_S \times 10^3$ [cm/h]
Camphor	9.66	0.26 ± 0.23	14.58 ± 0.49	1.51 ± 0.05
Carvone	3.97	0.26 ± 0.22	5.83 ± 0.07	1.47 ± 0.02
1,8-Cineole	4.92	0.22 ± 0.04	6.69 ± 0.10	1.36 ± 0.02
Linalool	4.74	0.23 ± 0.21	3.88 ± 0.07	0.82 ± 0.02
Menthol	3.80	0.46 ± 0.13	3.03 ± 0.05	0.80 ± 0.01
α-Thujone	2.52	0.21 ± 0.18	1.55 ± 0.02	0.62 ± 0.01
Menthone	2.01	0.30 ± 0.26	0.80 ± 0.01	0.40 ± 0.01
t-Anethole	4.44	0.61 ± 0.06	0.63 ± 0.06	0.14 ± 0.01

[a] Mixture of dibutyl adipate (90%), jojoba oil (2.5%), sweet almond oil (2.5%) and EO mixture (5%).
[b] Mean ± S.D. (n = 6).

All the investigated terpenes were found to cross SkinEthic® RHE, but had permeability coefficients significantly lower than the reference drugs that have usually been employed in validation studies. For instance caffeine and testosterone were found to have P_S values of 7.78 and 21.24 × 10^{-3} cm/h respectively in the same RHE type. To the best of our knowledge this is the first investigation evaluating the diffusion kinetics of essential oils through RHE, and no data is available in the literature for direct comparison. Among the rare data available on native human skin, camphor and menthol were reported to need 15–30 and 30–60 min respectively to saturate the stratum corneum (SC) and epidermis, following application of a commercial ointment containing 5.7% and 3.8% respectively of the two terpenes [15]. This is in good agreement with the values of lag-time determined in this work, which are indicative of the time necessary to saturate the skin and establish the diffusion gradient. Terpinen-4-ol (log P = 2.99; from tea tree oil) was found to have a P_S value of 2.28 × 10^{-3} cm/h in native human epidermis [14], i.e. of the same magnitude of our current values. Similarly a number of terpenes (D- and L-limonene, dipentene, terpinolene and eucalyptol) were found to have P_S values ranging from 0.2 to 1.8 × 10^{-3} cm/h in human epidermis from cadaver [22], i.e. the same range found in our investigation, suggesting that RHE could be a valid substitute for human epidermis *in vitro*.

Camphor and *t*-anethole showed respectively the highest and the lowest permeability among the tested terpenes, their P_S values differing by 1 order of magnitude. Interestingly they have also, respectively, the lowest and among the highest log P values, thereby contradicting the original Potts and Guy model that directly correlates P_S to log P [4]. Potts and Guy model was developed for "pure" chemicals applied on the skin, and the opposite trend observed here might be explained by the lower release of the most lipophylic compound from the oily donor. However no simple correlation is apparent in this study between permeability coefficient and liposolubility of the compound, possibly suggesting that specific interactions of the analytes with SC have a relevant role in the diffusion kinetics.

The precision in the determination of the flux and permeability coefficient was very satisfying, as can be judged from the quite low value of standard error obtained for all terpene markers. This is particularly encouraging when compared to the larger dispersion of data normally encountered in kinetic investigations with animal or human epidermis. Conversely a quite large variability was observed

for the lag-time, possibly reflecting some differences in the thickness of SC among different RHE specimens. This would conceivably require a different time for the impregnation of SC and the onsetting of the steady-state diffusion gradient. In this conjunction it should also be noted that the epidermis membranes are kept in aqueous medium before the assembling of the diffusion cells, and the oleolite donor would require time to equilibrate with the aqueous layer left on the surface.

Clearly the composition of the donor medium, hence of the cosmetic formulation, is expected to affect the percutaneous diffusion parameters. This has been observed for some molecules, e.g. α-tocopheryl acetate [23], and a few indications in this regard are available in the literature on essential oils administered *in vivo* [24] or *in vitro* [14,25]. This aspect would deserve systematic investigation.

4. Conclusions

In the present study a sensitive, accurate and reproducible analytical method is described to investigate the kinetics of diffusion of terpene compounds from essential oils contained in cosmetic formulations, through Reconstructed Human Epidermis *in vitro*. The described method and instrumental setting are sufficiently simple and cost-effective to be suited to the safety-efficacy screening of cosmetic formulations. Due to the very limited dispersion of experimental kinetic results (good repeatability), the use of RHE favourably compares with the larger variability normally encountered with native human epidermis, while overcoming the problems of limited availability. On the other hands the higher permeability of RHE compared to native epidermis, underlined by some investigators with other test molecules, suggests that care should be taken when transferring results to the clinical level. Possibly future investigation with RHE, and the extension of the set of available kinetic data to include a large variety of molecules will enable a reliable parameterization, to match simple and ethical *in vitro* studies to the behaviour *in vivo*.

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