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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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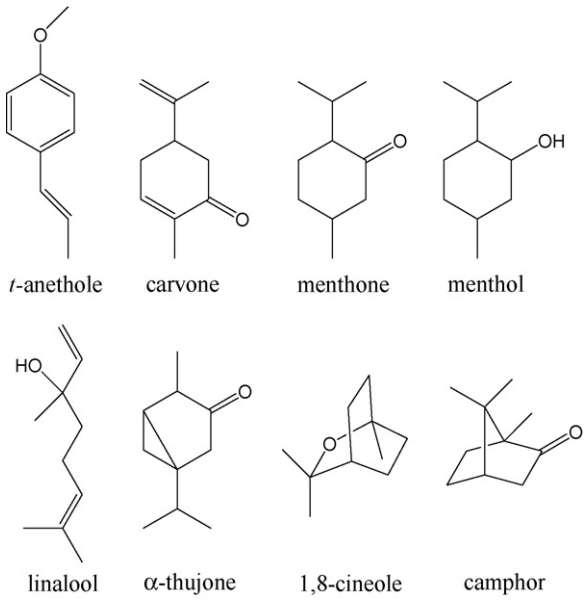

Chart 1. Terpenes investigated in this study.

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-