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In-vitro human skin penetration of the fragrance material geranyl nitrile

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Abstract

In-vitro human skin permeation and distribution of geranyl nitrile (GN) was determined using epidermal membranes following application (5 μl/cm^2) in 70% ethanol, under non-occlusive conditions, at maximum in-use concentration (1%). Permeation was measured (12 time-points over 24 h) using 6% (w/v) Oleth-20 in pH 7.4 phosphate buffered saline as receptor. Permeation of reference benzoic acid was assessed using the same skin donors. Overall recovery of GN at 24 h was low (14.1 ± 0.4%) due to evaporation. Evaporative loss of GN from polytetrafluoroethylene (PTFE) sheet, under the same conditions was rapid (93% over 24 h) although this overestimated loss during permeation where evaporation competed with uptake. At 24 h, 1.89 ± 0.15 μg/cm^2 GN, (3.74 ± 0.30% of applied dose) (mean ± standard error, SE, $n = 12$), had permeated. Following rapid initial permeation, the absorption plateaued due to depletion. Levels of GN in the epidermis (plus any remaining stratum corneum after tape stripping), filter paper membrane support and receptor fluid were combined (as per SCCNFP guidelines) to produce a total absorbed dose value of 4.72 ± 0.32%. Systemic exposure resulting from the use of GN as a fragrance ingredient, under unoccluded conditions, would be low based on the currently reported use levels.

Keywords: Fragrance; Geranyl nitrile; Dermal absorption; Human; Geranonitrile; 3,7-Dimethyl-2,6-octadienenitrile; CAS No. 5146-66-7

1. Introduction

Geranyl nitrile (geranonitrile, 3,7-dimethyl-2,6-octadienenitrile, CAS No. 5146-66-7) (GN) is widely used as a fragrance ingredient and is incorporated into a variety of consumer product types, including soaps, shampoos, cosmetics, perfumes, detergents and insect repellents. As a fragrance component, it is appreciated for its lemony-fresh odor similar to citral (Calkin and Jellinek, 1994). Its comparative stability under mildly alkaline conditions makes it particularly suitable to applications such as soaps (Arctander, 1969). Structurally, GN is part of a large group of nitriles used in perfumery. Annual use levels in this group range from <0.1 metric tons to between 100 and 1000 metric tons for GN (IFRA, 2000).

The permeability characteristics of human skin have been well established and have been extensively reviewed elsewhere (Roberts et al., 2002). The optimal characteristic for percutaneous absorption is that the permeant has relatively low molecular weight and is reasonably soluble in both hydrophilic and hydrophobic media, although the absolute amount of any compound permeating the skin will be dependent upon the vehicle in which it is applied. In in vitro investigations it is also necessary that the selected receptor phase is capable of solubilising all permeating compound and Oleth-20, a non-ionic surfactant, is routinely added to phosphate buffered saline at 6% (w/v) to improve the receptor phase solubility of hydrophobic compounds.

GN has a molecular weight of 149.24 Da. As reported by one manufacturer of this material (BASF), GN has a low calculated water solubility of ~45 mg/l, a calculated octanol/water partition coefficient, $\log P_{O/W} = 3.47$, a calculated

Abbreviations: BA, benzoic acid; GN, geranyl nitrile; IFRA, International Fragrance Association; LSC, liquid scintillation counter; PBS, phosphate buffered saline; PTFE, polytetrafluoroethylene; RSD, relative standard deviation; SE, standard error of the mean; SCCNFP, Scientific Committee on Cosmetic Products and Non-Food Products intended for Consumers.

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vapour pressure of 0.04 mmHg @ 20 °C and is liquid at room temperature (melting point −30 °C, boiling point 222 °C). All of these factors suggested that GN would permeate fairly readily through skin.

During skin permeation of volatile materials, such as solvents and fragrances, there can often be considerable competition between partition of the permeant into the skin and evaporation from the skin, with air-flow an important factor. It is common practice to attach absorptive traps above the donor chambers to retain evaporating material, improving mass balance (Reifenrath and Robinson, 1982). However, the presence of such traps can produce significant occlusion which has a very marked effect on skin permeation (Frantz et al., 1995; Lockley et al., 2002). In addition, trap absorption of the volatile material may be affected by competitive absorption of water vapour so that initially trapped material is subsequently lost. Less occlusive traps often use high skin surface air-flow, leading to evaporative loss which is unrepresentative of actual exposure conditions. Experimental conditions should alter the evaporation as little as possible compared to "in-use" unoccluded conditions (Yourick et al., 1999). Additionally, attempts have been made to predict evaporation from physicochemical properties of the test compound (Kasting and Saiyasombati, 2001) but the dataset used to validate this model to date is still small and limited in scope.

The purpose of this study was to determine the skin permeation and distribution of GN, applied at a maximum in-use concentration of 1%, using human tissue in vitro, in accordance with the SCCNFP in-vitro assessment of percutaneous absorption guidelines (SCCNFP, 2000). The permeation of a reference compound, benzoic acid (BA), was also assessed to confirm the validity of the test systems.

2. Materials and methods

GN (CAS No. 5146-66-7) and 2-^{14}C–GN (39.76 MBq/g, ethanolic solution) were supplied by BASF AG, 67056 Ludwigshafen. Water was distilled in all glass apparatus. Solvents (ethanol 99.7–100% (v/v), acetonitrile and tetrahydrofuran) and buffer salts were of AnalaR grade or better (VWR, Lutterworth, UK). Brij 98 (PEG 20 oleyl ether, Oleth-20) and BA (min. 99.5%) were from Sigma (Poole, UK). Tritiated water (^3H_2O) was from Amersham Pharmacia Biotech (Little Chalfont, UK) and (7-^{14}C)–BA in ethanol from American Radiolabelled Chemicals. OptiPhase 'HiSafe' 3 liquid scintillation cocktail and OptiSolve tissue solubiliser were from Wallac (UK) Ltd. Samples were counted using a Wallac 1409 liquid scintillation counter (LSC). Weight measurements were made on a Sartorius BP211D 5-place semi-micro analytical balance with a statistical printer.

2.1. Receptor phase preparation and assessment

Phosphate buffered saline (pH 7.4; PBS) was prepared by dissolving the following salts in water: 2.1 g/l $NaH_2PO_4 \cdot 2H_2O$, 19.1 g/l $Na_2HPO_4 \cdot 12H_2O$ and 4.4 g/l NaCl. The pH of the buffer was confirmed to be 7.4 ± 0.1. 6% (w/v) Brij 98 (Oleth-20) in PBS (BPBS) was prepared by dissolving 15 g Brij 98 in 250 ml PBS.

2.2. Preparation of test materials

The GN test vehicle was prepared by transferring 49.59 mg GN (unlabelled) and 185.97 mg (237.2 μl) ^{14}C–GN solution (containing 0.54 mg GN) to a 5 ml volumetric flask. Water (102 μl) was added followed by pre-mixed 70/30 (v/v) ethanol/water to volume. Final vehicle concentration was 1.003% (w/v) GN, with a measured density of 0.8700 g/ml at 25 °C. For the reference permeant, a 3.99 mg/ml BA solution containing 19.84 mg BA (unlabelled) and 50 μl ^{14}C-BA solution (0.11 mg BA) in 5 ml 50/50 (v/v) ethanol/water was prepared in the same manner as above. The density of this vehicle was 0.9199 g/ml at 25 °C. Samples (5–10 μl accurately weighed) from each vehicle showed uniform distribution (RSD < 0.5%) of label.

2.3. Assessment of solubility of permeants in receptor phases

Multiple sampling (five × 50 μl) of a radiolabelled 800 μg/ml GN solution confirmed complete miscibility (1.88% RSD). As the maximum possible receptor phase concentration of GN after permeation would be <20 μg/ml this receptor phase was suitable for use. For the reference BA, PBS was a suitable receptor phase (solubility 3.4 mg/ml at 25 °C).

2.4. Skin preparation

Full-thickness human female abdominal skin, obtained from cosmetic surgery with informed patient consent and stored at −20 °C, was thawed for processing. Following removal of subcutaneous fat by blunt dissection, individual portions were immersed in water at 60 °C for 50 s. The epidermis (comprising stratum corneum and viable epidermis) was gently removed from the underlying dermis. The latter was discarded and the epidermal membrane floated onto the surface of water and taken up onto aluminium foil. The membranes were thoroughly dried and stored flat at −20 °C until used. On the day of use, the epidermal membranes were floated onto water from the aluminium foil and taken up onto filter paper supports. The membranes were then mounted onto diffusion cells and trimmed to size. Four donors were used, distributed between the test groups.

2.5. Diffusion cells

Skin membranes were mounted as a barrier between the halves of greased (high vacuum grease, Dow Corning, USA) horizontal Franz-type diffusion cells, the stratum corneum facing the donor chamber. Cells were designed such that the area available for diffusion was about 1.0 cm^2, the exact area being measured for each diffusion cell. The diffusion cells were immersed in an unstirred Grant JB5 constant temperature water bath such that receptor cham-

bers were at 37.0 ± 0.5 °C which ensured that skin surface temperature was maintained at 32.0 ± 1 °C. Receptor chamber contents were continuously agitated by small polytetrafluoroethylene (PTFE)-coated magnetic followers driven by submersible magnetic stirrers (Variomag Telesystem 15.07). The water bath was placed on a bench in a small room (19 m^3) without forced ventilation, and was undisturbed, except at sampling intervals, to simulate typical in-use conditions where minimal evaporative loss would occur. Receptor chambers were initially filled with a known volume of water, capped, and allowed to equilibrate.

2.6. Membrane integrity assessment

The integrity of each membrane was assessed prior to dosing. Permeation of tritiated water was determined by applying 500 μl of 10 μCi/ml ^3H_2O to the skin surface and removing and counting a 200 μl receptor phase sample 1 h later. The skin surface was washed seven times and the receptor chambers three times with water, prior to refilling the receptor chamber with the selected receptor medium.

2.7. Application of test vehicle

The 1% GN solution was applied to the skin surface at a target dose of 5 μl/cm^2, in the following manner. The diffusion cell was placed on a 5 place electronic balance (supported on a glass beaker) and the balance tared. The appropriate volume was applied to the skin surface using an air-displacement digital pipette, and the actual weight applied immediately recorded. This weight applied was converted using the measured solution density (0.870 g/ml) to the exact volume applied. This weighing method was necessary as pipetting very small volumes of highly ethanolic solutions with air-displacement pipettes was inaccurate. Twelve replicates were dosed with GN solution. Additionally, three control cells were dosed with a 70/30 (v/v) ethanol/water solution (containing no GN). The average applied doses (mean ± standard error, SE) were 5.05 ± 0.09 and 5.36 ± 0.19 μl/cm^2 for the GN and control groups, respectively. The exact time of application was noted and that time represented zero time for that cell.

For the reference compound dosed cells, the same application technique was used to apply a target dose of 25 μl/cm^2 0.4% BA in 50/50 (v/v) ethanol/water. Diffusion cell donor chambers for this test group were immediately occluded using greased coverslips. The average applied dose (mean ± SE) for the six replicates in this test group was 24.24 ± 0.74 μl/cm^2. This dose and the occlusive conditions for this test group was necessary in order to allow direct comparison with published data from a multicentre study.

2.8. Determination of skin penetration

Receptor phase samples (200 μl) were taken (using a digital pipette) from GN dosed cells at 0.5, 1, 2, 3, 4, 6, 8, 10, 12, 16, 20 and 24 h and analyzed for ^{14}C. For BA dosed cells, receptor phase samples were removed at 1, 2, 4, 8 and 24 h and analyzed for ^{14}C. Liquid removed was replaced with fresh temperature equilibrated blank receptor medium.

2.9. Determination of skin distribution

For GN dosed cells, following removal of 24 h receptor phase samples, the remaining receptor phases were retained. Diffusion cells were dismantled, epidermal membranes secured onto small discs of thin plastic using cyanoacrylate adhesive, and GN remaining on the skin surface removed by gentle wiping with a dry cotton bud. The cotton bud wipe for each cell was extracted with acetonitrile (3 ml) and a sample (200 μl) analysed for ^{14}C by LSC. Each epidermal membrane was tape stripped 10 times using adhesive tape (D-Squame®, CuDerm Inc., Dallas, USA). Tape strips were grouped as follows: strip 1, strips 2 and 3, strips 4–6 and strips 7–10. Strips were dissolved in OptiSolve (1 ml) and a sample (200 μl) analysed for ^{14}C by LSC. Remaining samples of skin (epidermis plus any remaining stratum corneum) were dissolved in OptiSolve (2 ml) and a sample (200 μl) analysed for ^{14}C by LSC. Filter paper supports were extracted with acetonitrile (2 ml) and samples (0.5 ml) analysed for ^{14}C by LSC. Diffusion cell donor chambers were wiped using cotton buds to remove sealing grease, the grease dissolved with tetrahydrofuran (4 ml) and samples (0.5 ml) analysed for ^{14}C by LSC. Donor chambers were then washed with acetonitrile (15 ml) to recover any GN that had condensed on the donor chamber. A sample (1 ml) of each donor chamber wash was analysed for ^{14}C by LSC. During sample extraction/extraction processes, vials were tightly sealed to prevent evaporative loss of GN. Vials were vortexed (Genie 2, Scientific Industries) and shaken overnight using a Gyro Rocker (Stuart Scientific).

2.10. Assessment of evaporative loss of fragrance materials

The evaporative loss of GN under the experimental conditions was assessed in the following manner. Five ungreased diffusion cells were assembled with PTFE sheeting replacing the skin membrane. GN solution (1%) was applied at a target dose of 5 μl (mean ± SE = 5.01 ± 0.27 μl). One cell was dismantled at 1, 2, 6, 12 and 24 h, the PTFE sheet removed and washed with 10 ml, then 5 ml, acetonitrile. The donor chamber was washed with 15 ml acetonitrile. A sample of each wash solution was analyzed for ^{14}C to allow calculation of remaining radiolabel at each time-point.

2.11. Calculation of results

The amount of GN per unit area (μg/cm^2) and the percent of the applied dose of GN in the receptor phase and various compartments of the skin and diffusion cell were calculated. As per SCCNFP guidelines, the levels of GN in the epidermis, plus any remaining stratum corneum after

tape stripping, filter paper membrane support and receptor fluid were combined to produce a total absorbed dose value.

3. Results

3.1. Evaporative loss

The five PTFE cells dosed with the 1% GN solution were dismantled singly at 1, 2, 6, 12 and 24 h. The average GN dose was 50.3 ± 2.7 μg and the combined recovery from the PTFE membrane and donor chamber is shown in Fig. 1. At 1 and 2 h, only 35.7% and 10.4%, respectively, of the applied GN was recovered and remaining material then gradually reduced with a total of 7.1% applied dose recovered at 24 h. Although this method of measurement of evaporation would have overestimated loss from skin membranes, where evaporation was competing with skin absorption, it clearly identified evaporative loss as a major reason for the low recovery of applied GN in the skin absorption assessment.

3.2. Validation of skin integrity at time zero

In pre-use evaluation no skin membrane exhibited water permeability greater than 2.5×10^{-3} cm/h, indicating that the properties of all samples of skin tissue were within the normal range (Bronaugh et al., 1986).

3.3. Skin permeation of GN

The average GN dose was 50.7 ± 0.9 μg/cm^2 for the twelve active cells. No radioactivity contamination was found in any control cell sample. Overall recoveries of applied GN were low (14.1 ± 0.4%) due to the rapid loss by evaporation.

Permeation of GN is shown in Fig. 2. At 24 h, 1.89 ± 0.15 μg/cm^2 GN had permeated. Following normalisation for the exact dose applied to each cell, the 24 h permeation values corresponded to 3.74 ± 0.30% applied dose. Following rapid initial permeation through the skin (flux ~0.48 μg/cm^2 h), the rate decreased due to donor depletion (following evaporation of superficial GN).

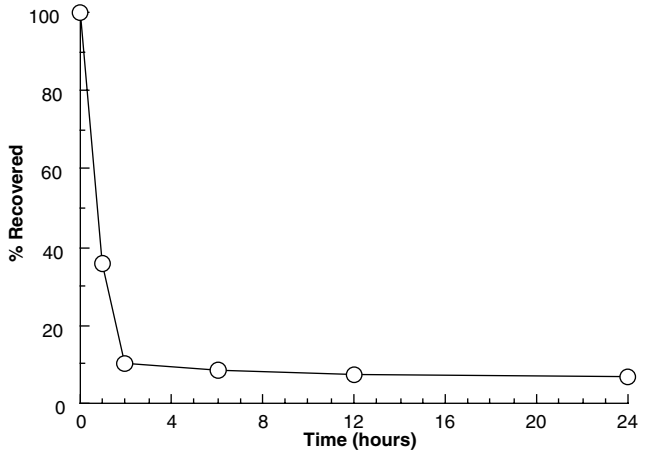

Fig. 1. Overall recovery (% applied dose) of geranyl nitrile from PTFE surfaces and donor chambers.

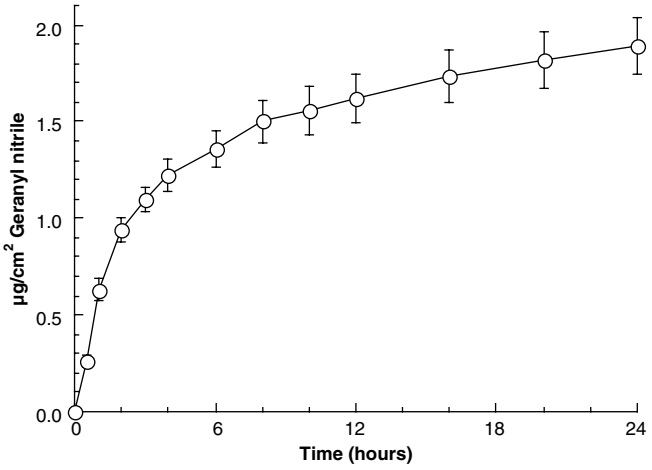

Fig. 2. Permeation (μg/cm^2, mean ± SE) of geranyl nitrile through human epidermal membranes from a 1% (w/v) solution in ethanol/water.

Distribution within the skin at 24 h was determined by measuring the levels of GN within the stratum corneum (tape strips) and the epidermis plus any remaining lower stratum corneum (following tape stripping). The skin was wiped prior to tape stripping to remove any GN remaining at the surface. Additionally, any GN in the filter paper epidermal membrane support, donor chamber sealing grease, and the cell donor chamber surfaces (due to evaporation from the skin surface, followed by condensation on the cooler, upper part of the diffusion cell) was quantified. Levels in the donor chamber wipe and wash were combined to produce donor chamber values. Recovery of GN from the surface wipes was relatively low (due to evaporation), with 6.23 ± 0.16% of the applied dose recovered. Total recoveries of GN in the tape strips (10 strips) and epidermis were 1.33 ± 0.22 and 0.416 ± 0.050% of the applied dose, respectively. The individual tape strip group data are shown, together with all other distribution and permeation data at 24 h, in Table 1.

Average amount of GN per tape strip data (μg/cm^2 per strip, Fig. 3) were generated by dividing the amount of GN (μg/cm^2) in each tape strip group by the number of tape

Table 1
Geranyl nitrile distribution data (mean ± SE) at 24 h

Compartment	GN (μg/cm^2)	GN (% applied dose)
Wipe	3.16 ± 0.10	6.23 ± 0.16
Donor chamber	0.935 ± 0.112	1.84 ± 0.22
Strip 1	0.222 ± 0.028	0.440 ± 0.056
Strips 2 and 3	0.248 ± 0.030	0.487 ± 0.058
Strips 4–6	0.140 ± 0.025	0.274 ± 0.048
Strips 7–10	0.064 ± 0.012	0.124 ± 0.022
Epidermis	0.210 ± 0.025	0.416 ± 0.050
Filter paper	0.285 ± 0.022	0.560 ± 0.040
Permeated	1.90 ± 0.15	3.74 ± 0.30
Overall recovery	7.15 ± 0.25	14.1 ± 0.4

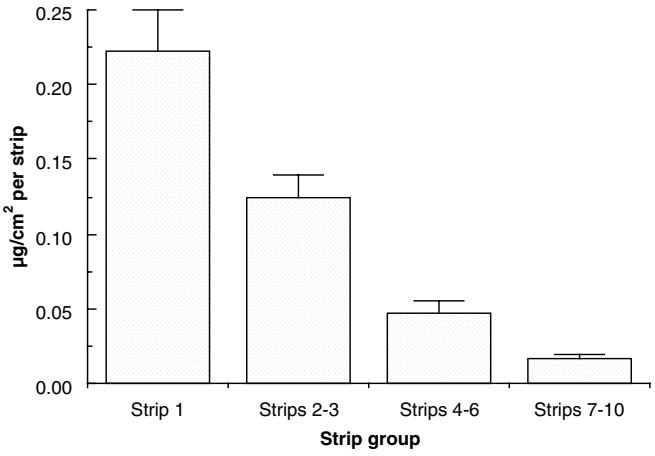

Fig. 3. Distribution (µg/cm^2 per strip, mean ± SE, $n = 12$) of geranyl nitrile within each tape strip group following normalisation for the number of strips.

strips in that group. These data showed that the amount of fragrance material recovered clearly decreased with skin depth. Although the contents of the first tape strip group could be regarded substantially as surface material, the general shape of this profile was a reflection of the concentration gradient of GN within the epidermal membrane.

The overall skin absorption of GN, defined by the SCCNFP as amounts that have permeated and amounts in the epidermis (therefore excluding tape strips), was 2.38 ± 0.16 µg/cm^2, or 4.72 ± 0.32% applied dose. Amounts in the stratum corneum (tape strips) at 24 h are not considered absorbed and contributing to the systemic dose.

3.4. Reference permeant data

Permeation of the reference permeant (BA) used in a recent multi-centre comparison study (van de Sandt et al., 2004) was assessed. pH 7.4 phosphate buffered saline (PBS) and epidermal membranes were used to match the GN dosed cells. The BA permeation data (32.4 µg/cm^2 h) fell within the range of measurements reported in the multi-centre study (2.87–38.20 µg/cm^2 h). Data from the multi-centre study showed highest absorption rates (30–32 µg/cm^2 h) from laboratories using the thinnest (0.3–0.5 mm) dermatomed membranes and the epidermal membranes used in this study provided very similar barrier properties. The reference permeant data confirmed the validity of the test systems.

4. Discussion

In the study reported here, the absorption of GN through human skin was low. The levels of GN in the epidermis (plus any remaining in the stratum corneum after tape stripping), filter paper membrane support and receptor fluid were combined (as per SCCNFP guidelines) to produce a total absorbed dose value of 4.72 ± 0.32%. Overall recoveries were 14.1 ± 0.4%, due to the rapid evaporation of superficial GN. For use in perfumery, the maximum skin level of GN in formulae that go into fine fragrances has been reported to be 0.24% (IFRA, 1997), assuming use of the fragrance oil at levels up to 20% in the final product. The 97.5%ile use level in formulae for use in cosmetics in general has been reported to be 0.7% (IFRA, 1997), which would result in a maximum daily exposure on the skin of 0.02 mg/kg for high end users of these products. The present study shows that when used as a fragrance ingredient under unoccluded conditions there would be little systemic exposure to GN.

Most nitriles historically used in perfumery have a low acute toxicity. There are a few exceptions, such as benzyl cyanide, which has a relatively high acute toxicity (RIFM, 1977). The toxicity of benzyl cyanide results from its propensity to undergo rapid metabolic breakdown to release a cyanide ion (Potter et al., 2001a,b; Guest et al., 1982). Due to the release of cyanide, benzyl cyanide is no longer used as a fragrance ingredient (IFRA, 2004). GN has been shown to have a low order of toxicity when administered by the oral or dermal routes. The oral LD50 in rats has been reported to be 4.15 g/kg (RIFM, 1974a). Dermal studies in rabbits have reported values of greater than 5.0 g/kg and 4.3 g/kg (RIFM, 1974b). Acute toxicity of geranyl nitrile is also low by the inhalation route, with a 4-h LC_{50} greater than 5.2 mg/L when administered to the rat (RIFM, 1989). A dermal biomarker study in rats indicated that only minute amounts of cyanide were released from GN following dermal application, as indicated by excretion of urinary thiocyanate (Potter et al., 2001a). Two reasons proposed for this were metabolism by alternative pathways that do not involve the significant release of cyanide and/or low dermal absorption. Kemper et al. (2005) investigated the in vitro metabolic clearance and biotransformation of GN in mice, rat and human primary hepatocytes. In each species, GN was observed to be rapidly metabolized with a $T_{1/2}$ (min) of 0.71 ± 0.13 in mice, 3.20 ± 0.22 in rats and 9.42 ± 6.72 in humans. The biotransformation pathways identified for GN were found to be similar across rodents and humans. The major pathways for phase I metabolism were found to involve epoxidation of the C6 double bond and oxidation of the terminal methyl groups leading to the corresponding acids. In all species, oxidation of the interior carbons occurred but to a lesser extent than the terminal carbons. Major routes of phase II metabolism included glucuronidation of the 8/9-hydroxylated species and conjugation of the epoxide moiety with glutathione. In humans, formation of an acid glucuronide was observed. The rapid metabolism following pathways that do not release inorganic cyanide and the low level of dermal absorption both contribute to the low order of acute dermal toxicity of GN.

In conclusion, the absorption of GN through human skin under unoccluded conditions is low. Systemic absorption of GN including the epidermis as a sink was observed to be 4.72 ± 0.32% of the applied dose. Systemic exposure resulting from the use of GN as a fragrance ingredient would be expected to be low based on the results of this study and the currently reported use levels. However,

further work is necessary to determine the effects of exposures under fully occlusive to semi-occlusive conditions.

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