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Absorption and Effects of Patch Test Preparations of Hydroperoxides of Limonene and Hydroperoxides of Linalool in Human Ex Vivo Skin

Aoife Clancy[1] | Lina Hagvall[2,3] | Per Malmberg[4] | Niamh M. O'Boyle[1]

[1]School of Pharmacy and Pharmaceutical Sciences, Panoz Institute, Trinity College Dublin, Dublin, Ireland | [2]Department of Dermatology and Venereology, Institute of Clinical Sciences, Sahlgrenska Academy, University of Gothenburg, Gothenburg, Sweden | [3]Department of Occupational and Environmental Medicine, Lund University, Lund, Sweden | [4]Department of Chemistry and Chemical Engineering, Chalmers University of Technology, Gothenburg, Sweden

Correspondence: Niamh M. O'Boyle (nioboyle@tcd.ie)

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ABSTRACT

Background: Limonene and linalool are fragrance terpenes that undergo autoxidation to sensitizing hydroperoxides. This study assessed the penetration of limonene hydroperoxide and linalool hydroperoxide from patch test preparations in ex vivo human skin samples, alongside analysing the effects of the patch test preparations on the composition of skin lipids, using Time-of-Flight Secondary Ion Mass Spectrometry (ToF-SIMS).

Methods: NativeSkin Access human skin biopsies were treated with commercially available patch test preparations 'hydroperoxides of linalool (1.0%)' or 'hydroperoxides of limonene (0.3%)'. ToF-SIMS analysis was performed to assess skin penetration of limonene hydroperoxide and linalool hydroperoxide and assess changes in endogenous lipids.

Results: ToF-SIMS analysis showed that limonene hydroperoxide and linalool hydroperoxide remain mainly confined to the stratum corneum. Multivariate analysis indicated that the chemical composition of the stratum corneum differed in the patch test exposed skin in comparison to control samples. There were significant changes in the intensity of several lipid species upon exposure to the patch test exposed skin.

Conclusion: Absorption of limonene hydroperoxide and linalool hydroperoxide occurs from patch test preparations. Skin exposure to these preparations can alter the skin lipidome.

1 | Introduction

Allergic contact dermatitis (ACD) is classified as a delayed type IVa hypersensitivity reaction, which is dependent on the activation of allergen-specific T cells and can lead to symptoms including redness, itching and blistering of the skin [1]. ACD arises from exposure to low molecular weight sensitising chemicals, known as contact allergens, like metals, preservatives and fragrances [2].

The International Fragrance Agency (IFRA) defines a fragrance as any substance used for its odour properties or malodour coverage [3] The European Commission reports that 1%–3% of the general population are affected by fragrance allergy [4]. Limonene and linalool are two fragrance terpenes. Limonene is the main compound in the essential oils of citrus fruits [5] while linalool is produced by *Lamiaceae* (mints) and *Lauraceae* (laurels, cinnamon, rosewood) plants [6]. They are added to many common household items and cosmetic products [7]. A study that used a novel smartphone application identified that 49.5% of tested products contained linalool and 48.5% of tested products contained limonene [8]. Limonene and linalool are pre-haptens and are classed as occasional contact allergens. Upon exposure to air, they undergo oxidation

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to their respective hydroperoxides, their primary oxidation products, which are significantly more allergenic [9]. The local lymph node assay (LLNA) categorises limonene hydroperoxides (LimOOH) and linalool hydroperoxides (LinOOH) as strong and moderate skin sensitisers, respectively, and they are the most potent sensitisers among the autoxidation products of the parent terpenes [10].

The potential involvement of lipids in the pathophysiology of ACD has become an area of increasing interest. Lipids are one of the fundamental components of skin. The outermost layer of the skin, the stratum corneum (SC), is composed of ceramides, cholesterol, cholesterol esters, and non-esterified fatty acids. In the lower layers of the epidermis, phospholipids are present in high abundance (up to 70%) [11]. The lipid composition of skin has been shown to be altered in many skin diseases. In psoriasis, for example, a decrease in ceramide content on the surface of the skin was observed, as well as a decrease in fatty acid content [12]. In atopic dermatitis, there was a reduction in the level of total skin lipids, in particular a decrease in total ceramide levels [13]. Little is known regarding skin lipid changes in ACD other than increases in the metabolites of some fatty acids and a reduction in ceramide levels [14]. Recently, the effects of limonene-2-hydroperoxide and linalool-6/7-hydroperoxides on the lipidome of a keratinocyte cell line using LC–MS and MS/MS techniques were determined, showing upregulation in several phospholipids, sphingolipids and triacylglycerols, and decreases in some polyunsaturated fatty acid-containing phospholipids (PE and PC) [15].

In previous studies, imaging mass spectrometry techniques were used to investigate the effect of contact allergens on skin. Matrix-assisted laser desorption/ionisation mass spectrometry imaging (MALDI MSI) recently showed that nickel ions were confined to the SC and had effects on several lipid species including phosphatidylcholine, sphingomyelin and ceramides in ex vivo porcine skin [16]. Time-of-flight secondary ion mass spectrometry (ToF-SIMS) is an imaging mass spectrometry technique in which a pulsed, energetic primary ion beam bombards the surface of a sample and induces a collision cascade, liberating secondary ions that are then sent to a time-of-flight (ToF) analyser for detection [17]. ToF-SIMS is a powerful imaging mass spectrometry technique for cell and tissue analysis [18]. ToF-SIMS has previously been used to visualise the penetration of metal allergens nickel, cobalt and chromium through the skin [19], as well as the allergenic preservative methylisothiazolinone (MI) [20]. In addition to localisation and tracking of ions, ToF-SIMS is well suited for the detection and analysis of lipid species in biological species. This is due to the instrument's high lateral resolution and sensitivity [21]. In this study, ToF-SIMS was used firstly to analyse the penetration of linalool hydroperoxides and limonene hydroperoxides from commercially available patch test preparations containing hydroperoxides of linalool 1.0% and hydroperoxides of limonene 0.3% into NativeSkin Access human ex vivo skin, and secondly to determine lipid profiles in the SC and viable epidermis (VE). Use of viable human full-thickness ex vivo skin samples together with patch test fragrance preparations aimed to simulate patient exposure to the allergens during patch testing and mimic the in vivo physiological interaction between allergens and skin.

2 | Methods

2.1 | Fragrance Allergens

Hydroperoxides of linalool 1.0% (Chemotechnique, H-031A, lot 21293A-5a) and hydroperoxides of limonene 0.3% (Chemotechnique, H-032A, lot 21441-5a) were obtained as a gift from the Department of Occupational and Environmental Dermatology, Sahlgrenska University Hospital, Gothenburg. These are commercially available products used for patch testing containing autoxidised linalool and limonene. It is important to note that the patch test preparations (H-031A and H-032A) contain oxidised linalool and oxidised limonene, respectively. The products contain the stated amount of linalool or limonene hydroperoxides, alongside other oxidation products. References to 'hydroperoxides of linalool 1.0%' or 'hydroperoxides of limonene 0.3%' throughout the manuscript refer to the commercially available patch test preparations H-031A and H-032A. The concentrations used in this study are the standard used for patch testing and were recommended for inclusion in the European Baseline Series by the European Society of Contact Dermatitis (ESCD) [22, 23].

2.2 | Ex Vivo Skin Tissue Exposure

NativeSkin Access (ex vivo skin samples of 11 mm diameter) (Genoskin, France) was cultured in sterile conditions as recommended by the manufacturers. Briefly, samples were incubated in the provided culture medium for at least 1 h at 37°C, 5% CO_2, and 95% relative humidity prior to treatment with patch test preparations. Hydroperoxides of limonene 0.3% wt/wt (10 mg) or hydroperoxides of linalool 1.0% wt/wt (10 mg) in Vaseline white Ph.Eur were added to 8 mm Finn Chambers on Scanpor, then applied to NativeSkin Access for 24 h, ensuring that the patch test mixture was in contact with the skin ($n = 2$). Vaseline white Ph.Eur was applied as the control ($n = 2$). The experiment was performed with skin samples from two different donors. Both donors were female, aged 42 years, with skin type 2 according to Fitzpatrick's classification guidelines [24]. The skin sample from both donors was taken from the abdominal area. Donor 1 had an allergy to latex and was taking omeprazole, triptan and Nurofen medication. Donor 2 had no allergies and was not on any medication. The Trinity College Dublin Faculty of Health Sciences Ethics Committee approved the study (ref: 220401).

2.3 | Sample Preparation and TOF-SIMS Analysis

The skin samples were sectioned vertically to a thickness of 10 μm using a cryostat (Leica CM1520, Nussloch, Germany). Two slices from the middle of the sample were collected, mounted on glass slides, freeze-dried, and kept at −20°C until ToF-SIMS analysis. The ToF-SIMS surface analysis was carried out using a ToF-SIMS 5 instrument (ION-ToF GmbH, Münster, Germany) equipped with a 30 keV bismuth nanoprobe and an argon gas cluster ion source (GCIB). Sputtering with the Ar-GCIB was performed using Ar1500 at 5000 eV with an ion current of 4.95 nA. Mass spectra in positive ion mode were acquired by using the bismuth primary ion sources. To acquire images with high

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spatial respective mass resolution, the delayed extraction mode was used [25] with an achieved mass resolution of at least M/ΔM = 4500 fwhm at *m/z* 500. The pulsed primary ion currents were in the range of 0.11 to 0.17 pA. Areas of ~200 × 200 μm on the skin section were selected with a raster of 256 × 256-pixels and analysed with 100 raster scans. Three different sections from the specimens of control and treated samples were analysed, respectively to determine the skin distribution of the specific compounds linalool hydroperoxide (LinOOH) and limonene hydroperoxide (LimOOH), which are components of the products H-031A and H-032A described above. The spectra were internally calibrated to signals of common fragments $[C]^+$, $[CH_2]^+$, $[CH_3]^+$, $[C_5H_{15}PNO_4]^+$, and $[C_{27}H_{45}]^+$. SurfaceLab (version 7.3, ION-TOF, Germany) was used to process, record, analyse and evaluate images and mass spectra.

2.4 | Data Analysis and Statistics

An automated peak selection was used to select *m/z* values of both the control and fragrance allergen-treated skin in SURFACELAB 7.1 software. Regions of skin (SC and VE) were analysed both together and separately using the software by use of appropriate SC (cholesterol ion *m/z* 369.3) and VE markers (PC headgroup ion *m/z* 86.1). The same mass range was selected (*m/z* 50 to 800) for the SC and the VE. Data were imported to SIMCA software (version 16.0, Umetrics, MKS Instruments Ltd.) as ion intensities normalised to the total ion count. Pareto scaling was performed, and analysis was carried out by principal component analysis (PCA).

3 | Results

3.1 | Identification of Limonene Hydroperoxide and Linalool Hydroperoxide Ions in Skin Using ToF-SIMS

Limonene hydroperoxide (LimOOH) and linalool hydroperoxide (LinOOH) ions produced using ToF-SIMS were first identified, allowing for the subsequent tracking of the fragrance allergens through the skin samples. Previous work using LC/ESI-MS/MS of LimOOH identified the most abundant fragment ions as the molecular ion peak at *m/z* 169.2, the $[M+H-H_2O]^+$ peak at *m/z* 151.2, and the $[M+H-H_2O-CH_2-CH_3COOH]^+$ peak at *m/z* 93.1 [26]. LC–MS analysis with ESI or APCI ionisation identified the ions at *m/z* 169.1 and 151.2 as the most abundant LimOOH fragments [26]. A study investigating the stability of LimOOH using LC–MS identified the $[M+H]^+$ peak at *m/z* 169.1 as a minor ion, and the $[M+H-H_2O]^+$ peak at *m/z* 151.1 as the most dominant peak [27]. These previously reported peaks were chosen and examined on the LimOOH spectra obtained from our ToF-SIMS analysis (Figure 1a,c). The mass spectrum peaks and distribution of ions within the skin sample were compared to the Vaseline-treated skin samples (Figure 1b,d). Differences in the mass spectra were identified in both the *m/z* 169.1 and 151.1 peaks, with both present in the hydroperoxides of limonene treated skin sample at higher intensities than in the Vaseline control treated sample. Examination of the ToF-SIMS images showed that both ions *m/z* 169.1 and 151.1 in the hydroperoxides of limonene 0.3% treated skin samples accumulated in the SC (Figure 1a,c). These differences in both the mass spectra and the ToF-SIMS images indicate that the ions at *m/z* 169.1 and 151.1 are representative of LimOOH.

A similar approach was taken to identify LinOOH ions in hydroperoxides of linalool 1.0% treated skin samples (Figure 2). LC/ESI-MS/MS of LinOOH identified the $[M+H]^+$ peak at *m/z* 187.2, the $[M+H-H_2O]^+$ peak at *m/z* 169.1 and the $[M+H-3H_2O-CH_2=C=CH_2]^+$ peak at *m/z* 93.0 [28]. The *m/z* 187.2 and 169.1 peaks were identified in the spectrum of the 'hydroperoxides of linalool 1.0%' treated skin samples (Figure 2a,c) and compared with the corresponding peaks in the spectrum for the Vaseline treated skin (Figure 2b,d). The ToF-SIMS images of the ions in both samples were also compared. There were differences in the distribution of the ions at *m/z* 187.2 and 169.1 in the patch test treated skin samples compared to those treated with Vaseline. The differences seen in distribution of the ions indicate that the ions at *m/z* 187.2 and 169.1 in the 'hydroperoxides of linalool 1.0%' treated skin samples correlate to LinOOH.

3.2 | Distribution of Limonene Hydroperoxide and Linalool Hydroperoxide in Skin

Following identification of LimOOH and LinOOH ions, the distribution of these specific allergens in the skin samples was assessed. To indicate the positions of the SC and the VE, lipid ions known to have primary residence in these areas were used. Cholesterol, at *m/z* 369.3, is found primarily in the SC [29] and phosphatidylcholine is primarily found around keratinocytes [30], and so the PC headgroup fragment at *m/z* 86.1 was used to identify the VE. The overlay images clearly identified the presence of clear skin structures (Figure 3a,d). The PC headgroup fragment at *m/z* 86.1 (blue, Figure 3) illustrates the phospholipid membranes of the keratinocytes in the VE. The circular shape and location of the ion *m/z* 124, surrounded by the *m/z* 86.1 (PC) ion, suggest that it represents a nuclear component.

Both hydroperoxides follow a similar pattern with regards to penetration through the skin; both were primarily confined to the SC with some penetration into the upper layers of the VE. Line scans of cholesterol were used to identify the SC, which was present at a depth of ca. 10–60 μm into the skin sample (Figure 3g). Line scans of PC were used to identify the VE, which was present at ca. 50–200 μm into the skin samples (Figure 3c,f). Line scans measuring ion intensity through the 200 μm depth of the skin sample confirmed where LinOOH and LimOOH were at their highest intensity (Figure 3b,e, respectively). The LinOOH ion (*m/z* 187.2) was at its highest intensity at a depth of 20–90 μm from the skin surface, which represents the SC (around 10–30 μm in thickness in normal healthy skin [31]) and the upper VE. Comparisons with the line scan for the LinOOH ion showed some overlap beginning at 60 μm into the skin sample, indicating that there is some penetration of LinOOH into the upper layers of the VE, with the intensity then decreasing further into the VE. Similarly, the LimOOH ion (*m/z* 151.1) was at the highest intensity between 30 and 50 μm into the skin sample, indicating the localisation of LimOOH primarily in the SC (Figure 3e). The intensity of the LimOOH ion decreases between 50 and 80 μM into the skin samples. The VE of the sample begins at 50 μm into the sample as indicated by the line scan for the

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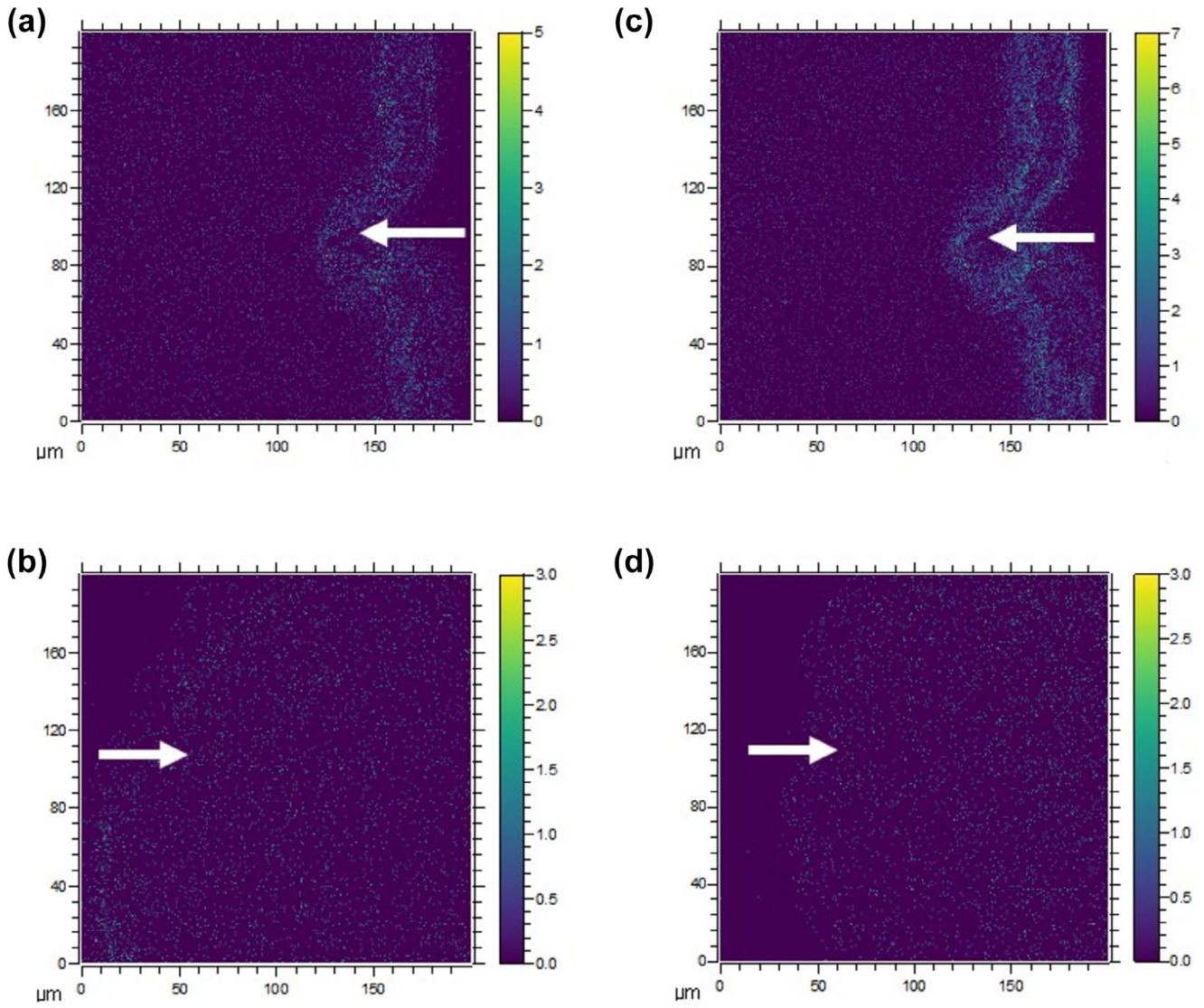

FIGURE 1 | ToF-SIMS data of the epidermis of human ex vivo skin cross-section (200 × 200 µm). $C_{10}H_{17}O_2^+$ $(M+H)^+$ ion at *m/z* 169.1 in skin treated with (a) hydroperoxides of limonene (0.3%) patch test preparation and (b) Vaseline treated skin for 24 h. $C_{10}H_{15}O^+$ $(M+H-H_2O)$ ion at *m/z* 151.1 in (c) skin treated with hydroperoxides of limonene (0.3%) and (d) Vaseline treated skin for 24 h. Images are representative of experiments carried out with $n=2$ donors, with four replicates in each hydroperoxides of limonene treated sample and three replicates in the Vaseline treated samples. Arrows indicate direction of penetration.

PC headgroup ion (Figure 3f), which indicates that LimOOH was penetrating the upper layers of the VE. The images of the hydroperoxide ions in the skin, coupled with the line scans, confirmed that, upon application to the surface of the skin in a patch test preparation, LinOOH and LimOOH remain primarily in the SC, with penetration into the upper layers of the VE.

3.3 | Skin Lipid Changes Induced by Hydroperoxides of Limonene and Hydroperoxides of Linalool

Lipid composition varies significantly throughout different layers of the skin, and thus to investigate the effect of hydroperoxides on the skin lipid composition, the SC and VE were analysed separately. To firstly identify whether there was any variability in the chemical composition of the SC of skin treated with 'hydroperoxides of linalool (1.0%)' patch test preparation, 'hydroperoxides of limonene (0.3%)' patch test preparation, and Vaseline, partial least squares discriminant analysis (PLS-DA) was carried out (Figure 4). Unsurprisingly, given the penetration depth of the hydroperoxides discussed above, more prominent lipid changes were seen in the SC rather than the VE, using principal component analysis (PCA). Skin samples analysed by PLS-DA showed clusters based on treatment, with the 'hydroperoxides of limonene 0.3%' treated samples grouped away from the 'hydroperoxides of linalool 1.0%' treated samples, and both sets of fragrance-treated samples grouped away from the Vaseline control-treated samples (Figure 4). The clustering of samples away from each other, without any overlap, indicates that there were differences in the chemical composition of the SC. PLS-DA has been previously used to analyse the effects of metal allergens on skin chemical composition, which indicated differences in the composition of the SC upon treatment with the

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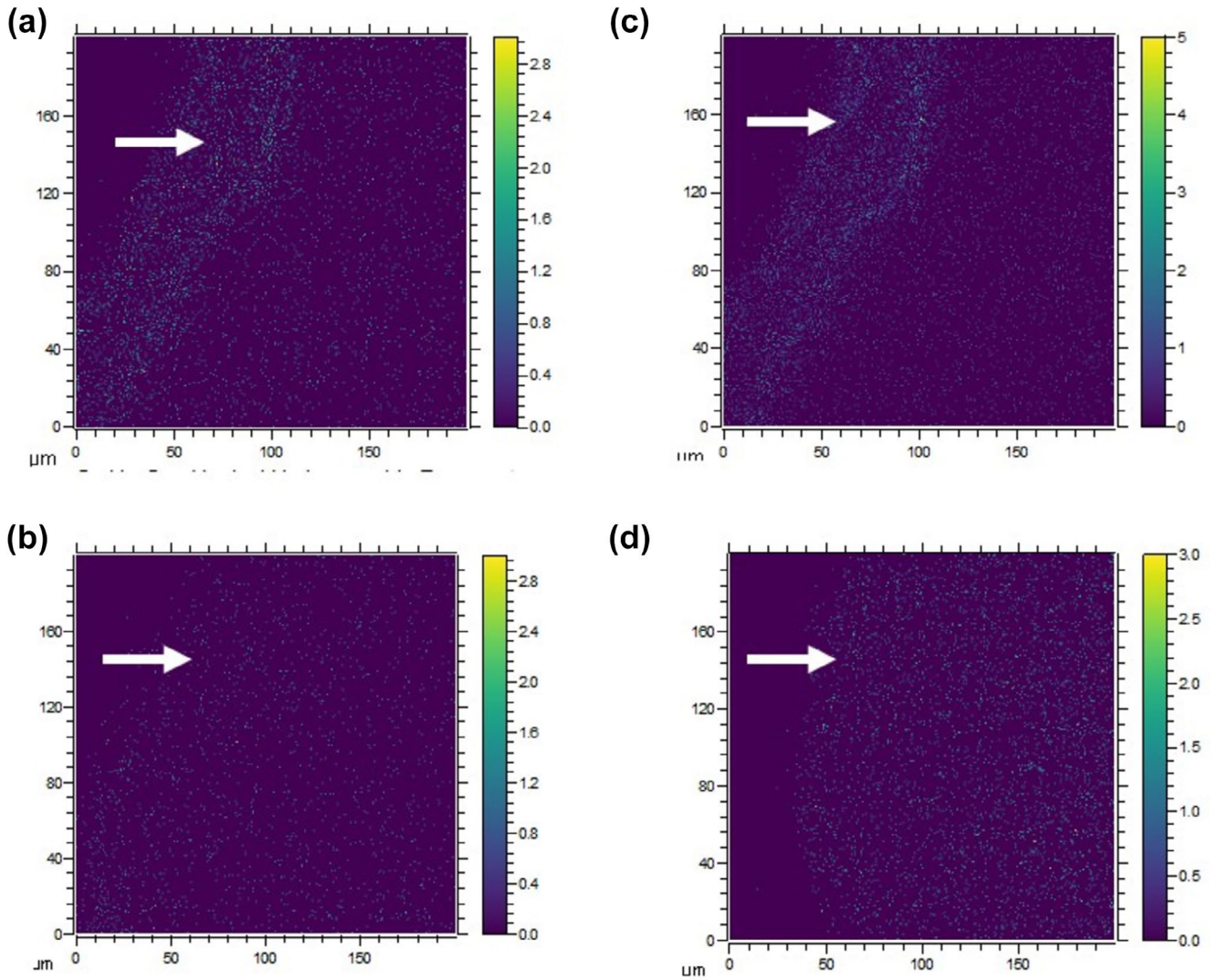

FIGURE 2 | ToF-SIMS data of the epidermal region of human ex vivo skin cross-section (200 × 200 µm). $C_{10}H_{19}O_3^+$ $(M+H)^+$ ion at *m/z* 187.2 in skin treated with (a) hydroperoxides of linalool (1.0%) patch test preparation and (b) Vaseline treated skin for 24 h; $C_{10}H_{17}O_2^+$ $(M+H-H_2O)^+$ ion at *m/z* 169.1 in (c) skin treated with hydroperoxides of linalool (1.0%) patch test preparation and (d) Vaseline treated skin for 24 h. Images are representative of experiments carried out with $n = 2$ donors, with four replicates in each hydroperoxides of linalool treated sample and three replicates in the Vaseline treated samples. Arrows indicate direction of penetration.

metal allergens nickel, cobalt, and chromium [32]. Our analysis indicated that lipid changes occurred in the SC upon exposure to patch test preparations of hydroperoxides of limonene and linalool. The same analysis did not show clustering by treatment when the VE was analysed, indicating that the lipids of the VE were unaffected by treatment with these patch test preparations (Figure S1).

A PCA scores plot also identified differences between the chemical composition of the SC of the Vaseline-treated skin samples (green) and hydroperoxides of linalool 1.0% treated (purple) skin samples (Figure S2A). Component 2 (t [2]) successfully separated the treated samples from the controls (Figure S2A). PCA analysis was also carried out to identify any variation in the composition of Vaseline-exposed skin and 'hydroperoxides of limonene 0.3%' patch test treated skin. As with treatment with 'hydroperoxides of linalool 1.0%', more prominent lipid changes were observed in the SC in comparison to the VE. A scores plot differentiated between the Vaseline-treated samples (blue), and the hydroperoxides of limonene 0.3% treated samples (green) (Figure S2B). Component 2 separated the samples, and the clustering of the blue samples away from the green indicates variability in the chemical composition of the SC of the control and 'hydroperoxides of limonene 1.0%' patch test-treated samples.

A PCA loadings plot was generated for each treatment condition and the Vaseline controls which indicated the *m/z* values at which there was the greatest variability within the samples (Figure S3). The analysis indicated variability between the 'hydroperoxides of linalool 1.0%' treated skin samples and control skin samples at *m/z* 603.5 and 577.4 which represented two diacylglycerol (DAG) ions, as well as in the PC headgroup fragment at *m/z* 86.1 and a cholesterol fragment at *m/z* 369.3. In the 'hydroperoxides of limonene 0.3%' treated samples, variability was also detected for ions at *m/z* 603.5, 577.4, 369.3 and 86.1.

To investigate the lipid changes in more detail, a selection of ion peaks corresponding to specific lipid species were selected and

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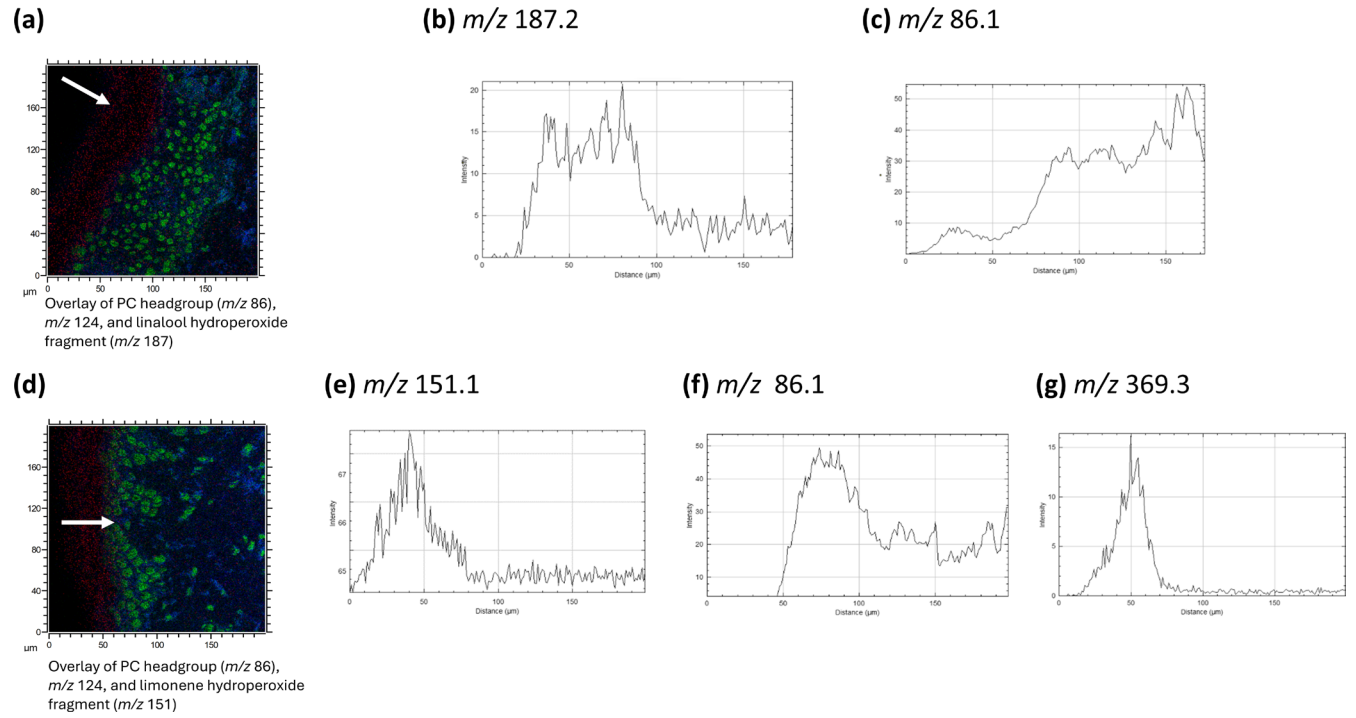

FIGURE 3 | Overlay Images and Depth Profiles of LimOOH and LinOOH treated skin samples (a) ToF-SIMS overlay images (200 × 200 μm) of ex vivo skin samples treated with hydroperoxides of linalool 1.0% patch test preparation. Red = LinOOH ion at *m/z* 187.2, green = unknown nuclear component at *m/z* 124, blue = phosphatidylcholine (PC) headgroup at *m/z* 86.1. White arrow indicates direction of penetration; (b) Line scan of LinOOH ion at *m/z* 187.2 showing intensity through the skin sample; (c) Line scan of PC headgroup ion at *m/z* 86.1 in ex vivo skin sample treated with 'hydroperoxides of linalool 1.0%' patch test preparationl; (d) ToF-SIMS overlay images (200 × 200 μm) of ex vivo skin samples treated with 'hydroperoxides of limonene 0.3%' patch test preparation. Red = LimOOH ion at *m/z* 151.1, green = unknown nuclear component at *m/z* 124, blue = phosphatidylcholine (PC) headgroup at *m/z* 86.1; (**e**) Line scan of LimOOH ion at *m/z* 151.1 showing intensity through the skin sample; (f) Line scan of PC headgroup ion at *m/z* 86.1 in ex vivo skin sample treated with 'hydroperoxides of limonene 0.3%' patch test preparation; (g) Line scan of cholesterol ion at *m/z* 369.3 in ex vivo skin sample treated with 'hydroperoxides of limonene 0.3%' patch test preparation. Images are representative of *n* = 2 donors with four sections of skin analysed for each donor.

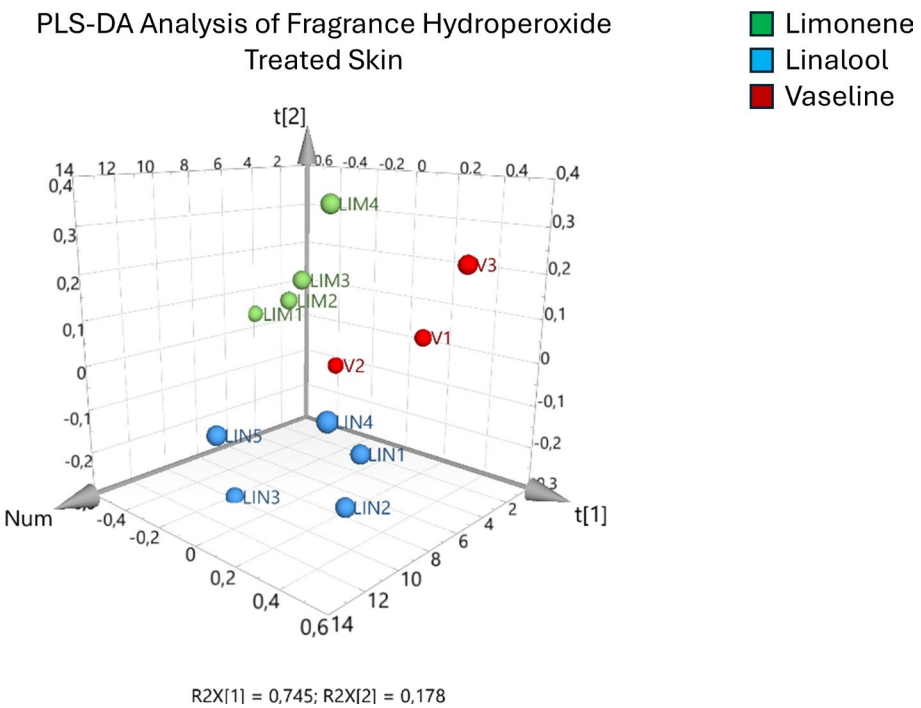

FIGURE 4 | Partial least squares discriminant analysis (PLS-DA) of SC composition of Vaseline (red), hydroperoxides of linalool 1.0% patch test preparation (blue) and hydroperoxides of limonene 0.3% patch test preparation (green) treated ex vivo human skin. Data were extracted and analysed as normalised intensities to total ion count. Range from *m/z* 50 to 605 analysed.

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analysed. The ions were selected based on indicated differences in the PCA loadings plot and additional ions of interest. The lipid species selected included PC headgroup (*m/z* 86.1 and 184.1), cholesterol (*m/z* 369.3), monoacylglycerols (MAG) (*m/z* 311.3, 313.3 and 339.3) and DAG (*m/z* 575.5, 577.4, 601.5 and 603.5). The SC for skin samples from two different donors was analysed (Figure 5).

An increase in the intensity level of the cholesterol fragment (*m/z* 369.3) was noted for both donor samples exposed to both patch test preparations, with a more pronounced effect for 'hydroperoxides of linalool 1.0%' treated skin in Donor 2 (Figure 5A). The baseline cholesterol levels in Vaseline-exposed skin were notably higher in Donor 2 than Donor 1. The most pronounced effects were on MAG and DAG for both donors, particularly

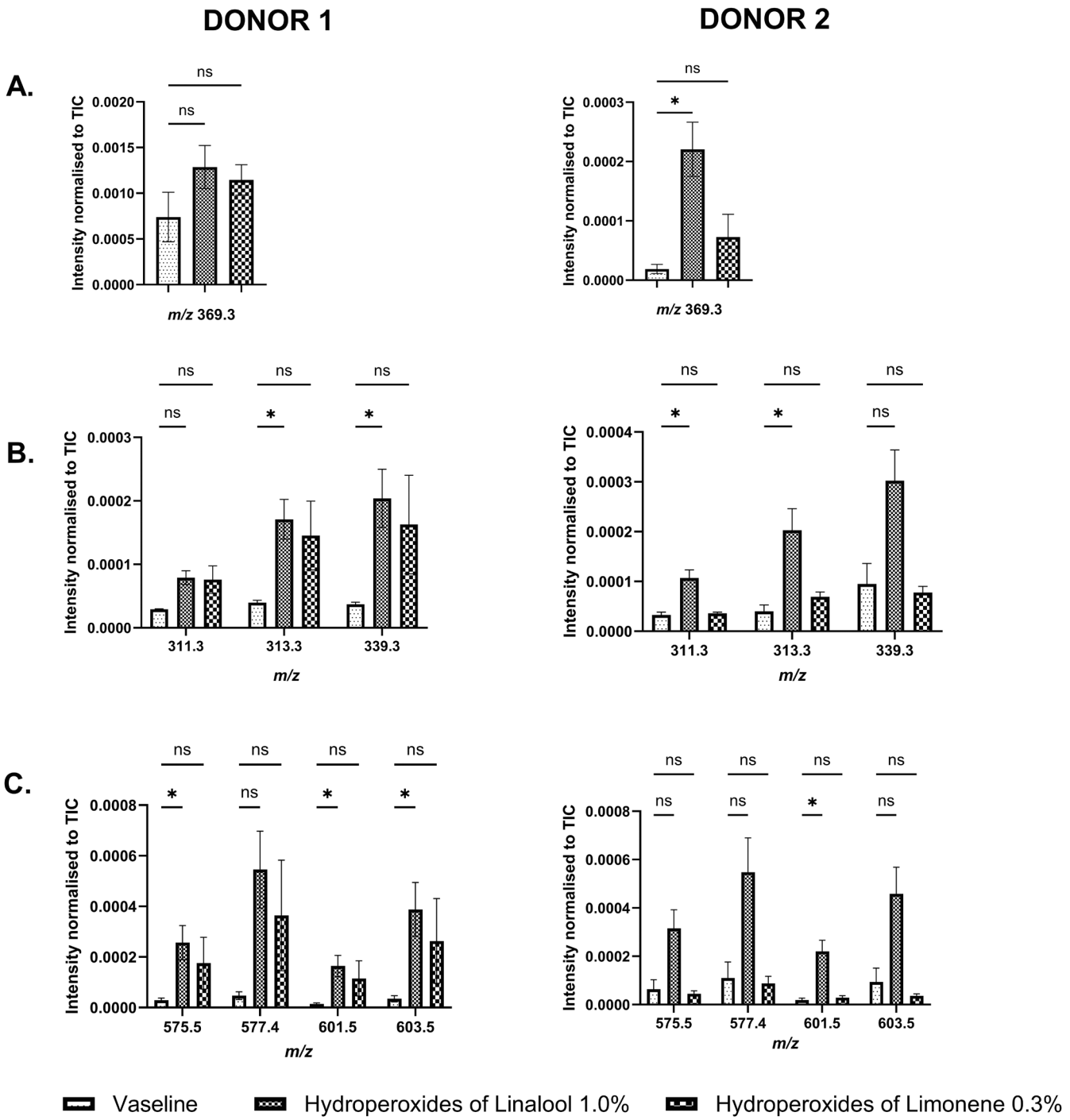

FIGURE 5 | Stratum corneum (SC) lipid composition of ex vivo skin from two donors treated with 'hydroperoxides of linalool 1.0%' patch test preparation, 'hydroperoxides of limonene 0.3%' patch test preparation and Vaseline (control). (A) Cholesterol (*m/z* 369.3); (B) monoacylglycerols (MAG; *m/z* 311.3, 313.3 and 339.3) and (C) diacylglycerols (DAG; *m/z* 575.5, 577.4, 601.5 and 603.5). (A) Cholesterol data were analysed by repeated measures one-way ANOVA with Tukey post hoc test. * = $p < 0.05$; (B and C). Data were analysed by repeated measures (RM) two-way ANOVA with Tukey post hoc test for comparison of means.

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Donor 1 for both patch test preparations, and Donor 2 for 'hydroperoxides of linalool 1.0%' exposed skin (Figure 5B,C). In Donor 1, exposure to the patch test preparation 'hydroperoxides of linalool 1.0%' induced a significant increase in MAG levels (*m/z* 313.3 and 339.3). While no significance was observed, there was an increasing trend in DAG levels in these samples, in comparison to control treated samples. There were significant increases in MAG (*m/z* 311.3 and 313.3), and DAG intensity at *m/z* 601.5. Non-significant increases in intensity were observed for other MAG and DAG ions.

The levels of phospholipids in the SC are negligible [33], so PC levels in the VE were only examined after skin exposure to 'hydroperoxides of linalool 1.0%'. In the VE, donor 2 showed a similar trend with a significant increase in intensity at *m/z* 86.1, but no significant difference was observed at *m/z* 184.1 (Figure S4). Similarly to Donor 1, in Donor 2 the 'hydroperoxides of limonene 0.3%' patch test preparation did not induce any significant changes in lipid composition of the SC. However, an increasing trend in PC intensity was observed, as well as increasing trends in the intensity of cholesterol and MAG ions in comparison to control-treated samples. A decreasing trend in DAG intensity was observed across all four *m/z* values analysed. This is in contrast with the observed increases in levels of these DAG in donor 1 upon treatment with 'hydroperoxides of limonene 0.3%', and the statistically significant increases observed in both donors after treatment with 'hydroperoxides of linalool 1.0%' patch test preparation.

4 | Discussion

LimOOH and LinOOH were both found mainly confined to the SC of ex vivo human skin following ToF-SIMS analysis. The localisation in the SC and poor penetration in the VE correlates to previous findings that documented poor skin absorption and low penetration potential of the related fragrance terpenes geraniol and citronellol [34]. An additional study investigating the penetration of terpenes from matrix-type transdermal systems concluded that terpenes including limonene have a high affinity to the SC [35]. It is also possible that LimOOH and LinOOH could bind to endogenous biomolecules, for example, proteins, in the ex vivo skin model [23, 36]. A previous study found that LimOOH can form adducts with cysteine thiols in many proteins and low molecular weight thiol-containing molecules, including *N*-acetylcysteine and glutathione, via free radical mechanisms [37]. This would produce a conjugate with a higher molecular weight that we did not search for. Additionally, fragrance hydroperoxides could be metabolised within the skin, as metabolic enzymes may still be functioning, leading to metabolites that were not detected. The site of the skin sample, from the abdomen, and interindividual variability also need to be considered as factors that contribute to absorption and skin lipid profile. Previous studies have concluded that the function and composition of the SC differs with location [38], for example, the SC is thicker in facial skin than in the limbs. The lipid composition of the SC also differs with age [39], with ToF-SIMS analysis identifying an increase in sterol cholesterol sulphate levels in the SC of elderly women in comparison to younger women.

Changes in skin lipids were observed after treatment with the patch test preparations 'hydroperoxides of linalool 1.0%' and 'hydroperoxides of limonene 0.3%'. The upregulation of DAG and MAG levels, and decrease in PC levels observed in 'hydroperoxides of linalool 1.0%' treated skin in comparison to control treated skin are consistent with previously reported findings, which used a lipidomic approach to investigate the changes in the keratinocyte lipidome upon treatment with fragrance allergens including linalool-6/7-hydroperoxide [15]. Decreases in phospholipid intensity indicate degradation, potentially through hydroperoxide-induced oxidative stress. Triacylglycerols (TAG) are crucial for the maintenance of the skin permeability barrier function [40], so increases in MAG and DAG intensity could be a response to fragrance hydroperoxide-induced SC disruption, and an attempt to restore skin homeostasis. Notably in ToF-SIMS, DAG ions may represent TAG, as TAG is effectively fragmented in ToF-SIMS yielding mainly DAG ions [41]. Exposure to the 'hydroperoxides of limonene 0.3%' patch test preparation did not induce any significant differences in lipid composition of the SC, but there were decreasing trends in PC intensity seen at both *m/z* values, and an increasing trend in the intensity of cholesterol, MAG and DAG in comparison to the control (Vaseline) treated skin samples. Increases in DAG were previously noted in the SC in chromium-treated human skin samples (from breast tissue that had been previously frozen), while MAG were increased in the SC of both chromium- and cobalt-treated skin [32].

Lipid alterations upon the application of patch test preparations of oxidised fragrance terpene mixtures indicate disruptions to the skin barrier, particularly within the SC. Although much of the LinOOH and LimOOH from the patch test preparations remained in the SC, as indicated by the depth profile analysis, the two hydroperoxides also penetrated the VE in this skin model. The role of lipid SC disruption in the pathophysiology of ACD remains to be determined. SC integrity has been linked to the immune response in other skin diseases such as atopic dermatitis, in which an increase in Th2 inflammation and the secretion of IL-4 affects the integrity of the SC barrier [42]. In psoriasis, the compromise of the SC leads to activation of innate immune responses which include the release of cytokines including TNF-α and IL-1 [43]. It has been hypothesised that skin barrier damage may promote activation of innate immunity signals crucial for the development of sensitisation [44]. The role of SC and VE lipid disruption in this process requires further research.

4.1 | Conclusions

ToF-SIMS analysis showed that LimOOH and LinOOH penetrate through the skin and are mainly confined to the SC, with lower levels of penetration into the VE. Through analysis of differing lipid species in the SC, it was shown that application of a commercially available patch test preparation 'hydroperoxides of linalool 1.0%' had a significant effect on the levels of cholesterol and DAG in ex vivo human skin. Decreases in the intensities of cholesterol and DAG ions were observed in both donor samples; however, a varying change in PC was observed. PCA analysis showed differences in the lipid composition of skin which was exposed to the patch test preparation

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'hydroperoxides of limonene 0.1%'; however, changes to individual lipids were shown to be non-significant compared to skin exposed to Vaseline. Oxidised fragrance terpenes can penetrate through the skin and influence skin lipid composition, which could have further implications on the pathophysiology of fragrance ACD.

Author Contributions

Lina Hagvall: investigation, resources, writing – review and editing. **Per Malmberg:** investigation, project administration, visualization, resources, writing – review and editing. **Aoife Clancy:** investigation, methodology, visualization, writing – review and editing, writing – original draft. **Niamh M. O'Boyle:** writing – review and editing, conceptualization, funding acquisition, investigation, supervision, resources, project administration, formal analysis.

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Ethics Statement

The Trinity College Dublin Faculty of Health Sciences Ethics Committee approved the study (ref: 220401).

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section. **Figure S1:** Partial least squares discriminant analysis (PLS-DA) of viable epidermis (VE) composition of ex vivo human skin treated with Vaseline (red), 'hydroperoxides of linalool 1.0%' patch test preparation (green) and 'hydroperoxides of limonene 0.3%' patch test preparation (blue). Data were extracted and analysed as normalised intensities to total ion count for range *m/z* 50–605. **Figure S2:** (A) 2D score plot of the SC of hydroperoxides of linalool 1.0% treated ex vivo human skin (purple) and control (Vaseline treated) ex vivo human skin (green); (B) 2D score plot of the SC of hydroperoxides of limonene 0.3% treated ex vivo human skin (green) and control (Vaseline treated) ex vivo human skin (blue). Data were extracted and analysed as normalised intensities to total ion count. **Figure S3:** (a) 2D loadings plot of the SC of hydroperoxides of linalool 1.0% treated ex vivo human skin and control (Vaseline treated) ex vivo skin and (b) 2D loadings plot of the SC of hydroperoxides of limonene 0.3% treated ex vivo human skin and control (Vaseline treated) ex vivo skin. DAGs at *m/z* 577.44 and 603.46 are highlighted in blue and red respectively. Data were extracted and analysed as normalised intensities to total ion count. **Figure S4:** Stratum corneum and viable epidermis phosphatidylchloine composition of ex vivo skin from two donors treated with 'hydroperoxides of linalool 1.0%' patch test preparation, 'hydroperoxides of limonene 0.3%' patch test preparation and Vaseline. The effect of 'hydroperoxides of linalool 1.0%' patch test preparation and 'hydroperoxides of limonene 0.3%' patch test preparation treatment on phosphatidylchloine (PC) in A. stratum corneum and B. viable epidermis of the ex vivo human skin of two donors, in comparison to Vaseline treated (control) samples. Significance was assessed by a repeated measures two-way ANOVA and a Tukey's Post hoc test. * = $p < 0.05$.

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