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MALDI MSI analysis of lipid changes in living skin equivalents in response to emollient creams containing palmitoylethanolamide

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ABSTRACT

Mass spectrometry imaging (MSI) is a powerful tool for the study of intact tissue sections. The use of matrix-assisted laser desorption/ionisation (MALDI) MSI for the study of the distribution and effect of emollient treatment on sections of reconstructed living skin equivalents during their development and maturation is described. Living skin equivalent (LSE) samples were obtained at 14 days development, re-suspended in maintenance medium and incubated for 24 h after delivery. The medium was changed, the LSE treated with either Physiogel A.I.® or Oilatum Junior® emollients and then re-incubated and samples taken at 4, 6 and 24 h time points. Mass spectra and mass spectral images were recorded from 12 μm sections of the LSE taken at each time point for comparison using MALDI mass spectrometry (MS). It was possible to detect ions characteristic of each emollient in the LSE. In addition a number of lipid species previously reported as being significant in the maturation of the LSE were observable. At the 24 h time point, the images revealed what appeared to be differences in the organisation of the skin cells observed across the Physiogel A.I.® treatment group tissue sections when directly compared to the untreated tissue group.

1. Introduction

Whilst many immunohistochemistry, autoradiography and spectroscopic methods are routinely used in dermatological research, the application of MALDI MSI is still relatively new. One of the key features of MALDI MSI that makes its use appealing is the ability to detect and study the distribution of multiple compounds simultaneously in a label-free manner. Additionally by using tandem MS analysis, molecules can be identified directly on the tissue sections. In the initial proof of concept study which demonstrated MALDI MSI of biological tissue [1] the technique was introduced for the MS analysis of large biomolecules. Since then however it has been applied to the analysis of a wide range of pharmaceutical compounds in situations ranging from whole animal sections to drug eluting stents [2–4].

A fine line exists between medicinal and cosmetic skin care products. In light of the seventh amendment to the EU directive 76/768/EEC, (which proposes a ban on the testing of cosmetics ingredients and products on animals), the use of *in-vitro* skin models, often described as living skin equivalent models (LSE), has become increasingly important in toxicity testing. Such models are an attractive alternative to the use of both animals and *ex-vivo* human skin for a number of reasons; (i) they allow for the topical application and testing of products used in daily life and can overcome the ethical constraints through being utilised as a substitute to mammalian *ex-vivo* tissue and (ii) the metabolism of skin models during an experiment can be sustained over time if they nurtured in ideal conditions [5,6]. This is not always possible with excised mammalian tissue. Once mammalian tissue is removed from the host, the cells slowly lose their viability, unless metabolism is quenched immediately. One known issue with LSE is however a reduced barrier function compared to human skin.

Emollients are multifunctional formulations for which multiple claims of efficacy are often made. Examples of emollients include: skin-hydration agents, hygroscopic agents, skin permeation enhancers, skin protectants against the external environments, particle coating and suspension stabilizers and essential lipid supplements

Abbreviations: MALDI MSI, Matrix-Assisted Laser Desorption Ionisation Mass Spectrometry Imaging; PCA, Principal Component Analysis; PEA, Palmitoylethanolamide; LLP, Light liquid paraffin.

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for barrier restoration [7]. For chronic skin diseases, many of the emollient treatment constituents are directed towards restoring epidermal differentiation and barrier function via the passive and active functions that they exert. Oil-based formulations containing, *e.g.*, petrolatum, paraffin, or mineral oil (known for their passive functions) can form a water-impermeable film over the surface of skin to decrease evaporation of physiological water [8,9]. Whilst the improvements in skin barrier function arising from treatment with emollients/moisturisers are well documented [10] it is still the case that not much is known on the underlying pharmacodynamic/toxicodynamic effects of many commercially available emollient treatments. Palmitoylethanolamide (PEA) (the endogenous cannabinoid agonist) and light liquid paraffin (LLP) incorporated into emollient formulations have been shown to reduce the clinical signs and symptoms of atopic dermatitis (AD) in children and in adults [11,12]. A recent study carried out using Physiogel A.I.®, the same PEA-containing emollient studied here, supports a role of PEA in the enhancement of lipid production in the stratum granulosum. However, the mechanisms proposed for this effect are at present speculative [13].

In order to study the effects of emollients on skin an untargeted lipidomic study of skin equivalent tissues treated with emollients containing PEA or LLP via MALDI MSI analysis was performed. The aim of the study was to examine endogenous lipid responses to treatment over a time course and to observe treatment dispositions in the skin models. This study follows on from a previous investigation that explored changes within untreated skin models over time [14]. Changes in response to treatment were weighted via multivariate statistical analysis so that lipids contributing to the variances could be identified.

2. Materials and methods

2.1. Materials

Alpha cyano-4-hydroxycinnamic acid (CHCA), acetonitrile (ACN), trifluoroacetic acid (TFA), carboxymethylcellulose (CMC) haematoxylin, eosin and xylene were purchased from Sigma-Aldrich (Gillingham, UK). Dulbecco's phosphate-buffered saline and Dulbecco's modification of Eagle's medium, used for tissue washing and incubations of living skin equivalents, were purchased from Invitrogen (Paisley, UK). The active ingredient of Physiogel A. I.®, palmitoylethanolamide (PEA), of Oilatum Junior®, light liquid paraffin and the emollient treatments themselves were provided by Stiefel Laboratories (GSK, Stevenage, UK).

In addition to the active ingredient the formulated Oilatum Junior® was stated to contain macrogol 1000 monostearate (*i.e.* polyethylene glycol monostearate), cetostearyl alcohol, glycerol, potassium sorbate, benzyl alcohol, citric acid monohydrate, povidone and purified water. In addition to the active ingredient the Physiogel A. I.® formulation was stated to contain *Olea europaea*, glycerin, pentylene glycol, palm glycerides, Olus, squalane, betaine, palmitamide MEA (PEA), acetamide MEA, sarcosine and water.

Labskin™ living skin equivalent (LSE) samples were provided by Innovenn (York, England).

2.2. Treatment and preparation of tissue

LSE samples were provided after 14 days of development. They were delivered as 4.5 cm^2 surface area inserts within transport culture medium. On delivery the LSE samples were partially suspended in LabSkin™ maintenance medium so that the cells were nourished and subsequently incubated overnight for 24 h within 5% CO_2 at 37 °C to normalise their metabolism.

In the second experiment, three LSE samples were treated with the Oilatum Junior® cream and three were treated with the Physiogel A. I.® cream emollient (5 mg per cm^2). For the control group, three skin samples were left untreated. All of these samples were then incubated for 4 h, 6 h or 24 h to create a time course across the treatment groups.

After incubation, the surface of all the samples was carefully washed with deionised water to remove excess formulation and then left to dry in ambient temperature. All samples were then frozen to −80 °C using the Grant Asymptote EF600 Control Freezer and stored at −80 °C until ready for analysis.

Tissue sections with a thickness of 12 μm were cut using a cryostat (Leica 2000 UV, Leica Microsystems, Milton Keynes, UK) and thaw-mounted onto indium tin oxide glass slides for MALDI MS analysis. Sections from each treatment group (at one specific time point) were thaw-mounted onto the same glass slide, so that they could be directly compared. The mounted sections were carefully washed for 20 s with deionised water to remove any excess salts and other impurities. Excess water was taped off and the mounted tissue sections were left to dry at ambient temperature.

The emollients' active ingredients palmitoylethanolamide (PEA) and light liquid paraffin (LLP) were made up in 70% MeOH/30% water (PEA) and in water (LLP) as 500 ng/μl standards. Standards were spotted (1 μl) on the same tissue slide but slightly away from the tissue sections to act as positive controls for imaging and profiling experiments. The spots were left to dry in ambient temperatures.

2.3. MALDI matrix application

A matrix solution of 5 mg/ml CHCA dissolved in a 70% MeOH/30% water/0.2% TFA solution was made up. This was deposited onto other tissue sections and over the compound standards surface using an Image Prep® matrix application device (Bruker Daltonics, Bremen, Germany).

2.4. Mass spectrometry

Analyses were performed using an UltrafleXtreme time-of-flight MALDI mass spectrometer (Bruker Daltonics) with a high-repetition Smartbeam™ laser operated at 2 kHz. Prior to experimentation, the instrument was calibrated using mixed lipid calibration standards (Bruker Daltonics). The instrument was set to reflectron mode to reach its highest mass resolving power (40,000 FWHM). The laser spot diameter was set to 30 μm (as a compromise between spatial resolution and sensitivity with the instrument having a minimum possible spot size of 10 μm) and data was acquired at a spatial resolution of 30 μm × 30 μm, with the laser energy set to 20% arbitrary units. The data was then processed using FlexImaging™ 3 software (Bruker Daltonics). All images were normalised to the total ion count.

For MS/MS profiling, the collision energy was adjusted between 2 eV and 60 eV and the laser energy adjusted between 185 and 200 arbitrary units to produce a signal-to-noise ratio of >4:1 for parent ions and product ions respectively. MS and MS/MS and MSI experiments were conducted on the epidermis and dermis of the different samples within the cohort and were conducted on the spotted compound standards to support the tentative identification of small molecules.

2.5. Data processing

All spectra used for multivariate analysis were re-calibrated by aligning the measured CHCA $[M+H]^+$ mass with the expected mass (*m/z* 190.050). Spectral data lists from the main cohort of the study were examined by principal component analysis (PCA). To perform

this, MS spectra (five acquisitions) were obtained from the epidermis and the dermis region of the tissue sections representing the three treatment groups (Physiogel A. I.®, Oilatum Junior® and the control untreated group) in combination with the incubation time point groups (4, 6 or 24 h). Data lists were exported from FlexAnalysis™ (Bruker Daltonics) as text files and imported into MarkerView™ software 1.2 (Applied Biosystems/MDS Sciex, Concorde, Ontario, Canada), where they could be tabulated. An exclusion process to remove known CHCA peaks was applied to the datasets.

2.6. Principal component analysis (PCA)

Tabulated text files were imported into MatLab® (The MathsWorks Inc. Natick, MA, USA). Once the dermal region, treatment and time-course data combinations had been labelled, the Eigenvector PLS Toolbox 7.0 was used to perform PCA. PCA plots were processed via data normalisation and mean-centred scaling. The PCA scores plot(s) enabled similarities and differences to be discerned between the sample groups. The loadings plot(s) distinguished the *m/z* agents that were contributing to the variances shown in the principle component space scores plot(s).

3. Results

Physiogel A. I.® cream was applied to the surface of LSE samples and allowed to penetrate for 4, 6 or 24 h within the incubation chamber respectively (n = 3 for each time point). Direct comparisons could be made between the control and the Oilatum Junior® treatment groups of the same incubation time point. It was possible to detect and plot the distribution of a number of the ions associated with the Physiogel A. I.® emollient including *m/z* 300.3 which had previously been identified in the standard (Fig 1a). This species was confirmed by MS/MS (Fig. 1b) as arising from the active lipid ingredient of Physiogel A. I.® *i.e.* palmitoylethanolamide (PEA). The signal at *m/z* 300.3 was distributed across the epidermis of the Physiogel A. I.® treated skin sections as shown in Fig. 1c. When comparing the 4 h and 24 h time point images there appeared to be no major differences in the ion distribution. Similar data were obtained from each biological replicate with all Physiogel A. I.® treated tissue sections displaying the same signal along the epidermis of the tissue.

Oilatum Junior® cream was applied to LSE samples and allowed to penetrate for 4, 6 or 24 h within the incubation chamber respectively (n = 3 for each time point). There were a number of prominent species that were associated with the Oilatum Junior® treatment group tissue section and the Oilatum Junior® emollient standard. These signals could be assigned to components of the formulation, *e.g.* polyethylene glycol monostearate species, which were clearly observable as two series of peaks 44 Da apart (Fig 2a) corresponding to sodium and potassium adducts. It was possible to image the distribution of the ions *m/z* 981.5, 965.5, 937.5 and 921.5 from the observed polyethylene glycol monostearate species in the Oilatum Junior® treated tissue sections (4 h time point) and in the emollient standard which acted as a positive control (Figs. 2b–e). Examination of the images in regions of high abundance of these species allowed the detection of other emollient markers which were less abundant in the spectral data. Such markers included ions at *m/z* 373.3 (Fig. 2f) and *m/z* 265.5 (Fig. 2g); the identity of these ions was not determined. It was not possible to detect LLP within the Oilatum Junior® spotted standard nor in the Oilatum Junior® treated tissue section. Many of the signals appeared as low background signals. A number of different matrices were tried for the detection of LLP (dithranol, lithiated 2,5-dihydroxybenzoic acid and 9-aminoacridine); however no ions arising directly from LLP were observed.

MALDI MS images of treated and control LSEs (control, Oilatum Junior®, Physiogel A. I.® groups) were recorded at 4, 6 and 24 h

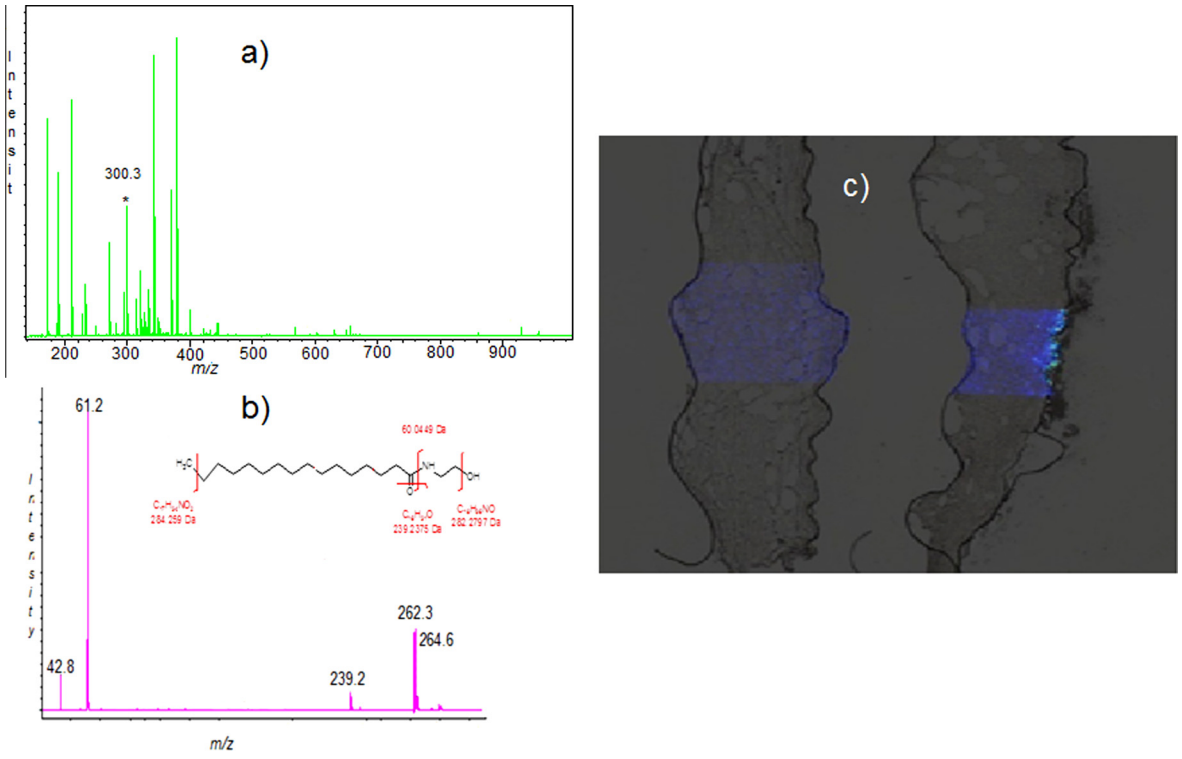

Fig. 1. (a) MALDI MS spectrum of Physiogel A. I.® with the peak at *m/z* 300.3 indicated. (b) MALDI MS/MS spectrum of the ion signal at *m/z* 300.3 along with the postulated structure of the relevant palmitoylethanolamide (PEA) species (c): MALDI MS images of *m/z* 300.3 associated with the palmitoylethanolamide compound across sections representing the LSE treatment groups.

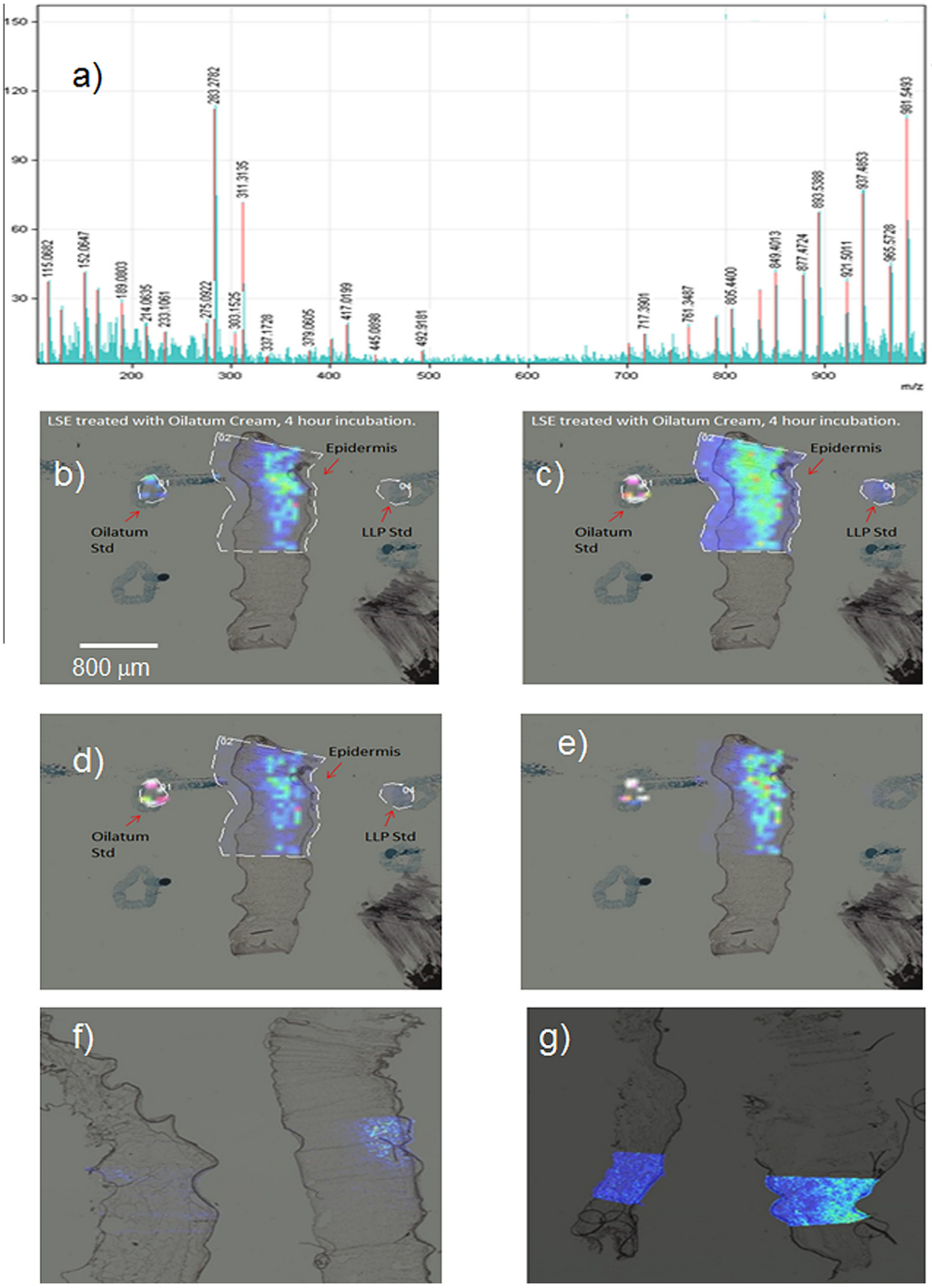

Fig. 2. (a) MALDI MS spectrum of Oilatum Junior® clearly shows series of peaks 44 Da apart assigned to polyethylene glycol. No peaks assignable to liquid paraffin were visible (b–e) MALDI MS images of ingredients of the Oilatum Junior® formulation detected in sections of the LSE (b) *m/z* 981.5, (c) *m/z* 965.5, (d) *m/z* 937.5, (e) *m/z* 921.5, (f) *m/z* 373.3 and (g) *m/z* 265.3. Note the signals shown in (f) and (g) were of lower abundance compared to the polyethylene glycol signals shown in (b)–(e) but were still clearly detectable in the LSE sections.

after treatment. The aim of these experiments was to observe the effect of time and treatment on the tissue. Ion maps were produced at a spatial resolution of 30 µm × 30 µm and normalised against the total ion count. Ions arising from endogenous lipids (*m/z* 759.5, 757.5, 732.5, 703.5) which had been previously identified as biomarkers of the organisation of the model into distinct dermal and epidermal layers [14] were employed to visualise differences between tissue sections as shown in Fig. 3. Here broad changes could be shown between the time points. The 24 h sections show a distribution of species primarily located towards the epidermis of the tissue. In the 4 and 6 h sections, the same species are distributed throughout most of the tissue section surface area. When comparing the control group sections at 4 and 6 h, there is some evidence of changes occurring with time which could be interpreted as early stage migration of the lipids from the dermis' basal regions toward the regions of the epidermis, a phenomenon we have previously reported [14]. At 24 h the emollient treatment group sections appear to show a reduced amount of change when

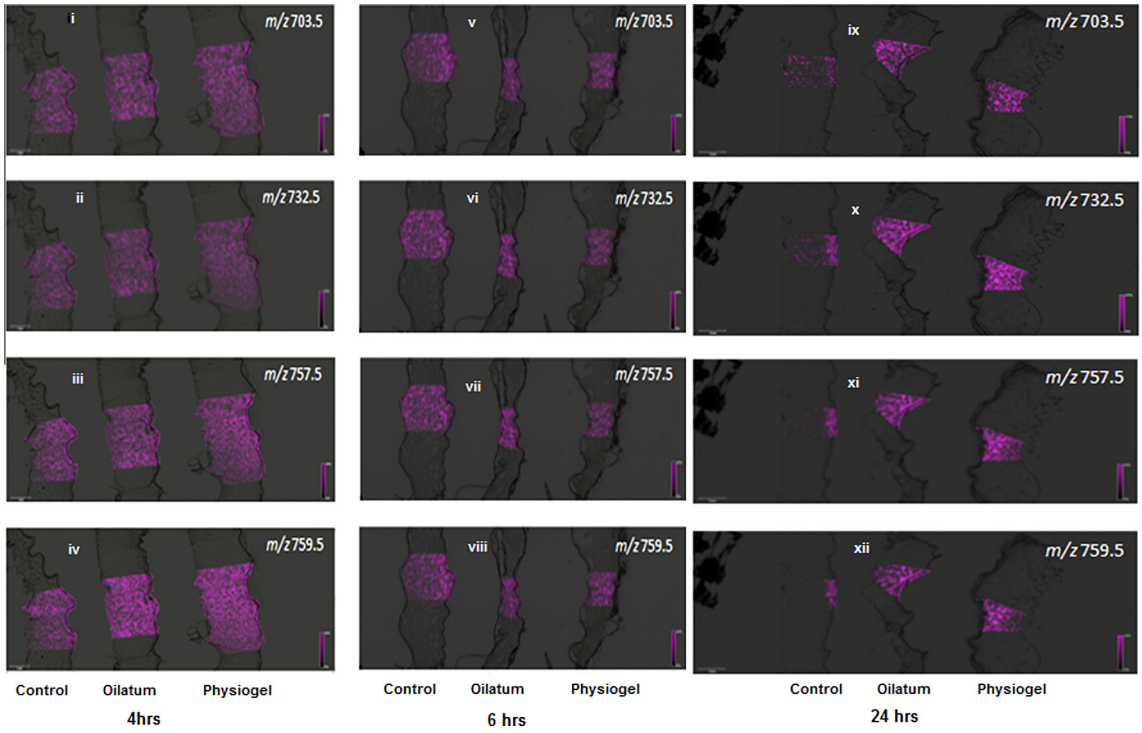

Fig. 3. MALDI MS images of representative phospholipid signals from LSE sections for intra-image treatment comparisons and inter-image time-course comparisons; (a) 4 h time point images, (b) 6 h time point images and (c) 24 h time point images. Of note is the less clearly defined formation of an epidermal region in the treated samples compared to the controls.

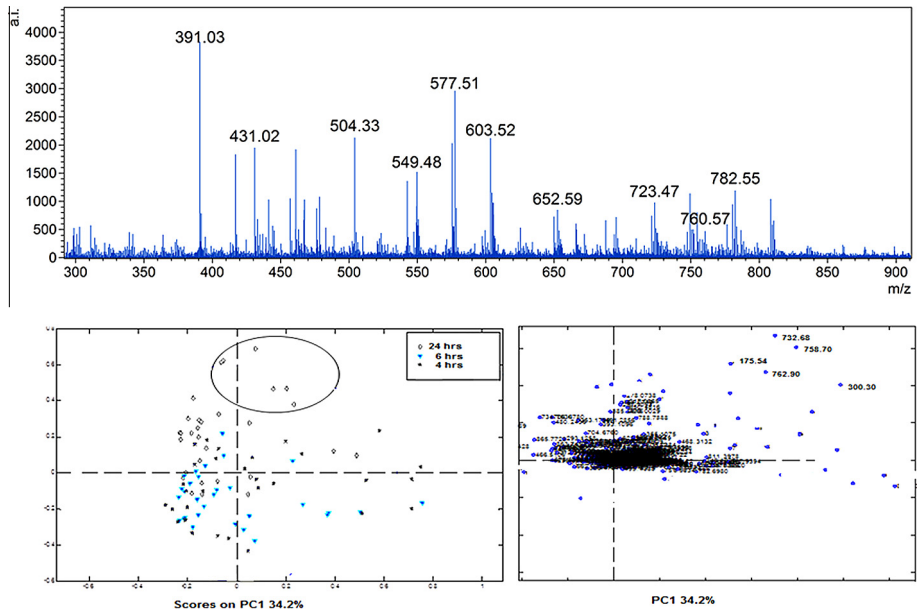

Fig. 4. Representative MALDI MS spectrum for the epidermal region of all LabSkin® LSE samples and PCA analysis of spectra taken from the images shown in Fig. 3. Five MALDI MS spectra were obtained from the epidermis and the dermis region of the tissue sections representing the three treatment groups (Physiogel A. I.®, Oilatum Junior® and the control untreated group) in combination with the incubation time-point groups (4, 6 or 24 h).

compared to the control group (Fig. 3 ix–xii). These differences could be interpreted as the action of the emollients being able to decelerate events that occur in the control group. This was particularly visible in the Physiogel A. I.®-treated skin group.

A representative mass spectrum for all LSE samples is shown in Fig. 4a; as can be seen a complex mixture of lipid species were observed. The output from a search of the LipidMaps database (http://www.lipidmaps.org/) carried out at 10 ppm mass measurement tolerance is given in Supplementary Information S1. PCA was performed in order to see if further changes would be revealed by statistical rather than visual inspection of the data and to determine which variables were important for driving the changes. As can be seen in Fig. 4b, time had a noticeable influence that could be distinguished across the PC2 axis. Most of the 24 h plots were

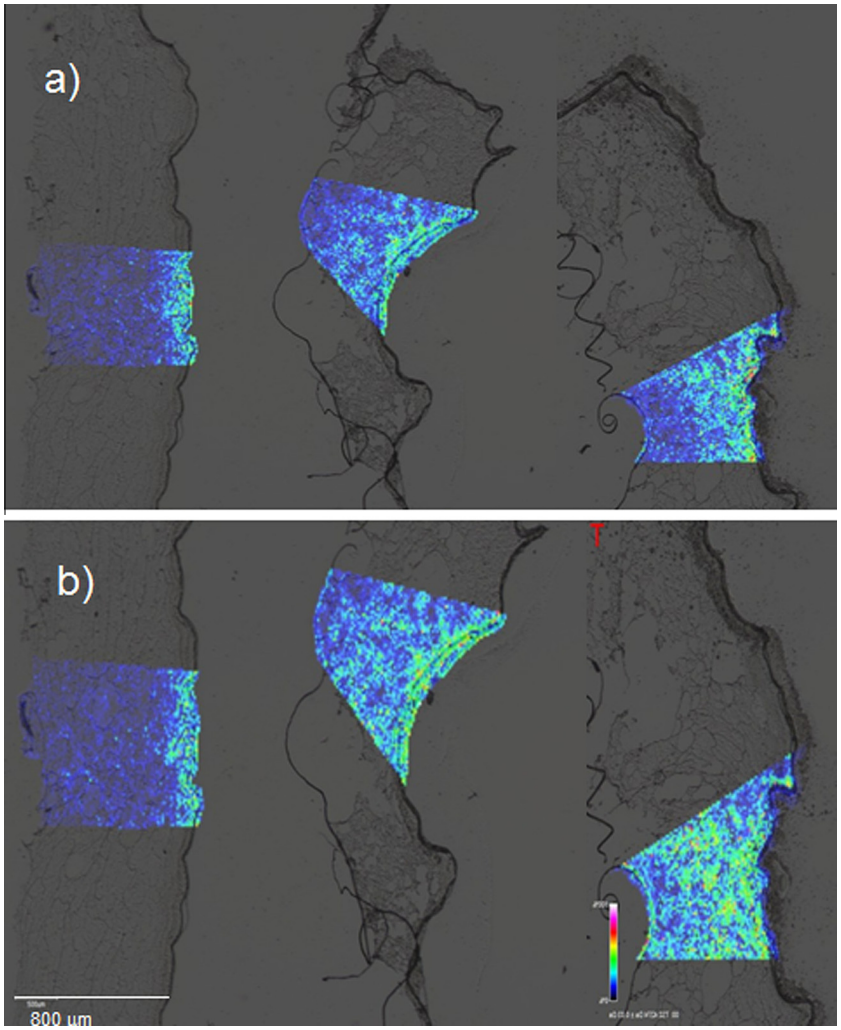

Fig. 5. MALDI MS images of LSE tissue representing the different treatment groups at the 24 h time point; mapping the ions at (a) m/z 758.6 and (b) 732.6 shown to be agents of variance from the PCA data. Similar to Fig. 4, less clearly defined formation of an epidermal region in the treated samples compared to the controls is observed and is a particular feature of those samples treated with Physiogel A. I.®.

predominantly located in the top quadrants whereas the 4 and 6 h plots were mostly positioned in the lower quadrants. From the loadings plot (Fig. 4c), the PEA ion *m/z* 300.2 and phospholipid ions *m/z* 758.6 putatively identified as a PC (36:1) and *m/z* 732.6 as a PC (32:1) were clearly distinguishable and within the cluster of interest. This association of the exogenous PEA and endogenous PC ions was thought to be possibly indicative of pharmacodynamic variances and so the phospholipids became targets in the imaging experiment.

Fig. 5 shows MALDI MS images of ions at m/z 758.6 and 732.6 from a 24 h time-point sample. As can be seen these species were mainly distributed throughout the epidermis of the control tissue; and in the epidermis and dermis of the treatment groups with more of a spread across the Physiogel A. I.®-treated tissue. The *m/z* 732.6 ion map had a wider distribution spread across the tissue in comparison to *m/z* 758.6 generally. When directly comparing the features of each image across the treatment groups, we could not distinguish any major ion intensity differences. The ions appeared to be more saturated across the treatment group tissue sections. The continued interpretation of the data was that the Physiogel A. I.® group showed a reduced extent of change compared to the changes observed in the Oilatum Junior® and the control treatment group.

4. Discussion

Observation of components of the emollient formulations and their absorption into LSE was achieved by MALDI MSI. The active lipid ingredient of Physiogel A.I.® (PEA) was detectable in the LSE and whilst the LLP component of Oilatum Junior® could not be detected other components of the formulation could, allowing its distribution in the LSE to be inferred.

We have previously reported the observation by MALDI MSI of normal biological changes in LSE in the absence of treatment [14]. This was highly informative for the present study. In agreement with the earlier study, we found that lipid changes could be shown across the three time points of the control untreated tissue with the distribution of some lipid signals increasing in intensity in the epidermis (compared to the dermis) over time. The 6 h time-point image clearly showed the possible early-stage lipid changes, particularly within the control untreated skin section. With an appreciation of the normal time-associated lipid changes in the LSE it became feasible to distinguish treatment associated changes.

In the PCA loadings plots, a cluster of the spectra plots located in the top right quadrant was found to represent the key variables of the cohort under interrogation; *i.e.* data points of the epidermal

Physiogel A. I.®-treated skin at 24 h. The spatial positioning of the cluster contributed significantly to the understanding of how the Physiogel A. I.® variances could be weighted in relation to time. The Physiogel A. I.® ions themselves, *e.g.* PEA at *m/z* 300.3, were probably inert drivers of the variance in the loadings chart (simply because the exogenous molecules that were present in one group were distinguishing factors in their own respect, since they were not present in the other groups). The endogenous lipids that were found to be active contributors to the variance were of direct interest and could be mapped in the MALDI MS image.

At the later time point, the MALDI MS images revealed what appeared to be 'lagging' effects of the lipid changes observed across treatment group tissue sections when directly compared to the untreated tissue group. The interpretations of decelerated changes derived from a number of observations. Firstly from mapping the features, we observed very little fluctuation in the relative image ion intensities between the tissue sections, but the differences in the ion distributions were striking and appeared regressive across the treatment groups in direction of the epidermis. Secondly, the MALDI MS images of *m/z* 758.6 and 732.6 suggest that the Physiogel A. I.® and Oilatum Junior® treatments had a varied impact on ion distribution, whilst the control untreated tissues changes did not vary between the two captions. Particularly for the Physiogel A. I.® skin section, the ion map of *m/z* 732.6 showed a signal that was distributed across the whole tissue section, whereas the *m/z* 758.6 ion showed ion distribution mainly across the middle and upper regions of the skin.

At present, there is little recent literature available on the active role of phospholipids in skin. Phospholipids are constituents of membrane bilayers in cellular organelles of viable cell layers. A proportion of the phospholipid content in the epidermis is likely to derive from the subcellular organelles. These phospholipids come in the form of precursor lipids contained within the lamellar bodies, along with glycosphingolipids and free fatty acids. It is known that at the stratum granulosum epidermal interface, the subcellular organelles release their contents into the extracellular spaces [15]. The current data is in support of these notions, as we observed a progressive localisation of PC species in the upper skin regions over time.

An increase in epidermal proliferation and disturbed differentiation (including some changes in lipid composition) is a known cause for impaired barrier function in atopic dermatitis. The topical use of emollients particularly creams might work to restore skin haemostasis by modulating or decelerating the processes of cell proliferation and differentiation. PEA is an endocannabinoid-like compound which is considered to be the parent molecule of the aliamide (autocoid local injury antagonist amides) family [16]. The changes observed may be linked to mechanisms coupled to or incorporated into an anti-inflammatory mediator pathway. Liquid paraffin or mineral oil is a transparent, colourless, odourless, oily liquid composed of saturated hydrocarbons obtained from petroleum [12]. The depth of absorption of the Oilatum Junior® as shown in the MALDI MS images was consistent with its mode of action to facilitate deep hydration. However, further exploration would be required to determine MALDI MS markers of trans-epidermal water loss; and to see if any associations in epidermal lipid perturbations could be drawn.

Skin equivalents can be engineered with specific genetic alterations in either dermal or epidermal compartments. An examination for the response of filaggrin-knockdown skin models would be insightful, as these could potentially present different phenotypic behavioural responses in a pathological setting and this could be related to the role of filaggrin in psoriasis. Although one recent study has shown that filaggrin-knockdown models express no differences in skin equivalent epidermal lipid profile [17], this is directly contradicted by a recent article [18] which does report an impaired lipid profile in a filaggrin-knockdown skin model. This is clearly a fruitful area for future research involving MALDI MSI.

5. Conclusion

The potential of MALDI MSI for investigating lipid changes of *in-vitro* skin equivalents following topical treatment is phenomenal. Great effort was made to conserve the experimental, technical and post-acquisition parameters between each image run to avoid inter-image run variability. The presence of exogenous and endogenous compounds could be clearly discerned across the treated skin samples by imaging the distribution of characteristic *m/z* values. As anticipated multiple signals provided spatial information to show the disposition of each cream within the model. In this study we propose a novel interpretation of skin changes visualised via MALDI MSI. When interrogating each image, the lipid distributions mapped across the sections significantly varied between the emollient treatment groups. Rather than showing volatile fluxes in ion intensities (suggestive of lipid up/down-regulation), the data appeared to show more spatial changes of the lipids particularly in the Physiogel A. I.® treatment group when compared to the control untreated group; which resembled lagging/decelerated changes over time. The skin equivalents were proficient as *in-vitro* systems to model some lipid changes that probably occur *in-vivo* in native skin.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ymeth.2016.02.001.

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