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Sample preparation and data interpretation procedures for the examination of xenobiotic compounds in skin by indirect imaging MALDI-MS

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Abstract

Aspects of the indirect examination of xenobiotic distribution on the surface of and within skin sections by imaging matrix assisted laser desorption ionisation mass spectrometry (MALDI-MS) have been examined. A solvent assisted blotting technique previously developed for the examination of the absorption of agrochemicals into leaves has been examined for the analysis of the distribution of hydrocortisone on the surface of skin. It was found that by careful control of the extraction and blotting procedure an 80-fold sensitivity improvement could by obtained over dry blotting with only 10% lateral diffusion of the image. However, in contrast it was found that the use of a hydrophobic blotting membrane was more suitable for the examination of the transdermal absorption of the pesticide chlorpyrifos.

The potential of incorporating a derivatisation step into the solvent assisted blotting procedure was investigated by blotting isocyanate treated skin onto a methanol soaked blotting membrane. This served the dual purpose of derivatising the isocyanate to a stable substituted urea derivative and extracting it from the skin. Preliminary data indicate that this approach may have some merit for field sampling for such compound and clearly derivatisation also offers the potential for sensitivity enhancements.

Finally, the use of principal components analysis with an ion species specific normalisation procedure is proposed to identify regions of drug treated skin where the ion abundance of the compound of interest is low.

Keywords: Imaging MALDI; Skin; Indirect imaging; Principal component analysis

1. Introduction

The skin is the largest organ of the body, it is constantly exposed to xenobiotic compounds from the environment and one of its major functions is the protection of tissue and organs from them. Despite its function as a barrier to xenobiotic absorption the skin is an attractive route for the delivery of therapeutic agents, not only for drugs where the skin is the therapeutic target, but also for drugs intended for delivery into systemic circulation where, for example, it is a requirement is to avoid the first pass effect.

Methods for analysing dermal absorption include skin extraction measurements, horizontal sectioning (corneum tape stripping), quantitative skin autoradiography and spectroscopic methods. However, inherent problems such as the requirement for a radiolabelled compound, poor sensitivity or the loss of spatial information limit the usefulness of these techniques for the permeation depth profiling of drug compounds in skin.

Performing mass spectrometric imaging experiments using matrix assisted laser desorption ionisation mass spectrometry (MALDI-MS) is a technique developed by Caprioli et al. [1]. In this technique, the sample is imaged by moving it by set increments under a stationary laser. At each position the laser is fired for a pre-selected time or number of shots and a mass spectrum is acquired. Images are obtained by plotting the spatial dimensions of x and y versus the abundance of a selected ion or ions, which is represented as a grey or colour scale.

The initial work on the detection of pharmaceutical compounds directly from tissue using MALDI-MS was published by Troendle et al. [2]. The first reported examination of the distribution of a pharmaceutical compound in animal tissue using imaging MALDI-MS was carried out by the Caprioli group in 2003, here the distribution of anti-tumour drugs in mouse tumour

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tissue and rat brains were described [3]. Subsequent studies have been carried out to map a range of compounds in a number of different biological tissues [4,5]. Sample preparation methods have been reviewed for both direct and indirect (i.e., tissue blotting) MALDI-MS imaging analysis of biological tissue [6].

We have reported recently an imaging MALDI-MS methodology for skin absorption experiments [7]. In this work, we found that skin presents a challenge to direct imaging MALDI analysis since poor matrix coverage was observed for all application methods tried, hence an indirect approach was developed. The reported methodology was based on blotting a vertical section of skin onto a cellulose membrane that had been pre-coated with MALDI matrix. We were able to demonstrate the use of such an approach for the study of the absorption of the antifungal compound ketoconazole (as formulated into "Nizoral" shampoo) into porcine skin and our data indicated that the ketoconazole penetrated into the skin only as far as the dermis with no absorption into the underlying connective tissue. This is as would be expected for a formulation intended for treatment of the dermal layers of the skin.

In a recent study, we have also reported the use of a "solvent assisted" blotting technique for the examination of the distribution of agrochemicals on soya plant leaves [8]. In this technique, in order to assist the extraction of the active compound from the surface of the leaf, the blotting membrane was pre-wetted with acetone prior to coating with the MALDI matrix and imaging MALDI-MS examination.

The work presented in this paper investigates sample preparation procedures for the study of a range of compounds of pharmaceutical and toxicological interest on and in skin by imaging MALDI-MS. These were chosen to represent compounds with a range of physical and chemical properties. In particular the use of cellulose membranes in conjunction with an extraction solvent and carbon loaded polyethylene membranes [6] are compared for the study of the dermal absorption of the pesticide chlorpyrifos (O,O-diethyl-O-[3.5.6-trichloro-2-pyridyl]-phosphorothioate). Some fundamental aspects of the solvent assisted blotting technique have also been investigated during its use for the examination of the distribution of the corticosteroid hydrocortisone (11β,17α,21-trihydroxy-4-(pregnene-3,20-dione)) on the surface of skin. The solvent assisted blotting technique has been further developed in the study of the distribution of isocyanates on the surface of skin. Here an alcohol-based extraction solvent was also able to act as a derivatisation reagent, serving the dual function of both stabilising the analyte and improving sensitivity. Finally, we report some preliminary data from the use of principal component analysis (PCA) to aid in the interpretation of small molecule imaging MALDI data where signals of interest are of low abundance compared to the matrix background.

2. Experimental

2.1. Materials

A generic hydrocortisone cream (0.1%, w/w, active ingredient) was obtained from a local pharmacist. Dursban 4 (480 g/L chlorpyrifos) was obtained from the Health and Safety Laboratory (Buxton, Cheshire, UK). 2,4-Toluene diisocyanate (2,4-TDI) and all other reagents were obtained from Sigma–Aldrich (Poole, Dorstet, UK).

2.2. Investigation of solvent assisted blotting

2.2.1. Assessement of extraction efficiency

Porcine skin was treated with 2 mL of a commercial hydrocortisone cream (0.1%, w/w, active ingredient) over a 2 cm^2 area and incubated at 37 °C for 1 h. At the end of this period the excess was washed away. Cellulose membranes (2 cm^2) were saturated in solvents and air dried for 60 s prior to blotting. Membranes were applied manually to treated areas under a pressure of approximately 1 kg/cm^2 for 60 s. Hydrocortisone was extracted from membranes in 1 mL of mobile phase (acetonitrile:water, 70:30) for quantitative HPLC analysis. HPLC analysis of the resulting extracts was carried out using a Spherisorb ODS 15 μm column, 25 cm × 4.6 mm at a flow rate of 1 mL/min with a mobile phase composition of acetonitrile:water 70:30). Detection was by UV at 242 nm. All experiments were performed in triplicate.

2.2.2. Assessment of lateral diffusion during solvent assisted blotting

In order to examine the lateral diffusion of analytes during solvent assisted blotting the degree of solvent saturation of the membrane and the extraction period were varied. Porcine skin was treated with a commercial hydrocortisone cream (0.1%, w/w, active ingredient) in a 6 mm diameter spot and incubated at 37 °C for 1 h. At the end of this period the excess was washed away with a 5 s water rinse. (In previous work, we have demonstrated this does not remove the analyte from the subsurface layers of the skin [7].) Ethanol saturated membranes were air dried for 30, 60 and 90 s prior to blotting. Each membrane saturation state was examined in conjuction with 30, 60 and 90 s extraction (blotting) periods. Following extraction all samples were coated with approximately 5 mL of matrix solution (acetone, 25 mg/mL α-cyano-4-hydroxy cinnamic acid (α-CHCA), 0.1% trifluoroacetic acid (TFA)) from a distance of 10 cm. Imaging the distribution of the hydrocortisone on the membrane was carried out as described in Section 2.6. Surface area measurements were taken from the metric scale of the imaging software. Experiments were conducted under ambient conditions of temperature and pressure in the laboratory on the same day. Drying times given are intended to be relative rather than absolute and indicative of a methodology for optimising blotting.

2.3. Examination of the surface absorption of chlorpyrifos

Porcine ear skin was used as a model of human skin for the investigation [9]. Five microliters of suspensions containing the commercial pesticide formulation Dursban 4 (480 g/L chlorpyrifos) in water at dilutions of 1:10, 1:50, 1:100 and 1:500 was applied to separate areas of a 2 cm × 2 cm piece of tissue. The tissue was incubated at 37 °C for 1 h. A cellulose membrane was saturated in methanol and air dried for a period of 60 s prior to blotting. The membrane was applied to the tissue surface

for 60 s. Approximately 5 mL of matrix α-cyano-4-hydroxy cinnamic acid 25 mg/mL, in acetone containing 0.1% TFA, was applied to the membrane by airspray at a distance of 10 cm. The experiment was repeated using a carbon filled conductive polyethylene membrane (0.08 mm thickness, Goodfellow, UK). Dursban exposed tissue was blotted directly onto the carbon membrane without the use of methanol. Matrix was applied as for the previous experiment.

2.4. Profiling chlorpyrifos absorption into skin

Suspensions of 200 μL of Dursban in water (1:10 dilution) were applied to 2 cm × 2 cm pieces of tissue. After 1 h incubation at 37 °C surface bound Dursban was lightly rinsed from the tissue with water for 5 s. The tissue was sectioned and blots of the cross-sections performed onto cellulose (saturated in methanol and air dried for 60 s) and carbon conductive membranes. Approximately 5 mL of α-CHCA, at 25 mg/mL in acetone/0.1% TFA, was applied to the membranes by airspray at a distance of 10 cm.

2.5. Preliminary study of the incorporation of a derivatisation stage into a blotting experiment

In order to examine the products of reacting 2,4-TDI with methanol 1 mg of undiluted 2,4-TDI (purity 95% containing 4% 2,6-TDI isomer) was added to 1 mL of methanol. The resulting products were analysed by MALDI using the particle suspension matrix graphite TiO_2 (50:50, total concentration 25 mg/mL in methanol containing 1% etheylene glycol). One microliter of this mixture was pre-spotted onto a 100 well target plate and allowed to dry. One microliter of the reacted isocyanate was pipetted onto the dry matrix and left to airdry before analysis.

In order to evaluate the derivatisation/extraction approach, 2 μL of undiluted 2,4-TDI (purity 95% containing 4% 2,6-TDI isomer) was pipetted onto the surface of a 2 cm × 2 cm piece of porcine skin. The tissue was incubated at 37 °C for 1 h. A cellulose membrane was saturated in ethanol and allowed to airdry for 60 s. The membrane was applied to the tissue surface for 60 s. Approximately 5 mL of the matrix α-cyano-4-hydroxy cinnamic acid 25 mg/mL, in methanol containing 0.1% TFA, was applied to the membrane by airspray at a distance of 10 cm.

2.6. Mass spectrometric analysis

All analyses were performed using an Applied Biosystems/MDS Sciex API "Q-Star" Pulsar i hybrid quadrupole time of flight instrument, fitted with an orthogonal MALDI ion source and "o-MALDI Server 4.0" ion imaging software. The Nd:YAG laser used has an elliptical laser spot of approximately 150 μm × 100 μm. The laser was used at an energy of 30% (3.2 μJ) and a repetition rate of 1 kHz. The laser was rastered over the membrane surface and mass spectra were acquired at 0.2 mm increments, the laser firing for approximately 2 s per spot.

2.7. Examination of imaging MALDI data by principal components analysis

Spectra taken from areas inside and outside the drug treatment area were examined by principal component analysis. Ion species specific (i.e., protonated molecule, Na^+/K^+ adduct) normalisation was carried out on spectra after assignment of ion species to all peaks in the image spectra. Each ion species was normalised across all spectra against the average ion intensity of the respective ion species of the matrix. PCA was carried out using a pre-release version of Applied Biosystems/MDS Sciex 'MarkerView' statistical analysis software with Pareto scaling. The use of data pre-treatment in multivariate analysis of MALDI MS image data is important in reducing the emphasis that large intensity changes have on the overall outcome of PCA. The intensity changes observed in matrix related peaks often mask variation in the intensity of smaller peaks when data is left untreated. Pareto scaling uses the square root of the standard deviation as a scaling factor and functions to lessen the dominance of intensity changes observed in the matrix. This facilitates detection of intensity changes in smaller peaks of interest [10].

3. Results and discussion

3.1. Investigation of solvent assisted blotting

Fig. 1 shows the results from the assessment of potential solvents for blotting hydrocortisone from skin in terms of their extraction efficiency, determined by HPLC analysis. As can be seen from the graph the extraction efficiencies displayed for hydrocortisone follow the expected trend, directly correlating with the solubility of hydrocortisone in the blotting solvent. These results indicate that compound solubility in the extraction solvent is a determining factor for success of the blotting process. From these data, ethanol was chosen for further study. More than an 80-fold increase in extraction of hydrocortisone from the tissue to the membrane was observed with the use of ethanol as opposed to relying on moisture within the tissue.

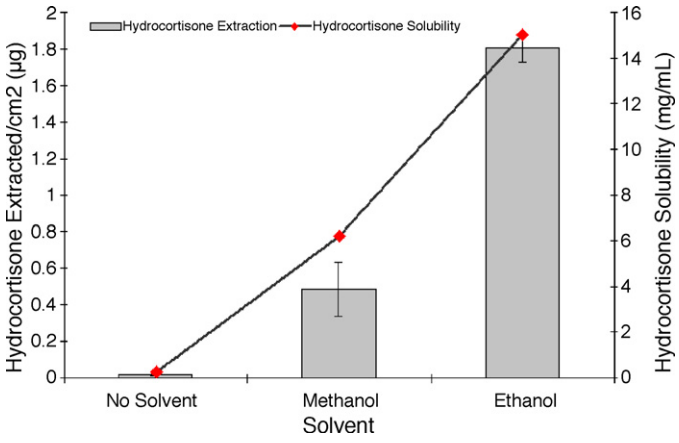

Fig. 1. The correlation between extraction efficiency of hydrocortisone from porcine skin by solvent assisted blotting and the solubility of hydrocortisone in the extraction solvent.

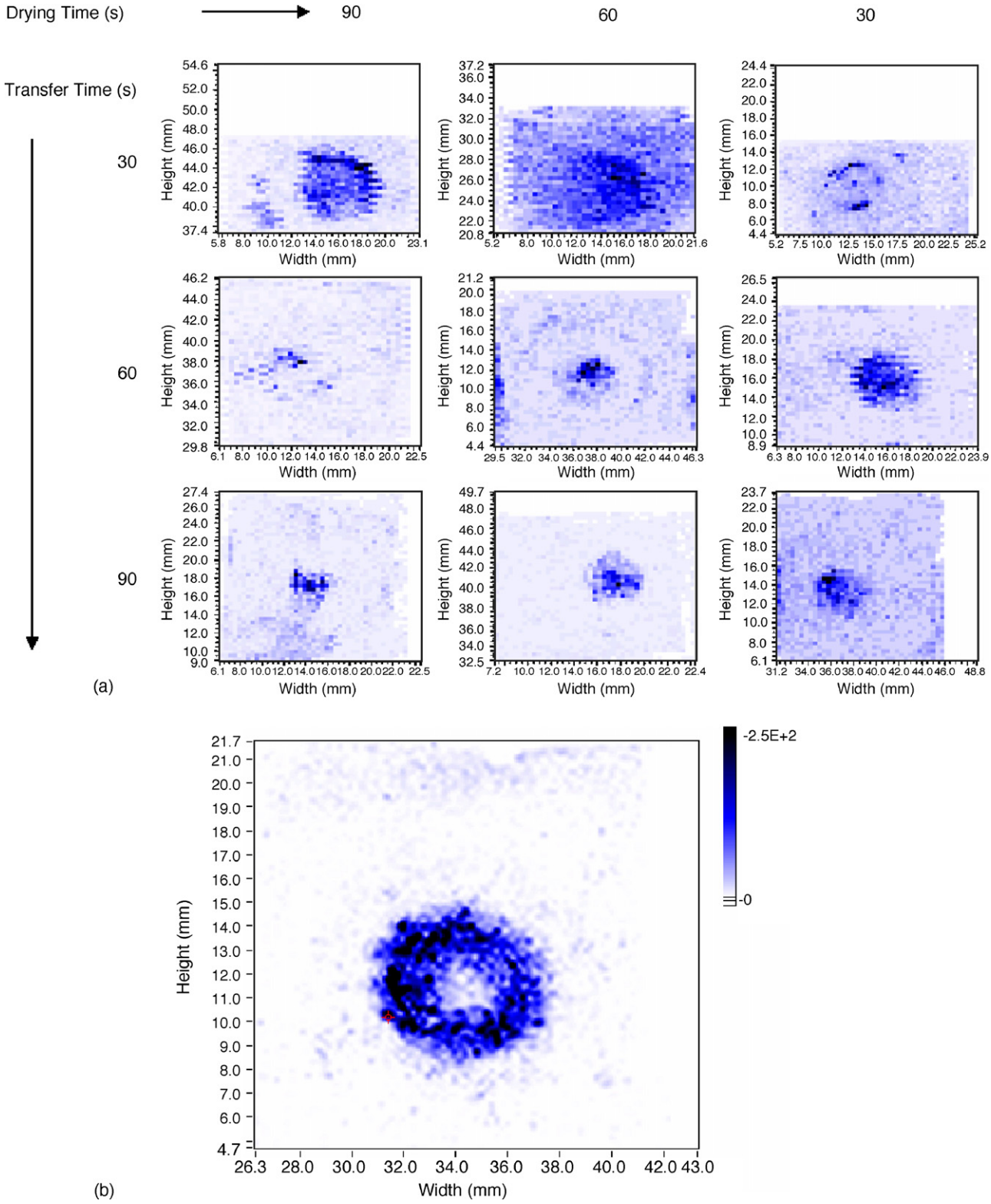

Fig. 2. (a) The optimisation of drying time (i.e., degree of wetness of the extraction membrane) and the blotting time for the imaging of the distribution of a 6 mm spot of 0.1% hydrocortisone cream on the surface of porcine skin using an indirect solvent assisted blotting approach with ethanol as the extraction solvent. Each box represents an image obtained for the distribution of the hydrocortisone $[M+H]^+$ ion at *m/z* 363.28 on the surface of the blotting membrane. (b) Expansion of the optimum results obtained with 60 s drying time and a 90 s blotting time. The degree of lateral diffusion indicated by this image is 10% (±4%). *Note*: The ion intensities for each of the images are normalised individually with the intention to best display lateral migration of the analyte.

Fig. 2 shows the data obtained from the experiments conducted in order to investigate and minimise lateral diffusion. The two major parameters that lead to lateral diffusion in a solvent assisted blotting experiment are the wetness of the blotting membrane, here represented by drying time, i.e., how long after the membrane was soaked in ethanol was the blotting carried out and the blotting/pressing time. It was found that at the optimum drying time (60 s) and the optimum blotting time (90 s), lateral diffusion could be limited on average to approximately 10% (±4%) ($n = 3$) (Fig. 2), i.e., an originally 6 mm diameter area yielded an image diameter of 6.6 mm. Note the 'donut' like appearance of many of these images is simply caused by analyte diffusion during the drying process.

Optimisation of membrane saturation and transfer period are therefore critical in allowing retention of spatial integrity and sufficient extraction of analyte to produce contrasting areas of high and low analyte abundance. Further work is required to investigate whether this degree of analyte spreading is acceptable. In situations where the MALDI imaging technique is being used as an alternative to autoradiography, for imaging xenobiotic distribution, it is possible that this degree of spreading is not significant.

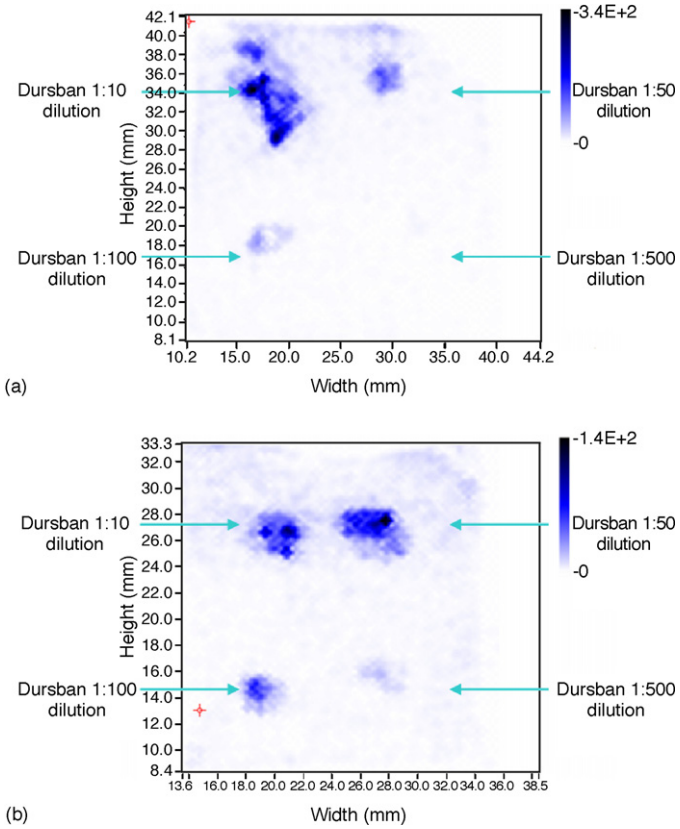

Fig. 3. (a) MALDI image showing distribution of the chlorpyrifos molecular ion (m/z 349.9) at a range of concentrations on the cellulose membrane surface following solvent assisted blotting. (b) MALDI image showing distribution of the chlorpyrifos molecular ion (m/z 349.9) on the surface of a carbon loaded polyethylene membrane also at a range of concentrations. Note the improved spatial resolution and sensitivity obtained by the use of the carbon loaded membrane in this case.

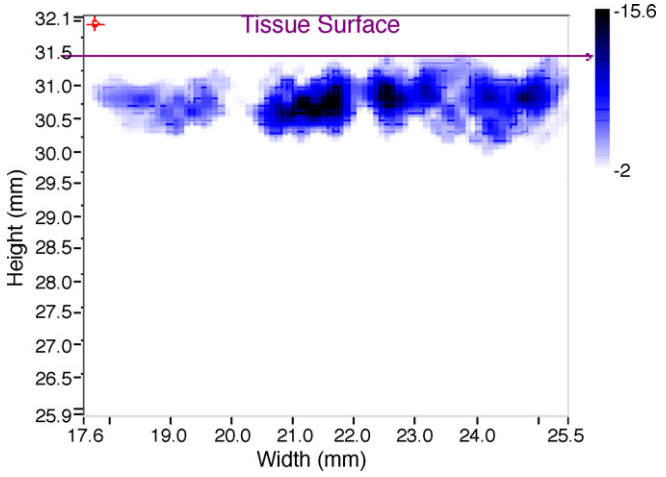

Fig. 4. Image showing the distribution of chlorpyrifos (m/z 349.9) within a vertical cross-section of porcine skin blotted onto a carbon membrane. A penetration depth of 1.7 mm is observed.

3.2. Profiling chlorpyrifos absorption into skin

Fig. 3(a and b) shows a comparison of two different approaches for surface blotting techniques for the imaging of chlorpyrifos on skin. Whilst both approaches proved successful as can be seen, the use of carbon membranes yielded higher quality images. In addition analyte spreading was minimised during the blotting process since there was no requirement for a transfer solvent.

The carbon blotting medium was also the most successful for imaging of the vertical section. Chlorpyrifos was observed to reach a penetration depth of 1.7 mm (Figs. 4 and 5). Chlorpyrifos was observed to penetrate through the epidermis and dermis within the 1 h exposure time with the highest concentration being observed in the dermis. Complete absorption of chlorpyrifos through skin is well documented with chlorpyrifos being detectable in the urine of exposed workers (J. Cocker,

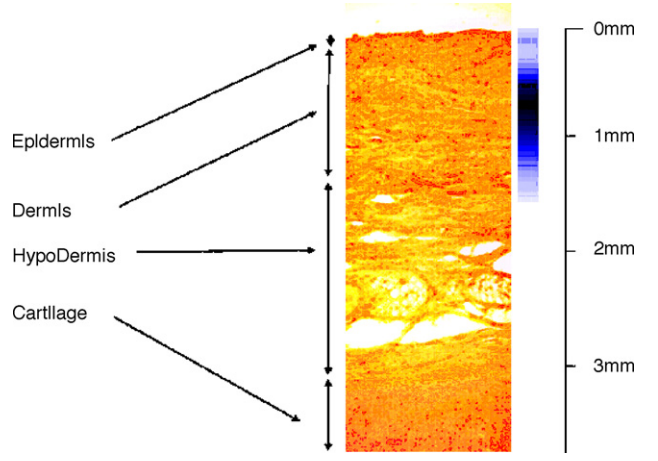

Fig. 5. Comparison of the histological image obtained from a skin section and the corresponding MALDI image taken from an adjacent section. The chlorpyrifos appears to have penetrated through the epidermis, dermis and into the hypodermis tissue. However, after the 1 h exposure time used in this experiment the highest concentration of chlorpyrifos is in the dermis.

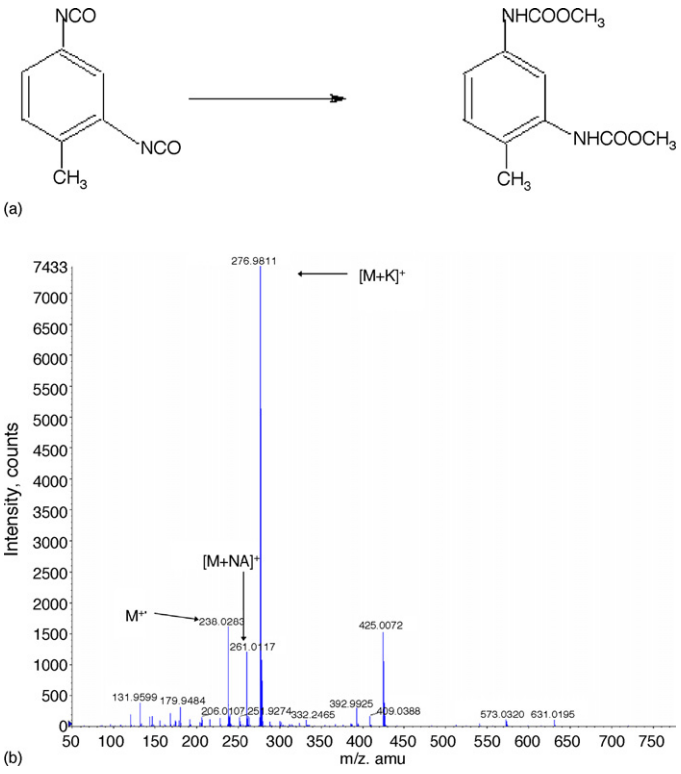

Fig. 6. (a) The reaction between 2,4-TDI and methanol to form a stable substituted urea derivative. (b) MALDI mass spectrum obtained following the reaction of 2,4-TDI with excess methanol using a titanium dioxide/graphite suspension matrix. Peaks are clearly observable for the molecular ion of the urethane derivative (*m/z* 238) along with the sodium and potassium adducts (*m/z* 261 and 277, respectively).

personal communication). A proposed reason for the improved performance of carbon membranes is due to the relative non-polar and highly hydrophobic properties of the analyte. From this limited study, it would appear that the choice of membrane is related to the hydrophobicity of the compound. However, the blotting procedure is not 100% reproducible with satisfactory images only produced from approximately 50% of the blots. The process also appears to be dependant upon the physical properties of the tissue. Fat and endogenous liquids including blood may be transferred during the blotting process causing analyte suppression and resulting in the production of a poor quality image.

3.3. Preliminary study of the incorporation of a derivatisation stage into a blotting experiment

The spectrum shown in Fig. 6 obtained from the initial spotting experiment indicates that 2,4-TDI was successfully derivatised by methanol. Peaks were observed for the molecular ion of the derivative (*m/z* 238) along with the sodium and potassium adducts (*m/z* 261 and 277, respectively). These ions were also observed in the combined derivatisation/blotting experiments and were used to construct the images shown in Fig. 7. Whilst the methodology is clearly not optimised, since there is noticeable lateral diffusion observable in these images, there is clear potential here for the development of a field sampling method. Following the taking of swab or blot samples (using an appropriate alcohol) the highly reactive isocyanates can be stabilised and hence be transported back to the laboratory for subsequent analysis. This will be the subject of further investigation.

3.4. Examination of imaging MALDI data by principal components analysis

Fig. 8 shows MALDI spectra obtained from the skin blots in regions inside and outside the area treated with the 0.1% hydrocortisone cream. The protonated molecule of hydrocortisone at *m/z* 363.28 is visible in the spectrum from the treated area although not a particularly prominent peak.

The use of principal components analysis in the analysis of MALDI image datasets has been reported previously by McCombie et al. [11]. In this work, the coefficients of principal components were plotted as images in order to identify regions of tissue that were different. Problems found using raw data in PCA stem from irreproducible ion intensities. This is particularly evident whilst attempting to apply PCA to small molecules where variations in mass and intensity of potential analytes are masked by more significant variations in the type and intensity of matrix peaks. This in-turn relates to the homogeneity of the matrix coverage and the distribution of salts within the tissue.

In order to apply PCA to small molecules, normalisation of ion intensity has to be carried out. The normalisation must take into account variation in ion intensity in relation to matrix coverage, as well as the effect that variation in salt content has on protonated molecules versus salt adduct formation. For this reason a method of normalisation was developed which functions to normalise each ion species of a compound against the respective ion species of the matrix. PCA carried out on data normalised in this way was able to give clearer indication of variants than was found when using the raw data. In Fig. 9a, the PCA scores plot obtained from our data spectra obtained from the treated (red dots) and untreated tissue (blue squares) are clearly differentiated, following the ion species specific normalisation procedure. In the loadings plot (Fig. 9b), *m/z* 363.28 is clearly an outlier and hence makes a significant contribution to the difference between the data groups in the scores plot. Therefore, it is a clear candidate for imaging despite not being a prominent peak in the original mass spectra. The possibility of enabling detection of hidden variables in image data sets has clear implications in clinical and biomedical research.

The findings of the blotting experiments presented here illustrate how the hydrophobicity of a compound determines the appropriate blotting approach. For example, it has been shown that hydrophobic compounds may be transferred from the skin to a cellulose membrane with the aid of an extraction solvent. Whilst useful for skin surface blotting, it is found that the lateral diffusion in solvent assisted blotting produces a loss in spatial integrity of the sample. In generating useful images for depth profiling purposes, solvent-free blotting is the preferred approach. It is thought that the carbon-based membrane approach is unsuitable for compounds of relatively low

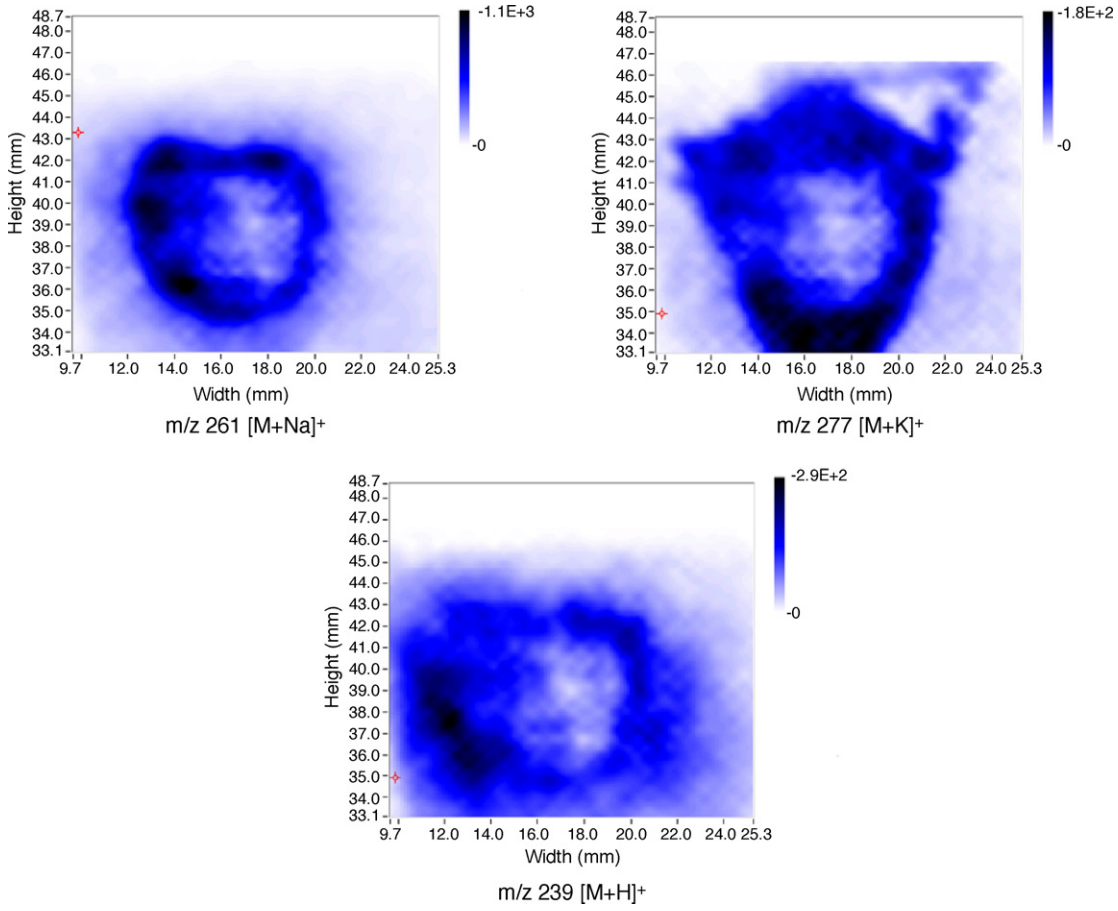

Fig. 7. MALDI images obtained of the distribution of the protonated molecule (*m*/*z* 239), the sodium adduct (*m*/*z* 261) and the potassium adduct (*m*/*z* 277) of methanol dervatised 2,4-TDI obtained from a blot of treated procine skin in a combined extraction/derivatisation blotting experiment.

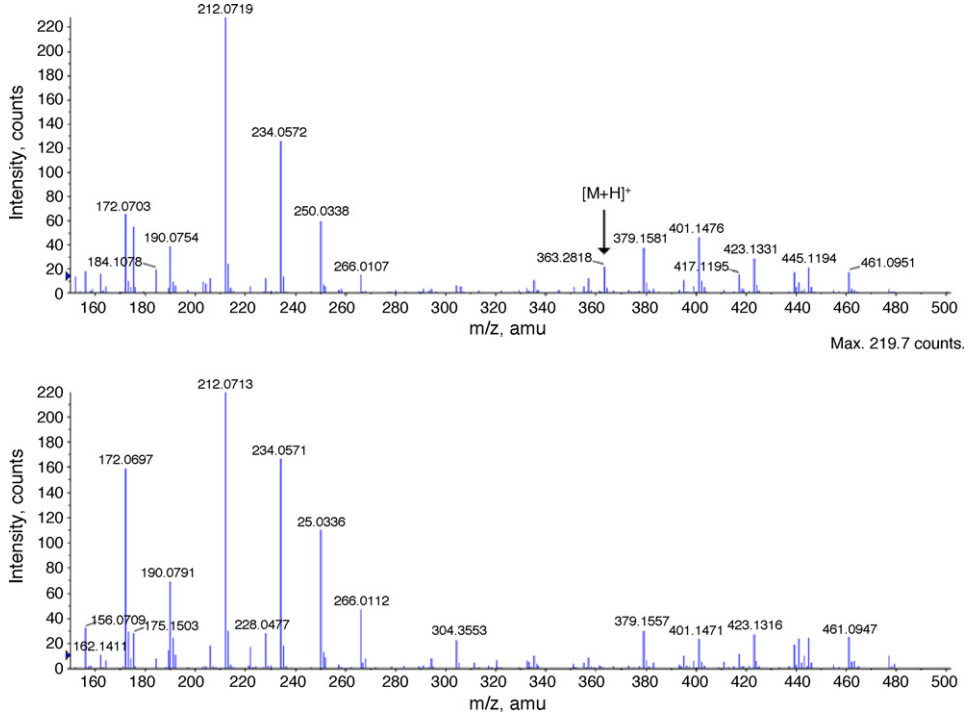

Fig. 8. MALDI mass spectra taken from two areas of a membrane used for a solvent assisted blotting experiment to examine the distribution of hydrocortisone on the surface of porcine skin. As can be seen, although visible, the $[M+H]^+$ ion for hydrocortisone (*m*/*z* 363.28) is not a particualry prominent peak compared to the matrix background.

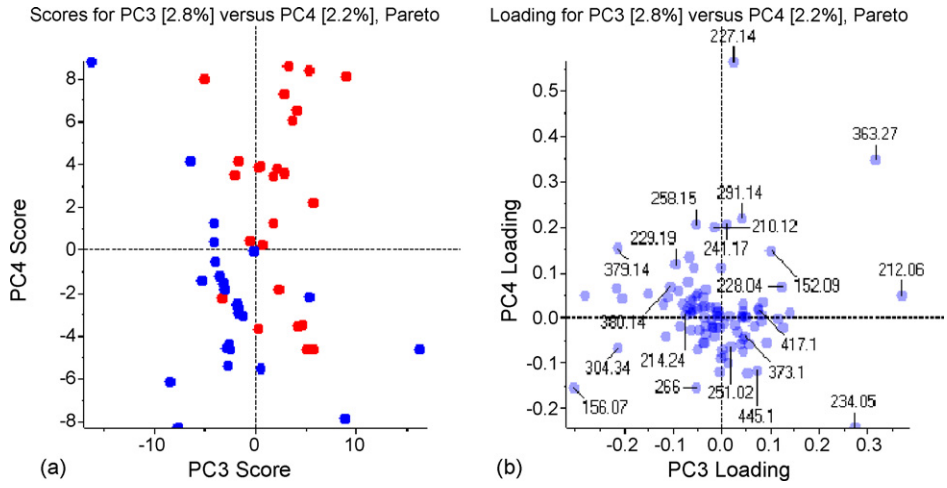

Fig. 9. (a) Principal components analysis of 25 spectra taken from inside and 25 from outside the drug treated area of porcine skin treated with a commerical 0.1% hydrocortisone cream. The ion intensities in each spectrum were normalised to the corresponding matrix species, i.e., peaks presumed to be non-alkali metal containing based on their fractional mass were normalised to the α-CHCA $[M+H]^+$ ion at *m/z* 190 and others to the $[M+Na]^+$ ion at *m/z* 212 or the $[M+K]^+$ ion at *m/z* 228 as appropriate. In the scores plot clear grouping in treated (red spots) and untreated (blue squares) is observed. (b) The loadings plot indicates that *m/z* 363.28 the $[M+H]^+$ ion for hydrocortisone is a major contributor to the differentiation of the groups.

hydrophobicity, such as hydrocortisone, as a result of insufficient hydrophobic interaction with membrane. On application of the matrix to carbon-based membranes, compounds which may not be adhered through hydrophobic interaction are washed from the membrane surface. In contrast, compounds with greater hydrophobicity, such as chlorpyrifos, remain static on the membrane during matrix application. These findings would suggest that ketaconazole should behave in a similar manner to chlorpyrifos, however experiments have shown [12] that this is not the case. Ketoconazole is found to be washed away from carbon membrane surfaces during matrix application, thus displaying similar behaviour to hydrocortisone. It is possible that this occurs through the altered hydrophobicity of ketoconazole in an acidic environment, as would be the case on application of an organic acid matrix solution.

4. Conclusions

Solvent assisted blotting is useful for generating images of xenobiotic distribution of compounds in biological tissue. It appears to be of particular value in instances where target analytes present low water solubility. More than an 80-fold increase in extraction of hydrocortisone from the tissue to the membrane was observed with the use of ethanol as opposed to relying on moisture within the tissue.

This method of blotting does, of course lead to some analyte spreading. It was found, however, that by optimising the drying time and the blotting time lateral diffusion could be limited to approximately 10%. In contrast this procedure has not been found suitable for the analysis of the absorption of chlorpyrifos into skin, here the loss in spatial resolution was unacceptable and the use of a hydrophobic membrane was found to be more appropriate.

Preliminary work carried out on the incorporation of a derivatisation step into indirect MALDI imaging by blotting isocyanate treated skin onto methanol soaked membranes has indicated the potential of such an approach for stabilising reactive analytes and enhancing sensitivity.

Examination of the MALDI mass spectra obtained from hydrocortisone treated skin showed that for "real" samples, peaks of interest could be of relatively low abundance compared to the matrix background. Normalising such data relative to equivalent species in the matrix facilitated the use of principal components analysis to both group the data into regions of treated and untreated skin and indicate components that led to those differences.

Overall the hydrophobicity of a compound appears to dictate the appropriate blotting methodology. However, other factors which may influence the success of the indirect imaging approach such as; the ability of lipids within the tissue to retain the compound; or the possibility that the compound may be located in intracellular regions, must also be considered in future work.

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