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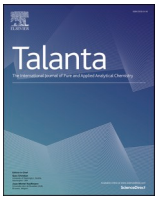

A proof-of-concept study utilising 2D NMR spectrometry for in *situ* characterisation and quantitation of key biomarkers and actives in tape stripped ex *vivo* human skin

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ABSTRACT

The development of a semi-automated and rapid analytical technique for dermatological analysis has become a key aim of many medical and commercial entities through greater awareness of people to skin health and its importance in the 21st century. We present a proof-of-concept methodology demonstrating the use of validated non-destructive, in-situ (Nuclear Magnetic Resonance Spectroscopy) NMR techniques for characterisation and quantitation of (Natural Moisturising Factor) NMF compounds and actives from topical formulations. This quantitation is crucial for appropriate diagnosis of atopic dermatitis severity due to its association with reduced NMF abundance. This study is the first to combine diffusion NMR, semi-automated quantitation and ex-vivo skin samples to measure NMF and permeation of actives. We have shown that diffusion NMR allows for resolution between formulation components through determination of self-diffusion coefficients. We also demonstrate how the metabolomics software chenomx^{tm} can be used to identify and quantitate individual NMF components. We show comparable results to previous literature on NMF layers in the skin, alongside reinforcing findings on permeation enhancers and heat effects on transdermal delivery of actives and formulation components. The presented methodology has shown great potential as an effective non-destructive, fast and versatile technique for dermatological analysis of physiology and actives, with future hardware and software developments in NMR making the future of dermatological analysis via NMR very promising.

1. Introduction

It has been demonstrated, through tape stripping methods, that the *Stratum corneum* (SC) varies between areas of the body, for example the cheek SC is less able to retain water. Elucidation of these differences was carried out by analysis of SC protein concentrations, enzyme activities and TEWL measurements [1]. Further studies measuring the SC proteins have also shown clear variation in the biochemistry of individuals who suffer from sensitive skin [2]. Natural moisturising factors (NMF) have largely been investigated for their role in retaining moisture in the skin and maintenance of the *Stratum corneum* surface. NMF is derived from filaggrin and correlates with changes in the development of the *Stratum corneum* from basal layer to the outer layers. Regional and temporal differences in NMF, alongside protease activities, have been suggested as a prediction tool for Atopic dermatitis in early childhood [3].

1.1. Modelling of the skin

Various experimental models are employed in dermal and transdermal research, in-vitro, ex-vivo and in-vivo. Correlations between in vitro and in vivo from animal and human models have been shown, however many models still show large deviations in results. Unfortunately, there are only a small number of reported correlations that have been validated, owing to the active nature of the skin [4].

The use of ex-vivo tissue for analysis has shown reliable results. The difficulty with ex-vivo use is in making sure that extraction, storage and

Abbreviations: Cr(AcAc)3, Chromium Acetylacetonate; DOSY, Diffusion Ordered Spectroscopy; HSQC, Heteronuclear Single Quantum Correlation; NMF, Natural Moisturising factor; NOESY, Nuclear Overhauser Effect Spectroscopy; PCA, Principal Component analysis; PBS, Phosphate Buffer Solution; SC, Stratum Corneum; TEWL, *Trans*-epidermal water loss; TSP, Trimethylsilylpropanoic Acid.

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acquisition of results is consistent between studies. Studies using ex-vivo samples from humans are ethically sounder than those from animal samples as informed consent is given. One issue of animal modelling in in-vivo studies is that the composition of free fatty acids, triglycerides and hair follicles must be considered when comparing the skin barriers of rodents compared to humans. This has more value in permeation models as these components are part of the main transdermal delivery pathway [5]. These reasons make ex-vivo tissue sampling a fruitful area for interrogation of skin features and permeation profiling of topical formulations [6].

1.2. Formulation design and analysis

The design of topical moisturisation formulations for the *Stratum corneum* aim to increase the ability of the skin to decrease *trans*-epidermal loss (TEWL) and increase delivery of actives to deeper layers of dry skin. One of the most well-known moisturising compounds is glycerol. Glycerol acts as a moisturiser and plasticiser of the *Stratum corneum*. It is now known that glycerol acts through the drawing of water to the surface of the skin, through regulation of osmosis within the intracellular milieu, alongside a role in the maintenance of the fluid structure of cell membranes and intercellular lipids (ICLs). Glycerol delivery is beneficial when it is kept near the surface of the skin [7]. Panthenol has demonstrated significant decreases in TEWL after 30-day application of down to 1% solutions. It also showed that panthenol containing formulations showed immediate reduction of TEWL when compared to controls and vehicle formulations [8].

Permeation of active compounds in topical treatments has been optimised in several ways, varying from formulation design to chemical and physical effects upon the skin. The most common permeation enhancers used in dermal treatments are glycols, specifically propylene glycol with Pentylene glycol being shown to demonstrate anti-microbial activity [9]. Longer chain glycols, such as 1,2-pentanediol have also shown improved penetration of caffeine in pig skin models [10].

Studies by McAuley et al. demonstrated the potential of using heat to increase delivery of actives into the skin. Heating of scalp skin showed finasteride penetration into the human scalp via targeted hair follicles being significantly increased. This research also showed that combinations of penetration enhancers can be used for targeting of permeation with heat application [11–13].

1.3. Analytical methodologies

The separation of complex mixtures in skin analysis by HPLC methods is extensively used in the characterisation and quantitation of NMF components in the skin. The extraction process requires tape strip washing in ammonia solution, evaporation and reconstitution in pure water. Solvent system and columns must be carefully chosen for specific systems and calibrated with reference standards [3]. It has also been shown that tape strips are extracted through centrifugation and shaking in KOH solution for at least 2hrs, with alkaline extracts neutralized with acid and shaken again for 2hrs followed by filtration [2]. This requires column, flow rate, mobile phase and retention time to be carefully set for each extraction.

Separation of complex mixtures by NMR methodologies can exploit the use of pulsed field gradients and spin echoes, which can calculate differences in translational diffusion through fitting of attenuation profiles of compounds to the Stejskal-Tanner equation alongside removal of large molecules through relaxation time filtering. Differences in diffusion coefficients gives resolution between single molecule peaks in NMR spectra and can be optimised using pseudo 2D and 3D acquisition techniques.

NMR analysis through separation, characterisation and quantitation gives multiple concentrations of several identified compounds of interest within a single sample that has not been chemically or physically treated beforehand. The use of NMR metabolomics software package Chenomx^{tm} has demonstrated the potential of NMR as a non-destructive analytical technique [14], with capacity to use a primary method for quantification through the integral ratio method, with tolerance for hydrophilic and hydrophobic elements within biological mixtures. In addition, NMR demonstrates high sample throughput by automation, with remote desktop applications for simplification of use.

For this analysis we limit our analysis to the surface, keratinized, anuclear cells. This limitation however does not obstruct the analysis of water retention potential and the most important aspect of the skin as a barrier to physiological, chemical and microbe attack at the surface *Stratum corneum* level.

2. Materials and methods

2.1. General

Volumetric measurements done using VWR single-channel pipettes, mechanical, variable volume, Ergonomic High Performance (EHP). A Mettler MT5 scale used for solid state mass measurements.

2.2. Tape stripping

Normal human skin samples collected by Genoskin from donors who underwent abdominoplasty procedures and had given informed consent. Healthy skin biopsy samples were obtained by Genoskin from unused material from dermatology department of hospital with informed consent of the patient. Full ethical approval for the study protocol was obtained from local research ethics committee, and authorization given from the French Ministry of Research (AC-2011-1443). All studies were conducted according to the Declaration of Helsinki protocols. The experiments in this study were conducted on samples collected from a single donor woman aged 42 years. Skin samples were collected immediately after surgery and processed immediately as nativeskin®. Upon receipt of the nativeskin®, culture dish was incubated for at least 1 h at 37 °C, 5% CO2, and 95% relative humidity before performing the first experiment. Skin samples were subsequently separated into 6 groups and treated respectively; Native skin with no topical treatment applied (Ctrl), Physiogel AI lipid formulation (AI), Physiogel formulaton (AI), BlueWater formulation vehicle (V), BlueWater formulation (BW) and BlueWater formulation heat treated at 70C^{o} (BW70). The skin was gently cleaned by swabbing with a cotton pad soaked in distilled water at ambient temperature and allowed to dry at room temperature. Skin sites marked with a surgical marker to ensure tapes were consistently applied to the same area. Fifteen consecutive standard D-Squame® tapes were taken from each nativeskin® sample. The standardised pressure device was used to apply 225 g cm^{-2} of pressure for 5s. Tapes were subsequently removed by a single rapid stroke. Tapes were stored in Eppendorf vials at −80 °C until further analysis [15]. SquameScan® densitometer was used to measure the total protein content present within the tape strip. The tapes, alongside native deuterated PBS buffer as control, were sonicated in a solution of deuterated PBS buffer 0.1 mM, pH 5.5 for a minimum of 10 min in order to remove 85–90% of total protein from the tape strip, and low rpm centrifugation removes any cell debris from supernatant containing proteins, peptides and active compounds of cells and extracellular matrix.

2.3. NMR

A Bruker Avance III 600 MHz FT-NMR Spectrometer at 300 K was used for all NMR acquisitions. NMR tubes used were 5 mm, Ultra-Thin Wall Precision NMR Sample Tubes 7″ L, 600 MHz, (545-PPT-7), from GPE-Scientific. Extracted deuterated PBS supernatant was added to NMR tube at a volume of 594 μL, followed by the addition of 6 μL TSP D2O solution (10 mM). TopSpin 3.6 was used for acquisition preparation and processing of raw data. Quantitative data was acquired with the pulse program noesygppr1d, with a scan number of 1024. D1 = 4–10s

after initial T1 relaxation experiments. Automatic fourier transformation, phasing and baseline correction were applied followed by manual phase correction and manual integration of peaks with bias and slope manually corrected.

Quantitation was achieved using the primary ratio method of integrals with an internal standard being spiked in samples, Trimethylsilylpropanoic acid (TSP), at a consistent concentration (100 μM). Triplicate and interleaved experiments for each sample were acquired to evaluate reproducibility and repeatability.

DOSY D20 and P30 values were calibrated through the acquisition of 1D experiments (ledbpgppr1d). Pulse sequence ledbpgppr2s was used with ns = 64, d1 = 5s, D20 = 0.1s, P30 = 0.75 ms, with 16 transients in the diffusion dimension (F1) using shape SMSQ10.100 for a linear gradient ramp with power starting at 2% amplitude and ending at 98% amplitude for acquisition of 2D DOSY spectra. The software package Dynamics centre (Bruker UK Ltd) was used to determine the diffusion coefficients using the diffusion fit function (f(g) = I_0*e-yˆ2*gˆ2*δˆ2*(Δ-δ/3)*D).

2.4. Data analysis

12 sample data sets for each treatment were used, apart from the control treatment where we only had 8 samples available [5–12]. Grouping of tape strips into layers 1–4, 5–8 and 9–12 was done.

2.5. Chenomx^{tm}

Metabolite detection software Chenomx^{TM}, was used to provisionally identify compounds present in the tape strips through reference spectra fitting and gave concentrations of identified compounds through fitting of database reference spectra and reference deconvolution in relation to set internal standard concentration [16].

2.6. Statistical analysis

Statistical analysis was done using Unscrambler X. Grouped NMF data averages were input into 11 × 15 matrix and normalized with area normalisation. The Principal component analysis model has a max of 7 components with mean centred data. Each variable was weighted equally with cross validation and calculated using the singular value decomposition (SVD) algorithm. Model warnings were set with ratio of calibrated to validated residual variance of 0.5, validated to calibrated residual variance of 0.75 and residual variance increase limit of 6%. Outlier limits were set with warnings at >5% and alarms at 0.5% F-residuals and hotelling T2. PCA overviews were produced displaying PC-1 against PC-2 and PC-1 against PC-3 with explained variance up to 95%.

3. Results and discussion

3.1. Observations

Standard ^{1}H 1D spectra from the two control groups and five treatment groups were initially visually compared to identify common elements of all spectra, namely PBS buffer, solvent peaks and tape strip peaks which were assigned to remove confusion with treatment elements and Chenomx^{tm} assignment of NMF compounds. One limitation found during this assignment was the overlap of fatty acid peaks from the skin's intercellular lipid matrix and those added in treatment groups.

We see component signals throughout the layers, especially in the treatment samples where peaks, surmised to be topical formulation components, drop at a much faster rate than peaks representative of skin and tape strip elements seen in the control groups. We can detect and quantify formulation peaks throughout the twelve layers of each treatment, alongside peaks corresponding to skin compounds in the tape strip control.

Initial quantitative comparison of layers was done by regional percentage abundance of the total signal with the sum of integrals normalized to 100 shown in SI. We observe large changes between adjacent layers, which can be explained by skin furrows and the inherent heterogeneity of skin layers, therefore results were grouped (see more in methods) like that in previous literature [15]. From the regional percentage abundance analysis, we see clear differences between the Aliphatic and Alcoholic regions of treatments when compared to the control tape strips with the abundance of alcohol being higher in treatments. This is primarily explained by glycerol and glycols. We see in treatments V, BW and BW70, an increased abundance in carboxylic peaks. This is explained by the presence of Niacinamide in these treatments, which adds peaks to the conjugated/non-conjugated alkenes region, the carboxylic, aldehyde and amide region.

This method of analysis allows for rough fingerprinting of the different topical treatments applied to our skin samples and compares their overall formulation permeation. This is however very limited in that it does not give information on specific compounds within the formulations or how the treatment affects the overall effectiveness of permeation.

Table 1
Calibration curve table with assigned compounds for BW70 tape strip layer 1, log diff coeff values mean ± root mean square error per spectrum.

Compounds	log diff coeff. ($m2s^{-1}$)	log FW	FW ($kgmol^{-1}$)
Methanol	−8.87 ± 0.034	1.51	32
Acetone	−8.95 ± 0.074	1.76	58
Acetate	−9.04 ± 0.08	1.78	60
Glycerol	−9.11 ± 0.048	1.96	92.1
Pentylene Glycol	−9.17 ± 0.11	2.02	105
Niacinamide	−9.12 ± 0.040	2.09	122
Proposed fatty acid	−9.27 ± 0.10	2.41	256
Panthenol	−9.28 ± 0.055	2.31	270

3.2. Characterisation by 2D NMR techniques

2D NMR acquisitions (DOSY, HSQC and COSY) identified individual molecules within the tape strip. Compounds of known molecular weights were then used to design a calibration curve which correlates calculated self-diffusion coefficients to the formula mass of specific molecules. Using DOSY we see eight compounds with distinguishable self-diffusion coefficients which are listed in Table 1. These identified compounds are then used to build a calibration curve which further validates assignments from COSY and HSQC experiments. In the COSY spectra we see cross peaks at 8 ppm that have coupling constants to each other, which alongside the 1D 1H NMR and 2D HSQC, corroborated by calculated self-diffusion coefficients of these peaks being equal, validates the assignment of Niacinamide. We then used this workflow to identify other compounds of interest in the different topical formulations applied to skin samples, specifically, Pentylene Glycol, Glycerol and Panthenol. These were detected for all the treatment tape strips. Alongside the known formulation components, we were also able to characterize a derivative, assumed to be formed through a reduction pathway as demonstrated in previous studies to produce piperidine-3-carboxamide, 1,4,5,6-tetrahydropyridine-3-carboxamide and 1,4-dihydropyridine-3-carboxamide [17]. Two doublet peaks with chemical shift and coupling constants matching literature values for these reduced forms of Niacinamide are seen [18]. Identification of breakdown products like this is important when we think of the changes in composition from bottle to skin, also it gives an opportunity to evaluate time from excision of sample to acquisition of data.

3.3. Calibration curve for RMM determination

Generation of the calibration curve in Fig. 1, with addition of methanol, acetone and acetate as references, of high abundance topical components Glycerol, Niacinamide, Pentlyene glycol and Panthenol

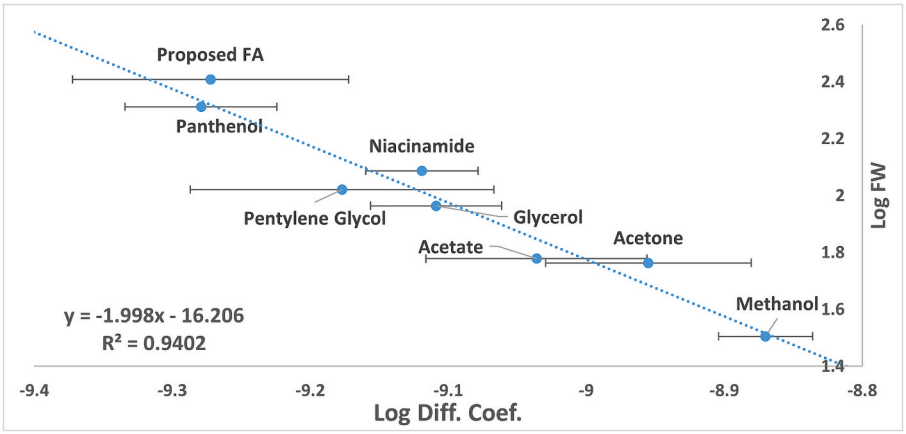

Fig. 1. Calibration curve from DOSY data of BW70 tape strip layer 1 with error ± spectra contour range Formula weight (FW) of compounds predicted through the method introduced by Li et al., 2009 [19].

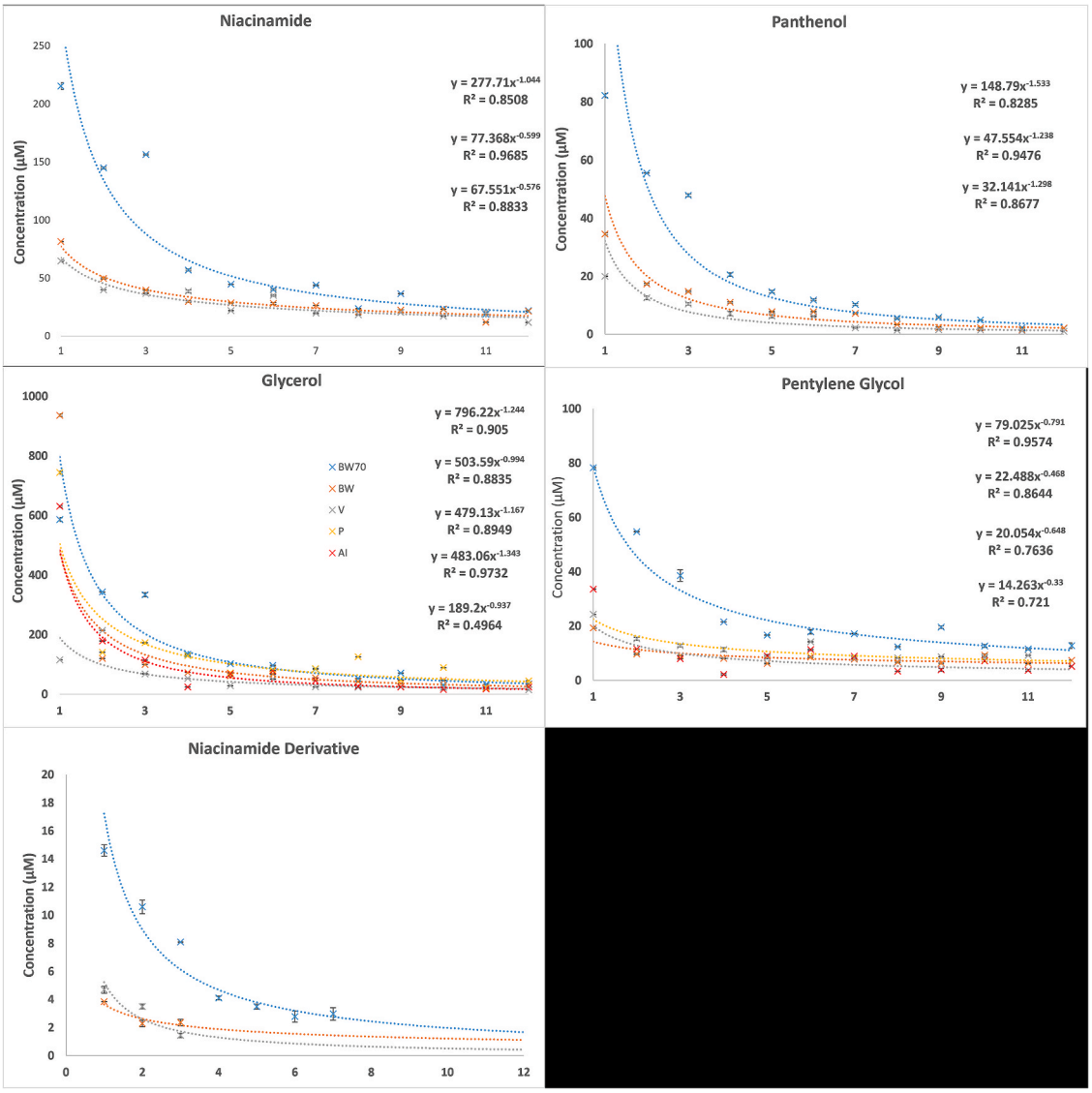

Fig. 2. Permeation profiles of Panthenol, Niacinamide, Pentylene Glycol and glycerol in treatment tape strips (BW70 = Blue; BW = Orange; Vehicle = Grey; Physiogel = Orange; AI = Red), mean ± SD. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

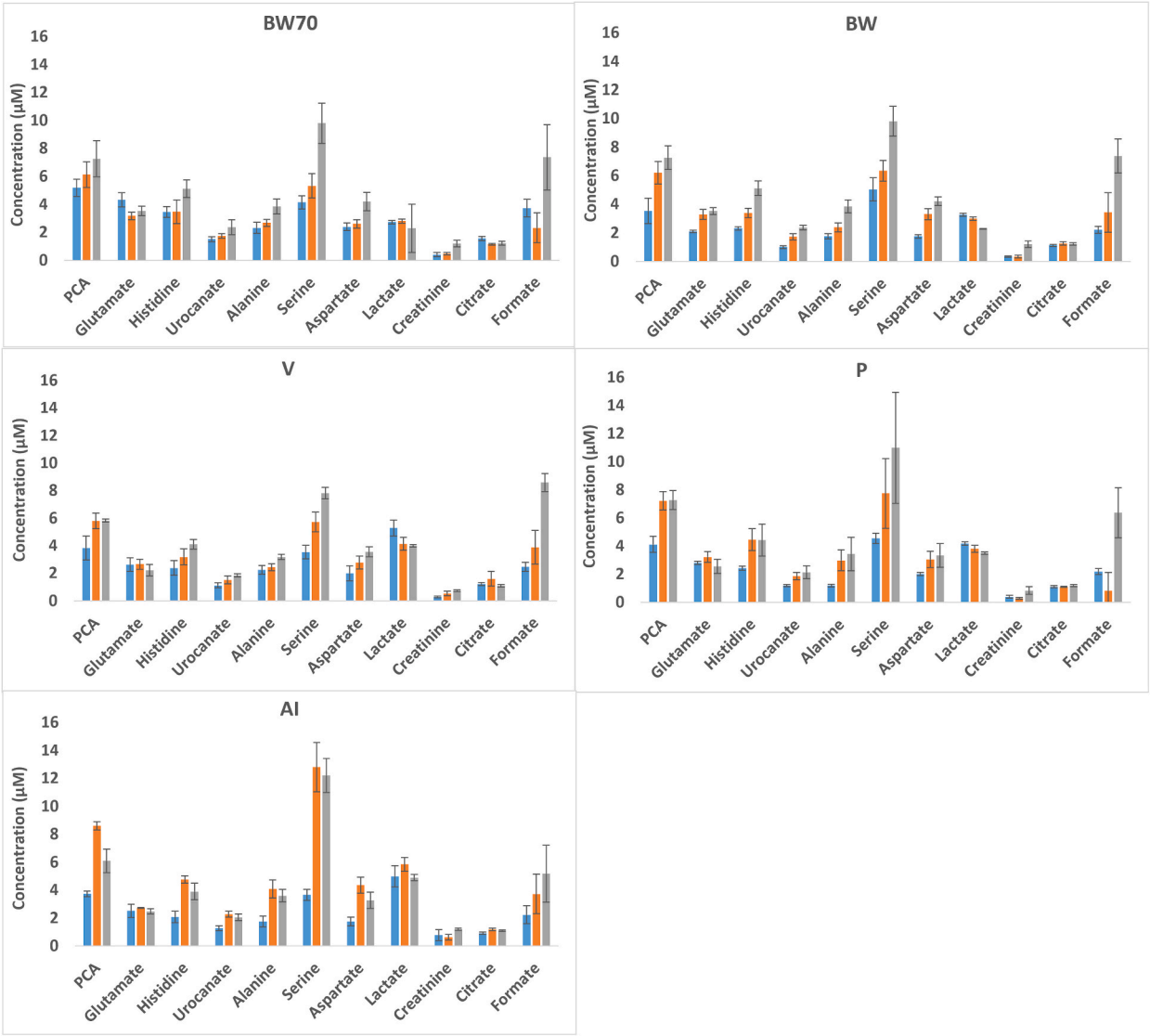

Fig. 3. Grouped tape strip (1–4 (Blue), 5–8 (Orange), 9–12(Grey)) quantitative data for NMF compounds in treatments, mean ± SD 95% CI. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

were added after determination of the linear correlation between log diffusion coefficient and log molecular weight. A linear correlation between log mw and log Diffusion coefficient with r^2 of 0.9402 was constructed, demonstrating satisfactory correlation for validation of selected peaks representing suspected topical components.

We do however see in Fig. 1 that the diffusion coefficient peak range (error) overlaps between individual molecules which shows that further optimisation of acquisition and processing would allow for greater resolution between self-diffusion coefficient values in more complex mixtures. The proposed fatty acid FW shown in Table 1 however cannot be validated as the free fatty acid (FFA) is a mix of palmitic acid and linoleic acid predominantly. Alongside this as previously stated, NMR suffers from overlap of peaks, which affects the calculation of self-diffusion coefficients introducing error. This composition uncertainty means that FFA can be compared between treatments and layers by relative peak intensities, but concentration calculations are limited by the variation in average FW.

3.4. *Permeation profiling of topical components*

In tape strips containing Niacinamide and panthenol (BW70, BW and V), where the formulation components are the same, we see in Fig. 2 a 3x increase in permeation of the top 3 layers for BW70 when compared to BW and V. There is also significantly higher levels of permeated compound throughout layers 4–7 for Niacinamide and panthenol. Comparing BW and V we see a slight increase in permeation for BW in the first 2 layers which matches the slight increase seen in panthenol and glycerol permeation. On the other hand, permeation of pentylene glycol between the two shows no significant differences. We see for glycerol a lower permeation in the top layer for BW70 and see greater permeation for BW and P, despite this we see the continuous permeation is increased in BW70 with layer 2 and 3 almost 2x more permeating than all other treatments which show no significant differences to each other. After tape strip 4 however we see no significant differences between any of the treatments and permeation of topical treatments has plateaued. The permeation of glycerol is an interesting marker of the effectiveness of pentylene glycol as a permeation enhancer, as we see that P permeates Glycerol at the same level as that of AI and significantly higher than V for the first layer. This can be explained by the P formulations being designed to stay and crystallize on the surface layers and keep moisture at the surface and is assumed to have higher levels of glycerol. The increases which are significantly seen in the BW70 samples, corroborates the findings found by McAuley et al. that thermal treatment can greatly increase the transdermal penetration of topical components.

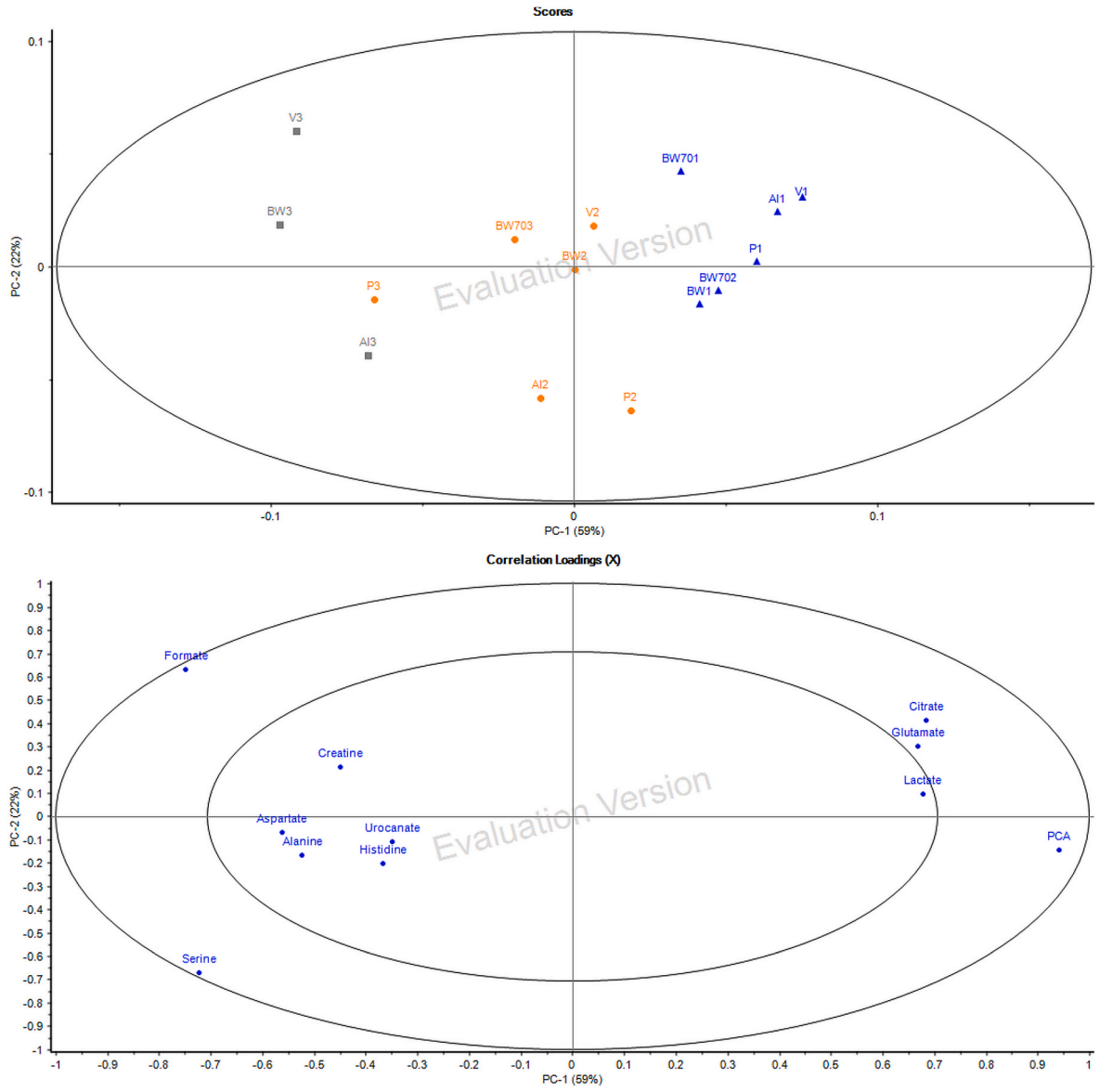

Fig. 4. Principal component analysis of grouped tape strip data, PCA overview, with symbols (Grey Square/Orange Circle/Blue Triangle) representing grouping of samples from variability of PCA (NMF) as the grouping factor, and correlation loadings for PC-1 against PC-2. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3.5. *Layer NMF quantitation by Chenomx*^{TM}

We see in the treatments the trends for each NMF component are very similar, as seen in Fig. 3, such that as the tape strips go from group 1 to group 3 layers, we see trends matching for each NMF. For PCA between group 1 and 3 we see significant increase in treatment P and BW. We also see in treatment AI a significant increase in group 2 when compared to group 1 and 3. The significant changes detected in PCA match that of previous findings that levels of PCA increase as we go deeper into the *Stratum corneum*, however at the depth that was done during this study this conclusion may be premature, and deeper layer analysis would be useful.

We see significant change in glutamate levels in treatment BW between groups 1 and 2/3. These results are not surprising considering the results for PCA did not show a universally significant result across treatments as these NMFs have been shown previously to correlate to each other. Histidine and Urocanate are the two other main NMFs that show a significant trend where both significantly increase from group 1 to group 3 in all treatments apart from treatment AI. We do see significant changes from group 1 to group 2, which is seen across most of the components with significant changes in the AI treatments group. This may represent the compounds increasing in abundance earlier in the layers than other treatments specific to the AI formulation.

3.6. *Statistical analysis*

We see in through the use of principal component analysis that we can group tape strip groups based upon the variability however it was unable to determine grouping of different treatment types from this statistical analysis. In Fig. 4 we see that samples become grouped with all group 1 samples being grouped together, however we also see BW70 tape strips 5–8 in group 1, which may be explained by the heat effects of the skin which make the surface have greater energy kinetics and higher diffusion of NMF's between layers. Apart from BW70 group 2 being grouped with group 1 samples, all other group 2 samples are grouped together. Group 3 samples is where we see the most variability, with both BW70 group 3 and P group 3 being categorised as group 2. This change could be explained by the drawing effect of treatment P to the

surface where formulisation has been applied and crystallised, with BW70 group 3 having a similar situation to group 2 where the heat treatment affected the heterogeneity of NMF compounds. The correlation loadings plot shows positive correlations between previously correlated NMF compounds, specifically PCA and glutamate, Histidine and Urocanate. It is however surprising to see no specific correlation metabolic markers, formate, citrate, creatine and lactate.

4. Conclusion

Here we present a proof-of-concept study demonstrating novel NMR methodologies in the characterisation and quantitation of NMFs and formulation components in tape stripped ex-vivo skin samples. This study corroborated previously reported findings about NMF values and how permeation of formulation compounds can be varied by formulation composition and thermal treatment. As an analytical method it demonstrated a relatively simple extraction procedure, quick sample preparation using a semi-automated approach to NMR quantitation and comparison. The benefit of this method is the time required for extraction to acquisition of NMR spectra which can minimise the effects of degradation and derivatization of target analytes but also allows for monitoring of derivatives as demonstrated by Niacinamide derivative permeation profile determination. It also presents analyte concentrations which are non-chemically treated and illustrate in-situ relative concentration. In future work this methodology can be used alongside *Stratum corneum*/vehicle partition coefficients (Km), maximum flux (J), enhancement factor (EF), and 24-h receptor concentration (Q24h) determination for formulation evaluation and permeation enhancement candidates. It can also be employed in the analysis of NMFs in different skin types, areas and models to validate previous findings and provide additional information that other methods cannot provide in a set time scale with in-situ conditions.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.talanta.2021.122980.

Credit author statement

Cameron Robertson: Conceptualization, Methodology, Software, Data curation, Writing – original draft preparation and creation, Visualization, Investigation, Formal analysis, Adam Le Gresley: Supervision, Writing – reviewing and editing, Conceptualization, Formal analysis, Nidhin Raj: Investigation, Writing – original draft presentation, Robert Lucas: Supervision, Software, Writing – reviewing and editing, Funding acquisition, Project administration, Tomris Coban: Writing – original draft presentation, Visualization, reviewing and editing.

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Data availability

Data-sets are available at https://doi.org/10.17632/bxhmvjmck7.1 an open-source online data repository hosted at Mendeley Data.

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