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Use of LC-MS analysis to elucidate by-products of niacinamide transformation following *in vitro* skin permeation studies.

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Pyridine-3-carboxamide, also known as niacinamide (NIA), is used in many pharmaceutical and personal care formulations for the improvement of skin barrier function, management of acne and amelioration of the symptoms of atopic dermatitis [1-3]. The widespread use of NIA (Table I) in skin care highlights the importance of understanding the percutaneous penetration and skin distribution of this molecule [4]. Previously, we have conducted several studies that have evaluated a wide variety of NIA formulations [4, 5]. The amounts of NIA recovered in mass balance studies for pseudo-finite (20 $\mu\text{L}/\text{cm}^2$) and finite (5 $\mu\text{L}/\text{cm}^2$) dose conditions fell below the Scientific Committee on Consumer Safety (SCCS) limits for dermal absorption studies (85–115%) [6]. The aim of the present work was to explore and identify the possible formation of molecular derivatives of NIA during dermal penetration experiments that may explain the previously reported recovery values. The major metabolites of NIA include niacin, nicotinuric acid and niacinamide N-oxide however none

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of these species could be identified in our previous investigations [7]. The present approach focusses on the detection and isolation of other liquid chromatography (LC) peaks that did not correlate to the known metabolites of NIA. The identification of such peaks using liquid chromatography mass-spectrometry (LC-MS) should thereby address the reasons for the lower recovery values of NIA in our earlier studies and ideally facilitate the development of more sensitive analytical methods to measure NIA following topical application.

NIA was purchased from Sigma Aldrich, UK. Commercial formulations used contained glycerin, niacinamide, 2-methylpentadecane, dimethicone, isopropyl isodecanoate, stearyl alcohol, panthenol, tocopheryl acetate, 1-hexadecanol, polyacrylamide, behenyl alcohol, titanium dioxide, C13-14 isoparaffin, 1,2-octanediol, hexamethylene glycol, phenoxyethanol, dimethylsilanediol, dodecylheptaglycol, sodium benzoate, sodium ascorbyl phosphate, peg-100 stearate, cetearyl glucoside, cetearyl alcohol, disodium EDTA, stearic acid, palmitic acid, butylated hydroxytoluene, zinc oxide and triethoxycaprylylsilane. All non-commercial formulations contained hydrogenated lecithin, capric caprylic triglyceride, isoamyl *p*-methoxycinnamate, diethylamino hydroxybenzoyl hexyl benzoate, bis-ethylhexylphenol methoxyphenyl triazine, shea butter, glycerine, olus oil, isostearyl isostearate, dicapryl carbonate, xylitol, panthenol, niacinamide, pentylene glycol and 1,2-hexanediol. All formulations were supplied by Glaxo Smith Kline (GSK, UK). LC-MS grade water, methanol and dichloromethane (DCM) were obtained from Fisher Scientific, UK. Phosphate buffer saline (PBS) tablets were purchased from Oxoid Limited, England. Porcine tissue was obtained from a local abattoir and kept at -20 °C until usage.

Studies of NIA in Franz diffusion cells were conducted using different doses (50 $\mu\text{L}/\text{cm}^2$ and 20 $\mu\text{L}/\text{cm}^2$) of formulations applied to full thickness porcine ear skin. Skin integrity was confirmed by evaluation of electrical resistance. Freshly prepared PBS (pH 7.3 ± 0.1) was used as the receptor solution in all studies. The skin temperature was equilibrated to 32 ± 1 °C and formulations were applied using an Eppendorf® Multipette Plus (Merck, UK). The donor compartment was occluded using Parafilm® after application of the formulations. A sample of 200 μL of receptor solution was removed from the receptor compartment at various time intervals up to 24 hours, with aliquot replacement using fresh temperature equilibrated PBS solution [5, 8]. Samples were analysed by LC and all unassigned peaks are shown in Fig.1. After the 24 hours, the PBS solution in the receptor compartment of the Franz diffusion cell was collected for further extraction. Two different approaches were devised to isolate any previous unassigned NIA related LC peaks, categorised as organic and

inorganic routes. The organic route involved the partitioning of different NIA by-products between a PBS:DCM mixture. The aqueous layer was extracted three times using 5 mL of DCM. After evaporation of the organic solvent, the residue was dissolved in a water:methanol (50:50) mixture and analysed by LC-MS. The inorganic route involved freeze drying the 24 hours PBS sample. The extraction of potential NIA by-products from the dry residue was carried out with 100 μ L of a water:methanol (50:50) mixture. Following sample extraction and filtration of undissolved residue, semi-preparative LC was performed. This was followed by LC-MS analysis of all previous unassigned peaks including that for NIA.

The analysis of NIA and NIA by-products was conducted using an Agilent Infinity II 1260 (Agilent Technologies, UK) system equipped with an Agilent G7129A vial sampler, G7111B quaternary pump and G7114A variable wavelength detector coupled to a single quadrupole mass spectrometer Agilent Infinity Lab LC-MSD XT. The mass spectrometer was set at positive ion mode operation at 350 °C and with an ion spray voltage set to 10000 V. Zero air was employed as the nebulizer gas and heater gas, with nitrogen used as the curtain gas (35 psi) and collision gas. Data were processed using OpenLab CDS ChemStation software (Agilent Technologies, UK). Analysis was performed using a Phenomenex Luna Phenyl Hexyl column (Phenomenex, UK) with a length, internal diameter and particle size of 250 mm, 4.6 mm and 5 μ m, respectively and a guard column. The mobile phase consisted of water:methanol (80:20). The pH of the mobile phase (7.4) was not adjusted to prevent possible chemical conversions of any NIA by-products formed during the permeation studies. The mobile phase was degassed using an ultrasonicator (VWR International) prior to use to remove air bubbles. The flow rate of the mobile phase was 1 mL/min and the column temperature was maintained at 40 °C. Chromatograms were acquired at a wavelength of 263 nm. Sample volumes of 100 μ L (for semi-preparative LC) and 10 μ L (for analytical LC-MS) were injected for a total run time of 10 min. Samples were lyophilised using a Thermo Scientific Heto Powerdry LL1500 manifold freeze dryer equipped with a RC 6 chemistry-HYBRID rotary high vacuum pump (Vacuubrand®, UK), with the condenser temperature set at -113 °C.

Isolation of NIA related by-products using the organic route was not successful as no significant LC-MS trace signals were found following sample separation and characterisation. This could have been the result of an extended PBS environmental exposure that led to possible salt formation of NIA and other more polar related structures. However, compound isolation using the inorganic route resulted in the appearance of two LC peaks

which were categorised based on run retention times as NIA and a more polar unknown compound (Fig. 2). Subsequent preparative LC separation was conducted and LC-MS analysis of these two isolates demonstrated the existence of NIA: m/z 123 for NIA+H and 145 for NIA+Na (Fig. 2, D1 and D2); in addition to the presence of unknown NIA by-products (Fig. 2, E1 and E2).

Following analysis and characterisation of the mass spectrometry data, an additional molecular structure was confirmed in the porcine skin permeation samples and was identified as 1,4,5,6-tetrahydropyridine-3-carboxamide (Fig. 3, compound 3) [9]. With an m/z of 126 (m/z 127 for M+H, Fig. 4), this molecular entity is thought to be a product of the chemical reduction of NIA as it permeates through porcine skin. This conclusion is supported by the highest ion abundance shown in Fig. 4 (i.e. m/z 173) which indicates the presence of this same molecule as its ion adduct for M+H+2Na. The presence of a m/z 149 value (Fig. 4) also correlates with the presence of the M+Na ion adduct for 1,4,5,6-tetrahydropyridine-3-carboxamide, confirming the existence of this NIA by-product. Moreover, the minor increase in polarity of this compound compared to NIA is shown by its lower LC run retention time. Results also showed an m/z 151 value (Fig. 4) which may reflect the presence of piperidine-3-carboxamide (Fig.3, compound 4) for M+Na in addition to an m/z 171 value (Fig. 4) that suggests the existence of 1,4-dihydropyridine-3-carboxamide (Fig. 3, compound 2) for M+H+2Na.

To confirm that these species were uncharacterised ion adducts of NIA by-products, skin permeation experiments and the same LC-MS analysis was conducted with control formulations with no NIA. Analysis of NIA in PBS alone was also conducted to evaluate possible chemical derivatisation of the compound in the Franz diffusion cell receptor compartment. However, none of the molecular structures reported in Fig. 4 could be detected following these experiments.

Lo and Sajiki previously reported the chemical reduction of the pyridine ring in NIA as well as NIA structural analogues [9, 10]. Our findings are consistent with these authors, namely the presence of a number of compounds following the reduction of NIA. The results further suggest that the low recovery of NIA following skin penetration and mass balance studies reflects chemical derivatisation of the molecule during skin penetration. The next steps of the work will be to synthesise the NIA by-products by reduction of NIA to facilitate further LC-MS molecular classification and quantification of relevant NIA derivatives. This

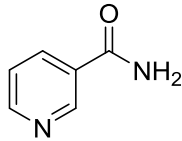
should enable the development of more sensitive methods to ensure full mass balance recovery of NIA following skin application.

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Table I – Physicochemical properties of niacinamide (NIA)

Molecular structure	
Molecular weight	122.13 Da [11]
<i>m/z</i>	122.05 (100.0%), 123.05 (6.5%) [11, 12]
Log P (<i>o/w</i>)	-0.37 [13]
pK _a	3.35 [14]
Solubility	1 g dissolves in about 1 mL water, in 10 mL glycerol, in about 1.5 mL alcohol [15]

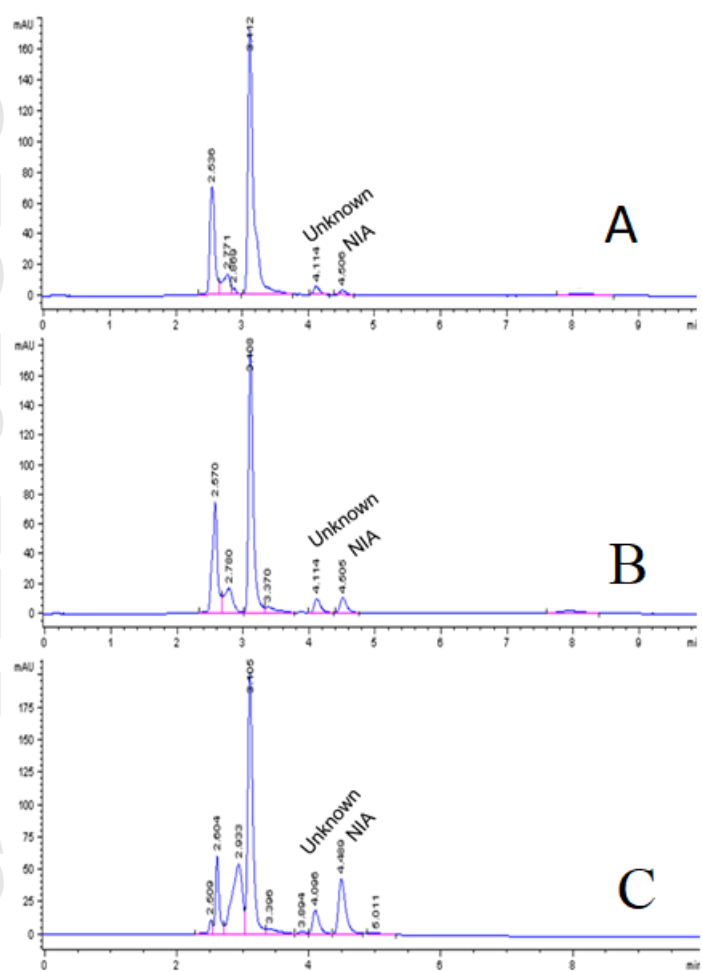


Figure 1 – NIA permeation study LC spectrums at different timepoints. *From top to bottom:* A - 2 hours; B - 12 hours; C - 24 hours

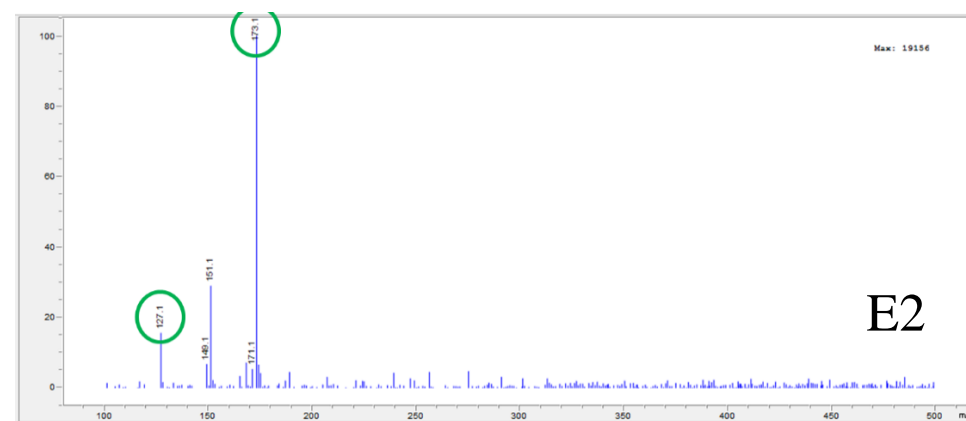
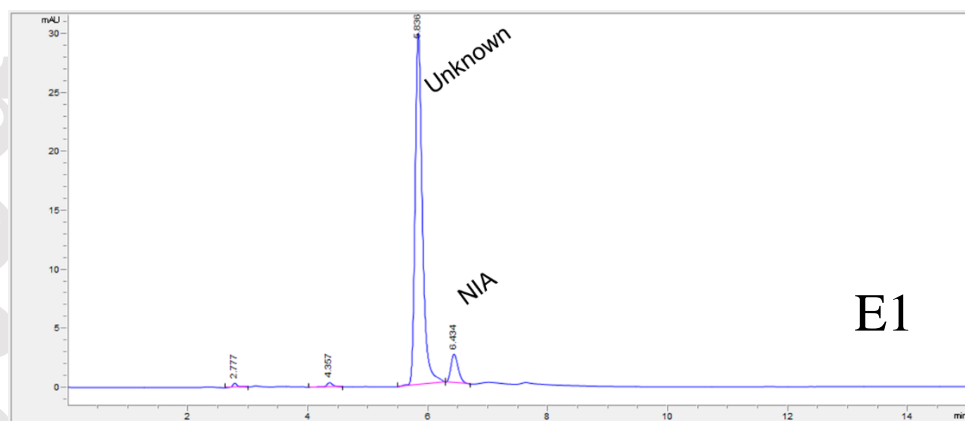
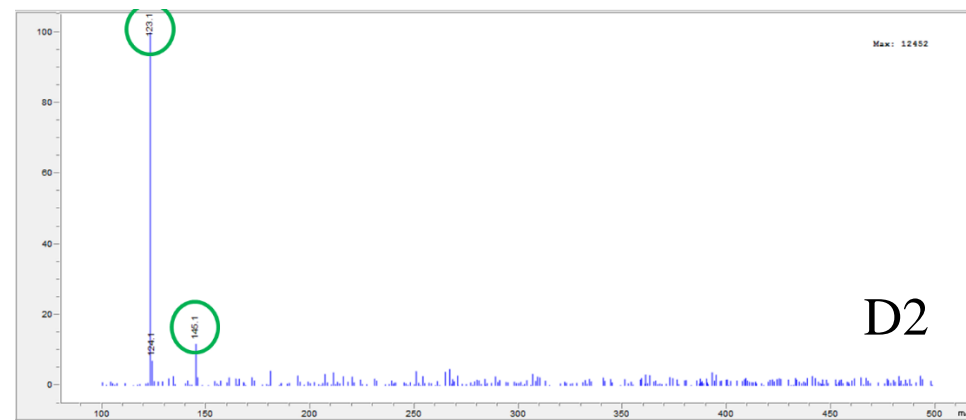
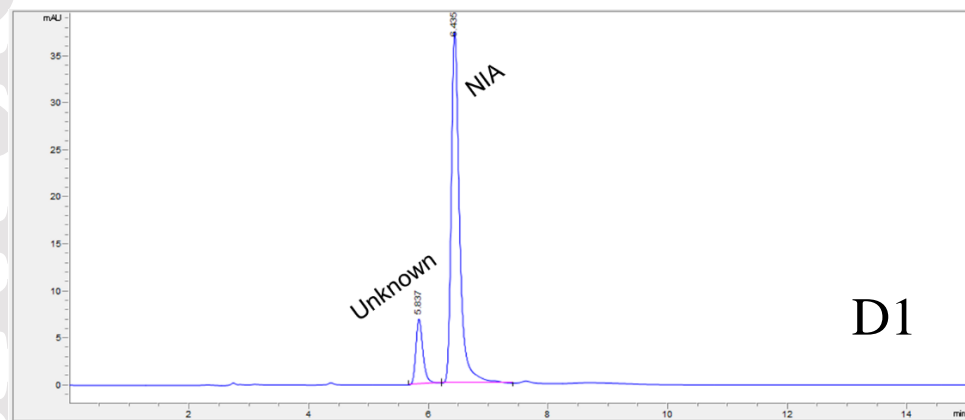


Figure 2 – NIA and unknown compound LC-MS spectrums. *Top*: D1 – NIA LC spectrum and D2 – NIA MS spectrum; *bottom*: E1 – Unknown compound LC spectrum and E2 – Unknown compound MS spectrum

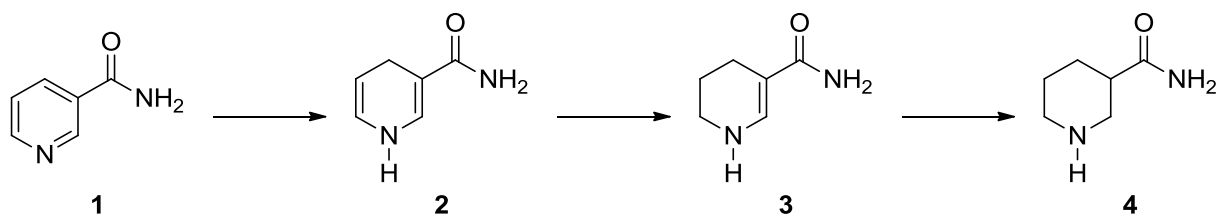


Figure 3 – Proposed NIA reduction pathway for the formation of its by-products

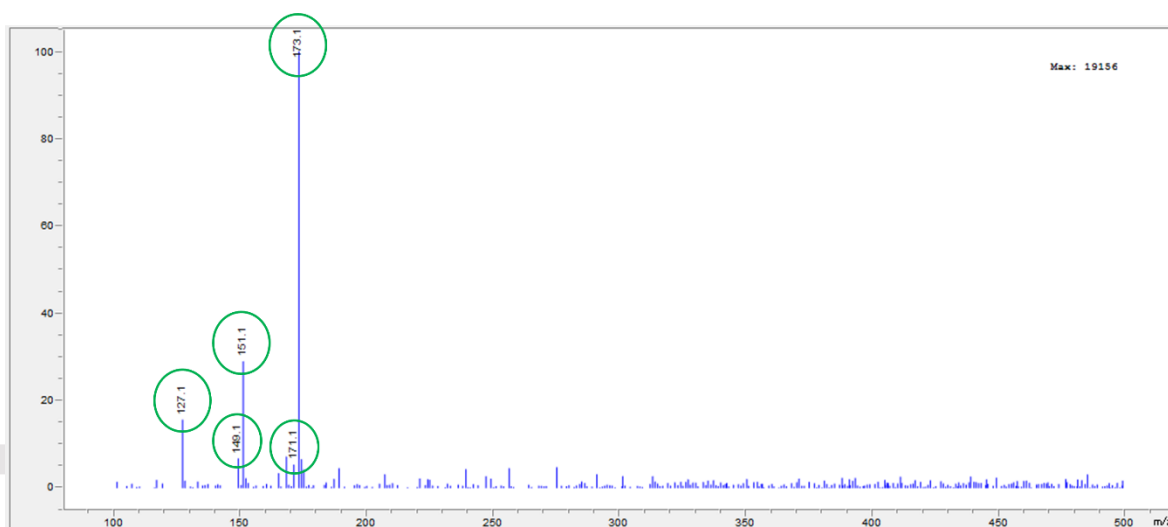


Figure 4 – Unknown NIA by-products MS spectrum