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The rational design of topical formulations

A thesis submitted in a partial fulfilment of the requirements for the degree of Doctor of Philosophy

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Declaration

I, Anna Małgorzata Duszyńska-Krupa, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Signature

Date

Abstract

This thesis addresses the development of topical formulations designed to treat atopic dermatitis (AD) using nicotinamide (NA). A rational approach to the development of topical formulations based on the physical and chemical properties of the drug and vehicle components is studied. This approach is an alternative to the model of formulation development where the drug is added into an existing vehicle without optimisation of the formulation in terms of the active delivery to its site of action.

The work encompasses preliminary pre-formulation studies, *in vitro* uptake and permeation studies using a model silicone membrane and pig ear skin. Moreover, the influence of topical formulations containing the model drug on the parameters indicative of skin health is tested in the *in vivo* studies.

The primary objective is to optimise the skin delivery of NA with the use of appropriate excipients. The solvents are chosen on the basis of their physicochemical parameters, namely solubility parameter (δ), mutual miscibility and ability to dissolve the model drug.

The performance of rationally developed simple formulations is tested *in vitro* and compared with prototype formulations containing more complex vehicles. *In vitro* uptake and permeation studies using silicone are performed to determine the influence of chosen solvents on NA permeation in a membrane which is less complex than skin. In addition NA skin delivery is evaluated with *in vitro* and *in vivo* techniques and the relationship between the physicochemical parameters of the solvents used and the drug percutaneous absorption is examined.

Finally, the efficacy of prototype NA formulations in improving the skin state *in vivo* is investigated. The performance of prototype formulations in terms of NA percutaneous absorption is determined with reference to their influence on the skin condition.

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I dedicate this work to Jakub, without him it would not be possible at all.

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List of Abbreviations

AD	Atopic dermatitis
AMC	Aminomethyl coumarin
CEf	Fragile corneocyte envelope
CEr	Rigid corneocyte envelope
CPE	Chemical penetration enhancer
D	Diffusion coefficient
DMSO	Dimethylsulfoxide
DPPG	Propylene glycol dipelargonate
DSC	Differential scanning calorimetry
FITC	Fluorescein isothiocyanate
FLG	Filaggrin
GLY	Glycerine, glycerol
HPLC	High performance liquid chromatography
IgE	Immunoglobulin E
IPM	Isopropyl myristate
J_{ss}	Steady state flux
KLK	Kallikrein
K_{oct}	Octanol/water partition coefficient
k_p	Permeability coefficient
LEKTI 5	Lympho-epithelial Kazal-type inhibitor 5
MO	Mineral oil, liquid paraffin
MW	Molecular weight
MSS	Multicomponent solvent systems
NA	Nicotinamide
NAD	Nicotinamide Adenine Dinucleotide
NADP	Nicotinamide Adenine Dinucleotide Phosphate
NMF	Natural moisturising factor
PA	Protease activity
PAR2	Protease-activated receptor 2
PARP	Poly-ADP-ribose polymerase
PE(20)OE	Polyoxyethylene (20) oleyl ether
PG	Propylene glycol

PGML	Propylene glycol monolaurate
PGMC	Propylene glycol monocaprylate
Q_A	Cumulative amount of a drug permeated per unit area
SC	Stratum corneum
SCCE	Stratum corneum chymotrypsin-like enzyme
SCTE	Stratum corneum trypsin-like enzyme
SD	Standard deviation
SEM	Standard error of mean
SLS	Sodium lauryl sulfate
SP	Serine protease
SPT	Serine palmitoyltransferase
TEWL	Transepidermal water loss
TGase	Transglutaminase
VE	Viable epidermis

1 Introduction

1.1 Skin anatomy and function

The skin is the largest organ of human body. It accounts for 15% of total body weight and has an average surface area of approximately $2m^2$. It allows for an effective communication between the endo- and exogenous environments and enables the maintenance of internal homeostasis of the human body [1-3].

Human skin is organized into three distinct layers:

- hypodermis – an insulator and protector, which consists of adipocytes specialised in fat storage,
- dermis – which nourishes skin appendages and connects all skin layers via capillary blood vessels and nerve endings; it is also responsible for mechanical resistance and elasticity of the skin thanks to the presence of collagen and elastin (noncellular connective tissue which accounts for about 70% of the dermis [4]),
- epidermis – the main barrier for exogenous substances permeation and endogenous water loss. The epidermis is described in detail in a later section.

The skin structure is represented in Figure 1.1.

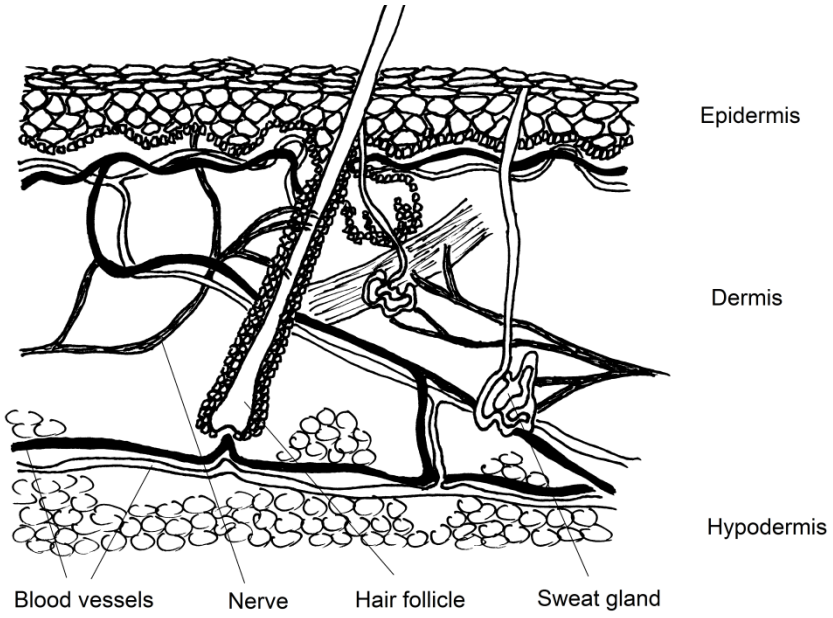

Figure 1.1 Schematic representation of the skin [adapted from 1]

1.1.1 Epidermis

The epidermis is the outermost, avascularised layer of the skin [5, 6]. The average thickness of the epidermis is about 0.1mm [7, 8]. However, depending on the site of the body, it varies from 0.06mm on the eyelids to 0.6mm on palms of the hands and soles of the feet [9, 10]. The epidermis can be divided into two layers: the stratum corneum (SC) and the viable epidermis (VE).

Epidermal cells, also called keratinocytes, are at various stages of differentiation progressing from the most inward layer of the epidermis (stratum germinativum) upwards to the stratum corneum [1]. A schematic representation of the stratified epidermal structure is shown in Figure 1.2.

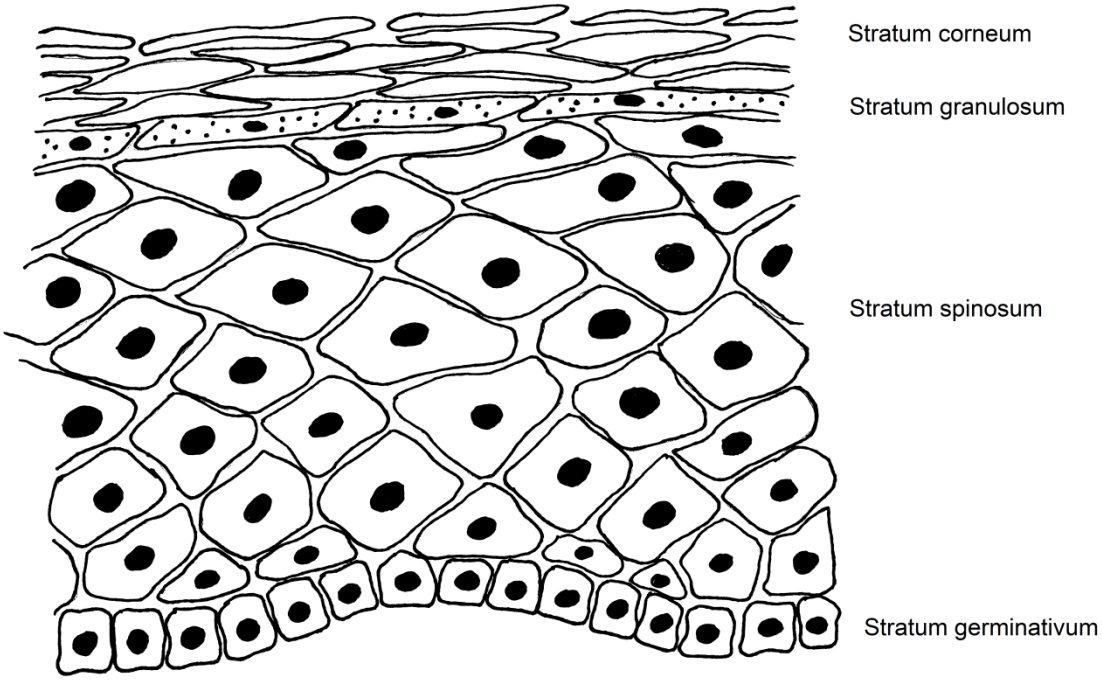

Figure 1.2 Structure of the epidermis

The adherence between the epidermis and the dermis is provided by the basement membrane connected to the stratum germinativum cells by proteinaceous hemi-desmosomes [11-13]. Adjacent cells in the VE are connected by desmosomes formed by transmembrane glycoproteins (desmoglein and desmocollin) associated with several cytoplasmic proteins [14].

Cells in the stratum geminativum are subject to mitotic division enabling renewal of the epidermis. It is estimated that in healthy skin total epidermal turnover time is approximately 40 days: 13 days of proliferation in the stratum germinativum, 10-14 days of differentiation and 14 days of desquamation from the stratum corneum [15-17].

1.1.1.1 Stratum corneum

Most of the skin barrier properties are localised in the stratum corneum (SC), the final product of epidermal cell differentiation [18]. Keratinocytes on their way to the SC lose their nuclei, become more flattened and transform into corneocytes. At the same time, keratin molecules are cross-linked and aligned in parallel inside the cell [19, 20]. The profilaggrin protein, processed into filaggrin (filament aggregating protein) is involved in the process of keratin aggregation [12, 21]. Further filaggrin digestion provides compounds responsible for skin moisturisation [12, 22, 23]. This mixture of free amino acids, their derivatives and salts is called natural moisturising factor (NMF) [22].

At the outermost surface of corneocytes, a 10-20nm thick cornified envelope (CE) is formed by protein and lipid components [24]. The main protein-bound lipids in the healthy skin (~50%) are ceramides connected to the extracellular proteins by ω-hydroxyester bonds [25]. Loricrin and involucrin are the main proteins involved in the formation of CE [12, 26]. The protein molecules are interconnected by γ-glutamyl links [27, 28]. The degree of cross-linking of the envelope proteins by the transglutaminases (TGases) increases during the SC maturation process. Thus, CEs can be divided into two types: fragile (CEf) and rigid (CEr). The latter type is characteristic for more mature corneocytes closer to the skin surface [28, 29]. The proteins, incorporated into the CE, are responsible for cohesion between the cells and the SC integrity [30, 31]. Also, a relationship between covalently bound ceramides and transepidermal water loss (TEWL) was found, implicating the importance of CE lipids in the skin barrier function [32].

In the process of epidermal maturation, lamellar bodies located in the stratum granulosum deliver key components for the permeability and antimicrobial barriers. These components include: lipid precursors, catabolic enzymes and antimicrobial peptides [33-35]. Lamellar bodies contribute to the formation of the lipid matrix by supplying enzymes involved in the synthesis of cholesterol, free fatty acids and ceramides [36]. They also secrete catabolic proteases which guarantee correct digestion of corneodesmosomes and epidermal desquamation [30, 37-39]. Two serine proteases from the kallikrein (KLK) family: trypsin-like KLK5 and chymotrypsin-like KLK7 are the main SC desquamatory enzymes [38].

The SC is perceived as a two-compartment structure. It consists of ten to fifteen layers of cells surrounded by a lipid matrix [40, 41]. This ‘brick and mortar’ model of the SC, proposed by Michaels et al. [42], is shown in Figure 1.3.

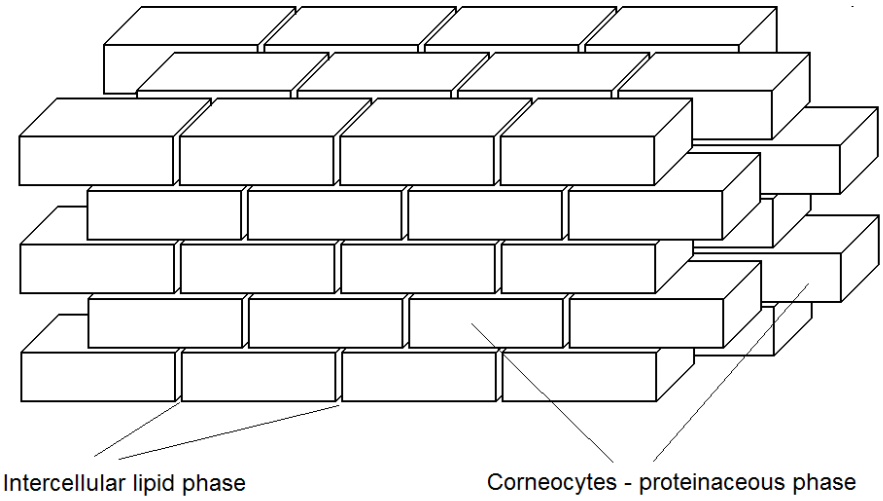

Figure 1.3 Idealised model of the stratum corneum [based on 42]

Each corneocyte is 0.5 to 1.5µm thick and has a diameter of approximately 35µm [43]. The thickness of the SC depends on the anatomic site, but usually it is about 10-20µm [1, 7, 44-46]. It is thickest on the palms and soles and thinnest on the lips [47, 48]. The SC mainly consists of protein (approximately 75% w/w) [49] while extracellular lipids account for 5-15% w/w of the SC dry mass [50, 51]. The unique composition of the SC extracellular lipid bilayers is reflected in the fact that many different ceramide types and no phospholipids can be found in this phase. The intercellular lipid matrix is composed of approximately 40% ceramides, 25% cholesterol, 18% cholesterol esters and 10% fatty acids [12, 52]. The lipid composition varies depending on the anatomic site and it influences epidermal barrier function [53-56].

1.2 Percutaneous absorption

Several steps are involved in the process of percutaneous absorption. The drug, after dissolution and diffusion in the vehicle, is subject to partitioning into the SC. The crucial role in this step is attributed to the relative solubility of the drug in the vehicle and the SC [57-59]. What follows is the diffusion across the SC and partition into the VE [60]. Further drug movement includes diffusion through the VE and the dermis, clearance through the blood and lymphatic circulation and partitioning into the subcutaneous fat and muscles [2, 61, 62]. On the way across the skin, the drug can interact with a receptor, be metabolised or form a reservoir [63, 64].

Although the SC is very thin (approximately 10µm) [46] compared with the viable epidermis and papillary dermis (both approximately 100µm), the highest diffusional resistance lies in the stratified structure of the SC. The role of other layers in impeding the diffusion process of a

drug is in most cases negligible [7]. The SC with its 'brick and mortar' organisation of corneocytes and intercellular lipids ensures skin function as a permeability barrier to topically applied penetrants [9, 65]. Skin impermeability can be particularly attributed to the high degree of molecular order in the extracellular lipids [9]. For example, water flux through SC was shown to increase with an increasing disorder of the lipids in the membrane [66].

The routes of percutaneous penetration were proposed in the 1960's. Tregear [67] postulated that the penetration of molecules through skin occurs 'through or around the horny plates' and that the appendageal route is most probably not significant in this process.

The molecule can penetrate the SC either intercellularly (by going around the corneocytes through the lipid matrix), intracellularly (or transcellularly, i.e. by taking the route across the cells and the lipids) or through the appendages (sweat glands or hair follicles) [9]. The three routes of penetration are shown schematically in Figure 1.4.

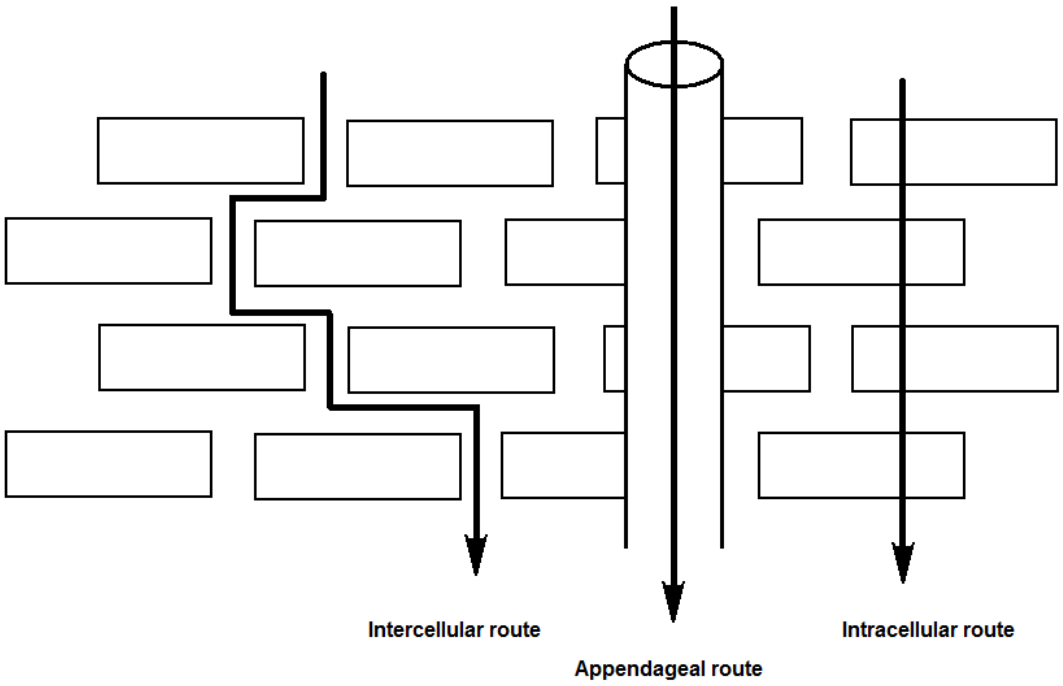

Figure 1.4 Possible routes of percutaneous penetration

The intercellular route of penetration is considered the most important for the majority of molecules [68]. First of all, the diffusional path length derived from Fick's laws of diffusion for model substances was found to be much higher than the actual thickness of the skin. The SC is approximately 10-20µm thick, but the estimated diffusional path length can be as long as 880µm [69]. Moreover, it was also possible to visualise molecular diffusion through this tortuous pathway [70, 71]. The intercellular route is rich in lipids which constitute ~10% of the

dry SC volume [51]. This volume may seem small, but it is the only continuous phase in the SC, thus it forms a preferential route for penetration of many substances [9].

The intracellular (transcellular) route requires the molecule to repeatedly partition and diffuse through the hydrophilic and very dense environment of corneocytes and the lipophilic intercellular spaces. Thus, this route, even though it is shorter, may not be the easiest and is not preferred by the majority of molecules [1].

Skin appendages cover only ~0.1% of the skin surface area. Thus, the appendageal pathway for skin penetration in most cases is considered insignificant. This route may be of importance for large polar molecules, ions or other slowly penetrating substances which would be able to diffuse faster through shunts, before steady state diffusion through and around the SC cells is established [1]. The hair follicles may be targeted by sub-micron sized particles as a result of massaging them into the lipids that surround the hair shaft.

Molecules penetrating through the superficial layers of the skin are removed from the dermis by the circulatory system. An abundant blood supply allows for the maintenance of 'sink' conditions for the penetrating compounds. Highly lipophilic materials may be transported away by the lymphatic system or protein bound within the blood plasma. This means that the concentration of the penetrating substance in the dermis remains close to zero allowing for maximal thermodynamic activity gradient across the skin. This gradient provides a driving force for diffusion and aids percutaneous absorption [1, 60, 72].

1.2.1 Interactions between drug, skin and vehicle

Drugs are rarely applied to the skin as pure compounds. Usually, the active substance is incorporated into a suitable vehicle which facilitates application and may also influence the skin barrier function [1]. The selective permeability of the skin depends not only on its physiological state but also on the properties of the vehicle and the drug [73, 74].

Percutaneous absorption depends on various physicochemical parameters of the permeant. The permeability of a drug can be estimated from such parameters as the octanol/water partition coefficient (K_{oct}) and molecular weight (MW) [75]. For systemic delivery, the drug needs to have a balanced water and lipid solubility allowing for the optimum partitioning behaviour into the lipophilic SC and the VE which is more hydrophilic [76]. Molecules with log K_{oct} between 1 and 3 are considered to be good candidates for transdermal drug delivery [76, 77]. On the other hand, the diffusion coefficient across the skin is related to MW [78]. Small molecules (i.e. the ones with lower MW) permeate faster across the skin [79]. Another

important factor is the drug melting point, which is inversely proportional to its transdermal permeability [80, 81]. In the case of weak acids or weak bases, permeation depends on the degree of ionisation. Ionised molecules have lower permeability coefficients than their unionised forms, which are the predominant diffusing species [82, 83].

Skin-drug interactions can include various forces: from weak Van der Waals attractions to strong chemical bonding, which may cause drug reservoir formation in the skin [1]. The skin is a hydrogen bond donor, thus a decrease in drug diffusion is expected with an increase in the number of hydrogen bonding groups [84, 85]. For some topical preparations, reservoir formation or adherence and binding of drug molecules to the skin components may be crucial for the achievement of the desirable effect [61, 86]. The metabolism of the permeant by the skin enzymes may also be important in determining the extent of percutaneous absorption [87-89].

Drug-vehicle interactions are equally important in the percutaneous absorption process [90]. The thermodynamic activity of the drug in the vehicle is crucial for the diffusion process and is related to the solubility of the drug in this vehicle [91]. Also, the release of the drug from the vehicle may be rate-limiting, and in this case, skin barrier is not the major factor which influences the diffusion of the drug [1].

The vehicle can also have an effect on skin hydration and temperature [92-94]. At the same time, body temperature and water present in the skin can influence vehicle components. Solvents present in the vehicle may reversibly or irreversibly alter barrier properties of the skin or damage it. These substances can enhance or retard penetration and their properties are exploited to achieve required skin delivery of the compound of interest [1].

1.2.2 Chemical penetration enhancers

Various techniques can be used to deliver the drug from the formulation to the skin (topical drug delivery) or across the skin to the systemic circulation (transdermal drug delivery) [95]. The use of chemical penetration enhancers (CPEs) is a widely investigated and commonly applied approach to increase percutaneous absorption [see the following patent literature reviews: 96, 97].

CPEs are the components of topically applied formulations that reversibly decrease the resistance and enable drug access into the deeper layers of the skin or to the systemic circulation [52].

The ideal penetration enhancer should:

- be non-toxic, non-irritant or non-allergenic (i.e. safe to use),
- not be pharmacologically active,
- have immediate onset of action,
- have immediate and full reversibility in terms of changes in the SC properties upon removal of the enhancer,
- be chemically and physically compatible with other formulation components,
- be cosmetically acceptable with good spreading and feel on the skin surface,
- be a good solvent for the drug,
- be inexpensive, odourless, tasteless and colourless [1, 98, 99].

Probably no enhancer, which combines all these properties, exists. Water is considered one of the most ideal compounds since an increase in the skin hydration leads to a decrease in the diffusional resistance of the skin [100]. In some cases occlusion can also enhance penetration by increasing the water content of the SC, but occlusive vehicles are not considered to be penetration enhancers, since they do not interact with the structures inside the SC [99].

The main mechanisms of action of penetration enhancers include:

- an increase of skin hydration [100],
- an extraction of intracellular lipids (e.g. ethanol) [101],
- an increase of drug partitioning into the skin (e.g. isopropyl myristate (IPM) and ethanol) [102, 103],
- an increase of drug diffusion through the skin (e.g. oleic acid and IPM) [104, 105]
- prevention of crystallisation of the drug on and in the skin (e.g. IPM and ethanol).

CPEs can act on different components of the skin interacting with SC lipids in their polar or lipophilic regions or influencing SC proteins [52]. A disruption in the packaging of the lipids fluidises the lipid domain and increases the water volume between the bilayers. Thus, the penetration of both lipophilic and hydrophilic molecules is enhanced [59]. Solvents such as propylene glycol are believed to increase drug solubility in the aqueous domain of lipid bilayers, by altering the solubility parameter of the membrane [106].

The solubility parameter (δ) is one of the values taken into account when choosing the penetration enhancer. This parameter was defined by Hildebrand and Scott [107] as the sum of the cohesive forces in the molecule. Hansen further developed this approach proposing a three-dimensional δ value where dispersion, polar and associative (hydrogen bonding)

attractions are the components of the total cohesive energy of a liquid [108]. When a solvent with an appropriate δ value is chosen, it can increase the drug solubility in the membrane, by shifting the δ value of the skin [109] towards the δ value of the drug. Increased solubility in the skin means increased affinity of the drug for the membrane and thus, the transport of the drug is increased [108, 110, 111]. It may also help in the identification of solvents, which prevent crystallisation of the drug on and in the skin.

1.3 Investigation of percutaneous absorption *in vitro*

There are several advantages of the use of *in vitro* techniques in the assessment of percutaneous absorption. One is the simplicity of experimental conditions which enables greater insight into the role of excipients in the transport of compounds through the skin. There is no need for extensive sample preparation, as opposed to the quantification of the drug in blood or excreta samples obtained during *in vivo* studies. *In vitro* techniques also enable the optimal use of human or animal tissue minimising the need for laboratory animals and human volunteers [112]. It is especially important when formulations exhibit toxic or irritant properties [113]. In comparison with *in vivo* investigations, *in vitro* methods require less space, have smaller variability and allow for correlation between human and animal tissue when the same experimental conditions are maintained [114].

A number of *in vitro* methods are used to study percutaneous absorption. The most common are diffusion methods, where a drug is applied to the donor compartment of a diffusion cell and permeation is evaluated by measuring the concentration of the drug in the receptor. According to the US Food and Drug Administration (FDA), the recommended *in vitro* method for evaluation of topical formulations is based on an open chamber diffusion system (e.g. Franz-type diffusion cell) [115]. Diffusion experiments without a barrier membrane give information on drug-vehicle interactions and release mechanisms. Permeation experiments using human, animal or artificial membranes may give more insight into the drug-vehicle-membrane interactions [1]. A Franz-type diffusion cell is shown in Figure 1.5.

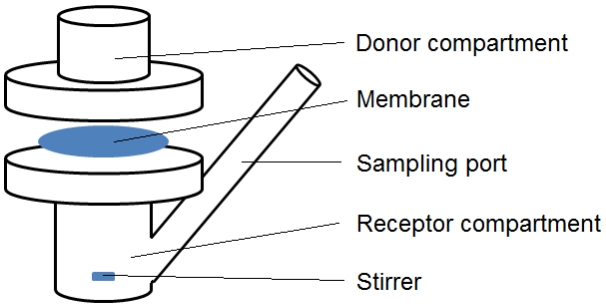

Figure 1.5 Franz-type diffusion cell

In permeation studies, the membrane is chosen based on its resemblance to the *in vivo* situation. However, human skin can often be difficult to obtain. Instead, polymeric membranes or animal skin have been used as mechanistic models of the skin.

The most popular and extensively studied artificial membranes are lipophilic silicone membranes [58, 116-125]. Dimethylpolysiloxane (silicone) membrane is a homogenous barrier, which can be used as a model for human skin because of its hydrophobicity [123]. The evaluation of silicone membrane as a relevant model for prediction of percutaneous absorption was attempted by several authors. A comparison of ibuprofen diffusion studies from supersaturated systems showed a linear correlation between skin and silicone data [126]. On the other hand, no correlation between silicone membrane and skin was found for caffeine permeation from saturated solvent systems and cosmetic formulations [127]. However, the authors concluded that diffusion studies across synthetic membranes can give valuable information in terms of quality and batch-to-batch variability of topical products. They also provide information about the thermodynamic activity of the active in the formulation.

Animal models for percutaneous absorption include several species such as rat, rabbit, monkey and guinea pig [128-130]. *In vivo* studies showed that skin permeability for a series of radioactive compounds is highest for rabbit, followed by rat, pig and human skin [128]. This and many other studies suggest that pig skin has a better predictive value for human skin permeation than the skin of rodents. Porcine skin is preferred to other animal membranes because of its similarity to human epidermis in terms of morphology and permeability [51, 131-133]. Although human skin is more robust to freezing and usually is less permeable many authors support the use of porcine skin, especially from the outer region of the ear [113, 134-138].

Comparisons between human and animal skin listed above showed that there are major differences in the skin permeability between species. Thus, human skin is postulated to have

the best predictive value for the *in vitro* assessment of percutaneous absorption [139, 140]. Human skin may be obtained from cadavers, biopsies or surgery and it usually maintains its integrity upon long-term storage (up to one year at -20°C) [141-143]. The permeability of human skin varies between individuals and within individuals depending on the anatomic site. Southwell et al. [144] reported the inter-individual and intra-individual variation in *in vitro* studies to be as high as 66±25% (n=45) and 43±25% (n=32), respectively.

1.4 Physicochemical models of percutaneous absorption

The level of molecular transport and absorption through the skin depends on numerous factors, such as the dose, vehicle, length of time of application, anatomic site and skin state [145]. Nevertheless, there have been attempts to analyse and understand the process, taking into account idealised conditions. Mathematical modelling of percutaneous absorption includes physicochemical and pharmacokinetic models. This section will focus on the physicochemical diffusion models of percutaneous absorption.

1.4.1 Fick's laws of diffusion

Diffusion is the transport caused by random molecular motion [146]. Since percutaneous absorption is the process of passive diffusion, it is possible to analyse permeation data and to predict permeation parameters using Fick's laws of diffusion [1, 147].

The driving force for passive diffusion is the chemical potential gradient across the membrane $\left(\frac{\partial \mu}{\partial x}\right)$. The chemical potential gradient is often simplified to the concentration gradient $\left(\frac{\partial C}{\partial x}\right)$, but strictly speaking, it is the difference in thermodynamic activity of the drug across the diffusional pathway that enables diffusion [91].

Fick's first law of diffusion [148] relates the concentration gradient to the diffusion coefficient (D) in the following manner [78]:

$$J = -AD\frac{\partial C}{\partial x}$$

Equation 1.1

where:

J - steady state flux [µg/cm^2/h],

A - diffusion area [cm^2],

D - diffusion coefficient [cm^2/h],

$\frac{\partial C}{\partial x}$ - concentration gradient across the membrane.

To describe the steady state diffusion Fick's first law is simplified to:

$$J = -ADK_{m/v}\frac{\Delta C}{h}$$

Equation 1.2

where:

$K_{m/v}$ - partition coefficient of the compound between the membrane and the vehicle,

ΔC - concentration difference across the membrane [µg/cm^3],

h - membrane thickness or diffusional pathlength [cm].

Usually the concentration of the drug in the vehicle, i.e. the concentration applied to the surface of the membrane is significantly higher than the concentration under the skin surface and $\Delta C \approx C_v$, where C_v is the concentration of the drug in the vehicle. Thus:

$$J = ADK_{m/v}\frac{C_v}{h}$$

Equation 1.3

The diffusion coefficient reflects the rate of penetration of a specific molecule under specific conditions. The partition coefficient describes the affinity of the drug for the vehicle and the SC. However, the diffusion coefficient (D) and the partition coefficient (K) may be difficult to deconvolute. What is more, flux calculated with these two parameters depends on the diffusion pathlength which in the case of the skin diffusion process through the tortuous route is difficult to determine. The permeability coefficient (k_p) [cm/h] is related to these three parameters according to the following equation:

$$k_p = \frac{KD}{h}$$

Equation 1.4

Hence, the flux calculation can be simplified to:

$$J = k_p C_v$$

Equation 1.5

According to Fick's laws of diffusion, for simple solutions, the flux shows a linear increase with C_v until it reaches the solubility limit in the vehicle. For suspensions (saturated solutions), which have enough undissolved drug particles to replace the diffusing molecules, flux is constant. This is because the thermodynamic activity (or chemical potential) of the drug in

these systems stays the same during the permeation process (as long as there is enough of undissolved drug to prevent its depletion from the solution). Drug that is diffusing through the membrane must be in solution. Hence, the concentration available for the process is equal to the solubility of the drug in the vehicle [149].

After about 3 times the lag time, a straight line of the cumulative amount of the drug permeated against time should be obtained, meaning that steady state conditions are achieved. Experiments with skin or artificial membranes can lead to achievement of pseudo steady state conditions from which flux values can be calculated. However, taking into account only the steady state part of the data can be erroneous and may be a serious limitation. Factors such as maintenance of sink condition and prevention of drug and solvent depletion have to be taken into account when designing an experiment. Thus, modelling of the non-steady and steady state data is attempted. The second Fick's law is used in this case [78]:

$$\frac{\partial C}{\partial t} = D\left(\frac{\partial^2 C}{\partial x^2}\right)$$

Equation 1.6

Fick's second law relates the change in concentration of the drug in the membrane to the rate of change in the concentration gradient relative to any point within the membrane, depending on time (t) and position (x). This equation is a second order partial differential equation, difficult to solve in a real time domain. In this case, full curve analysis by fitting all the experimental data to Fick's laws by an iterative least squares computer program can be used, utilizing for example the Laplace transform approach.

It should be noted that, the following assumptions hold to apply Fick's laws for studying percutaneous absorption [78, 150]:

- skin permeation is a simple passive diffusion process,
- the SC is the major, rate limiting step in the percutaneous absorption process,
- the diffusion coefficient is constant during permeation,
- the diffusion occurs in one direction across a homogenous and inert membrane of a defined thickness and cross sectional area.

1.4.2 Laplace transform

The Laplace transform is an integral transform method which enables solution of the ordinary and partial differential equations [78]. The Laplace transform $\bar{F}(s)$ of a function $f(t)$ is defined as:

$$\bar{F}(s) = \int_0^\infty e^{-st} f(t) dt$$

Equation 1.7

where s is a Laplace variable and the bar over the function symbol indicates that the function is written in the Laplace transform.

When the Laplace transform method is applied to the Equation 1.6, the time variable (t) is replaced with the Laplace variable (s) according to:

$$L\left[\frac{\partial f(x,t)}{\partial t}\right] = s\bar{L}[f(x,t)] - f(x,0)$$

Equation 1.8

where $C(x,0) = C_0$ in the region of $0 \leq x \leq h$ of the membrane thickness.

Thus, the Laplace transform reduces the partial differential equation to the following ordinal differential equation:

$$\frac{d^2\bar{C}}{dx^2} - \frac{s}{D}\bar{C} = -\frac{C_0}{D}$$

Equation 1.9

A general solution of Fick's law of diffusion is thus described by:

$$\bar{C}(x) = asinh(\lambda x) + bcosh(\lambda x) + \frac{C_0}{s}$$

Equation 1.10

where $\lambda = \sqrt{\frac{s}{D}}$ and a, b are constants determined from the boundary conditions.

The use of Laplace transforms for predicting percutaneous absorption began in the late 1970s [151]. However, it became more popular with the development of mathematical software, such as Scientist® (MicroMath Scientific, USA) [152]. The software inverts data from the time domain into the Laplace domain where it fits the experimental data to Fick's equations with appropriate boundary conditions. Modelling of the permeation data using Laplace transform and Scientist® software has been described by previous authors [119, 121].

1.4.3 Analysis of the permeation data

The analysis of the permeation data depends on the boundary conditions associated with the design of the experiment [150]. In general, two types of Franz cell diffusion experiments can be conducted: infinite and finite dose studies. The typical permeation profiles obtained for finite and infinite dose studies are shown in Figure 1.6.

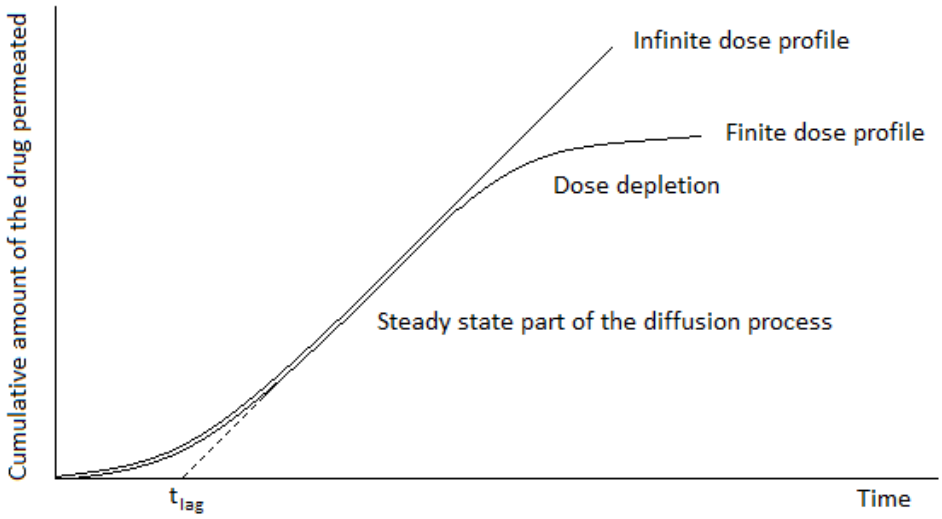

Figure 1.6 Typical permeation profiles for finite and infinite dose studies

1.4.3.1 Infinite dose studies

Infinite dose studies are commonly used to study the influence of CPEs on the skin permeation of a model compound. In this technique, a saturated suspension of a drug in a vehicle is applied to the donor compartment of a diffusion cell. The drug permeating from the vehicle is replaced by the drug dissolved from the crystals, allowing for the maintenance of a constant donor concentration. In the ideal situation, application of a saturated suspension of the drug in a range of vehicles should result in the same flux, provided that the vehicle components have no influence on the membrane [123].

The permeation data are often presented as the cumulative amount of the compound permeated per unit surface area of the membrane [µg/cm^2] against the collection time in hours [h]:

$$Q_A = \frac{V_R C_n + V_s \sum(C_1 + \cdots + C_{n-1})}{A}$$

Equation 1.11

where:

Q_A - cumulative amount of the compound permeated per unit area [µg/cm^2],

V_R - total volume of the receptor phase in the cell [cm^3],

C_n - concentration of the sample at the nth time point,

V_s - volume or the receptor phase sampled at each time point [cm^3],

A - diffusional area of the Franz cell [cm^2].

Permeation profiles are plotted as a $Q_A(t)$ curve. For infinite dose studies, the steady state flux can be derived from the slope of the linear part of this curve using linear regression, while t_{lag} can be expressed as the x-intercept of the steady state line. This is a simplified approach to permeation data analysis, where no iterative least squares calculations are necessary and simple straight line equations are used [153]. However, difficulties in visual determination of the steady-state data can be encountered. Also, the duration of the experiment might be insufficient to achieve steady state flux for slowly diffusing compounds and can lead to underestimation of the J_{ss} and lag time period (t_{lag}) [154]. The t_{lag} and the time needed to achieve the steady state (t_{ss}) can be described as [153]:

$$t_{lag} = \frac{h^2}{6D}$$

Equation 1.12

$$t_{ss} = 3\ t_{lag}$$

Equation 1.13

The following boundary conditions are used to simplify the solution of Fick's second law and to describe the infinite dose study [155]:

- No drug in the membrane ($0 \leq x \leq h$) at the beginning of the experiment ($t = 0$)

$$C_m\ (x, 0) = 0$$

Equation 1.14

- There is an equilibrium of the drug concentration between the donor and the membrane defined by the drug partitioning:

$$K_{m/v} = \frac{C_m}{C_v}\ (0, t)$$

Equation 1.15

where:

$K_{m/v}$ - partition coefficient between the membrane and the vehicle,

C_m - the drug concentration in the membrane,

C_v - the drug concentration in the donor.

- The receptor phase acts as a 'sink'

$$C_R(h,t) = 0$$

Equation 1.16

The boundary conditions for an infinite dose diffusion study across a homogenous membrane of a finite thickness (h) are shown in Figure 1.7. The donor phase has a constant concentration of the drug (C_v) and the receptor phase acts as a perfect 'sink'.

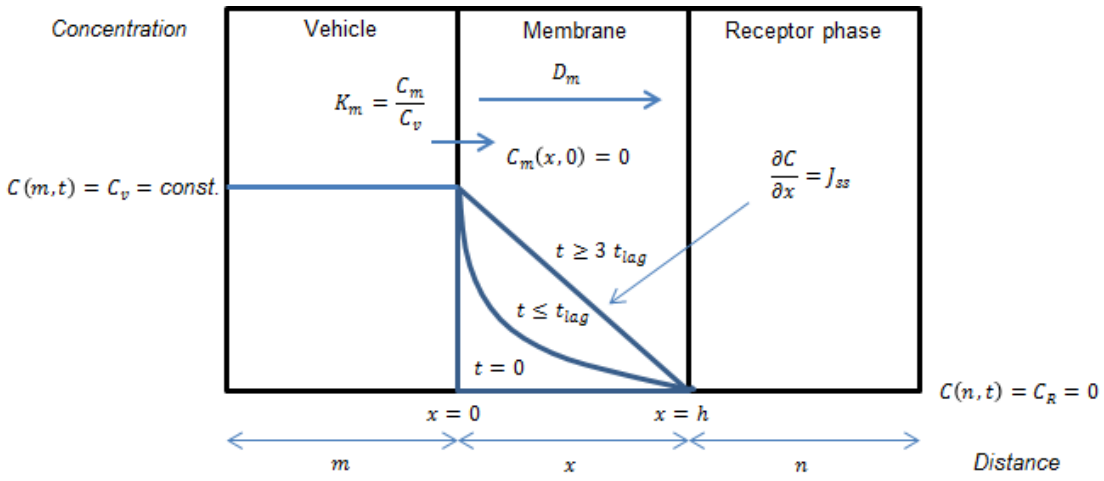

Figure 1.7 Schematic representation of an infinite dose study (the profile shown is for a partition coefficient of 1)

Applying the above summarised boundary conditions to the general solution of Fick's law (Equation 1.10) gives:

$$\bar{C}(0) = \frac{KC_v}{s}$$

Equation 1.17

$$\bar{C}(h) = \frac{C_R}{s} = 0$$

Equation 1.18

and allows calculation of the constants a and b in Equation 1.10:

$$a = -\frac{C_v K\ cosh(\lambda h)}{s\ sinh(\lambda h)}$$

Equation 1.19

$$b = -\frac{C_v K}{s}$$

Equation 1.20

After substitution of the constants into Equation 1.10 and appropriate mathematical transformations the equation which represents the concentration change at any point in the membrane is derived:

$$\bar{C}(x) = \frac{KC_v sinh[\lambda(x-h)]}{s\ \sinh(\lambda h)}$$

Equation 1.21

Thus, the transform for the flux (described in Equation 1.1) is:

$$\bar{J}(x) = \frac{AKC_v cosh[\lambda(x-h)]}{\lambda\ \sinh(\lambda h)}$$

Equation 1.22

The cumulative amount of the drug permeated over the time into the receptor solution can be derived as:

$$\overline{Q_A} = \frac{\overline{\bar{J}(s)}}{s} = \frac{AKC_v}{s\sqrt{\frac{s}{D}}\ \sinh\left(\sqrt{\frac{sh^2}{D}}\right)}$$

Equation 1.23

After fitting the steady and non-steady state permeation data to Equation 4.6 it is possible to determine D and K parameters for the best fit automatically inverting from Laplace to the real time domain. This equation is used to model the permeation data obtained from the silicone membrane studies, where the diffusion path length (h) is known.

Alternatively, parameters of P_1 [cm] and P_2 [hour^{-1}] can be obtained which are related to the diffusion (D) and partition (K) coefficients and h in the following manner [156]:

$$P_1 = Kh$$

Equation 1.24

$$P_2 = \frac{D}{h^2}$$

Equation 1.25

From these parameters, the permeability coefficient can be obtained:

$$k_p = P_1 P_2$$

Equation 1.26

P_1 (partition parameter) and P_2 (diffusion parameter) are used when the exact h is not known i.e. for the skin permeation studies [157]. In this case the equation for modelling of the permeation data is:

$$\overline{Q_A} = \frac{AP_1C_v}{s\sqrt{\frac{s}{P_2}}\sinh\left(\sqrt{\frac{s}{P_2}}\right)}$$

Equation 1.27

1.4.3.2 Finite dose studies

Finite dose permeation studies imply different boundary conditions to those encountered in the infinite dose studies. The three basic boundary conditions, listed for the infinite dose study, remain true also for the finite experiment. However, a finite dose of the drug is applied to the donor compartment and depletion of the drug from the vehicle occurs in the course of the study. Figure 1.8 shows a schematic representation of the boundary conditions for the finite dose study.

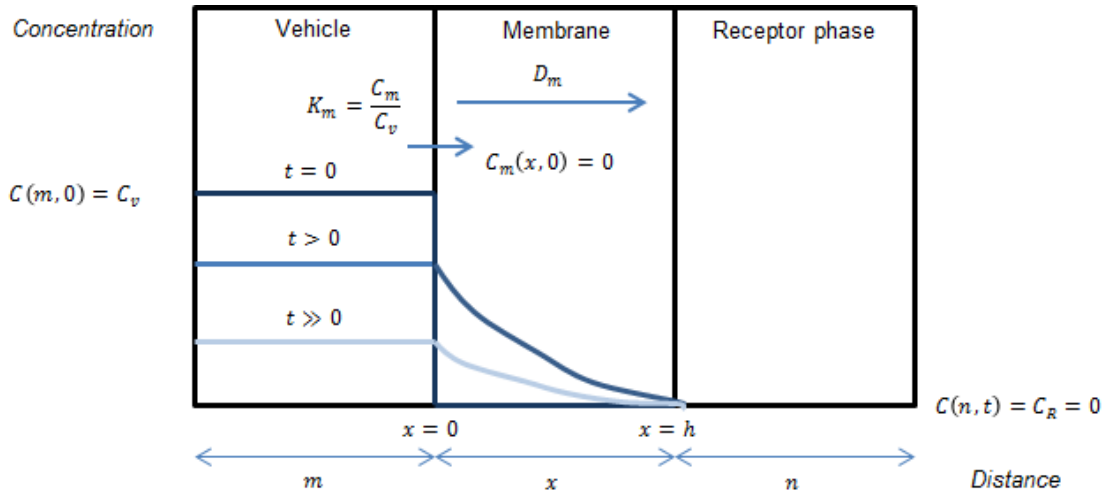

Figure 1.8 Schematic representation of a finite dose study

Mathematical models for finite dose studies take into account the depletion of the drug, thus the flux is not constant and changes with the changing concentration of the drug in the vehicle according to:

$$V\frac{\partial C_v}{\partial t} = -AD_m\frac{\partial C}{\partial x}(0,t)$$

Equation 1.28

where V is the volume of the vehicle applied to the membrane.

The Laplace transform of Equation 1.28 after appropriate mathematical transformations results in the following equation for the modelling of the finite dose studies:

$$\frac{\partial \bar{C}}{\partial x} = acosh(\lambda x) + bsinh(\lambda x)$$

Equation 1.29

Combining this equation with the general solution of Fick's law for the diffusion studies (Equation 1.10) and applying appropriate mathematical transformations the following equations are obtained for model membranes:

$$\overline{Q_A} = \frac{KC_vV\sqrt{\frac{D}{s}}}{s^2\left(AKcosh\sqrt{\frac{sh^2}{D}} + Vsinh\sqrt{\frac{sh^2}{D}}\right)}$$

Equation 1.30

and for the skin:

$$\overline{Q_A} = \frac{AP_1Q_0}{s\left(V\sqrt{\frac{s}{P_2}}sinh\sqrt{\frac{s}{P_2}} + P_1Acosh\sqrt{\frac{s}{P_2}}\right)}$$

Equation 1.31

where Q_0 is the amount of the drug applied to the donor compartment.

Modeling of both steady and non-steady state data according to these equations results in obtaining the permeation parameters: D and K or P_1 and P_2, for silicone membrane and skin finite dose permeation studies respectively.

1.5 Skin assessment *in vivo* – minimally invasive techniques

In vivo methods for prediction of percutaneous absorption and investigation of skin properties are considered to be more reliable than *in vitro* methods. However, whether humans or laboratory animals are involved, special care must be taken to perform the research ethically. The Declaration of Helsinki is considered to be the primary document listing the ethical principles for medical research involving human subjects [158].

When *in vivo* methods are further considered, the studies involving humans have the best predictive value for the performance of formulations tested. Animal skin is usually more permeable and thus, it is often not possible to predict percutaneous absorption in humans from animal studies [159].

In vivo penetration of the drug can be assessed by a variety of assays: from the quantification of the molecule in the blood, urine and faeces, to pharmacological response monitoring [160]. Depth concentration profiles of the drug can be obtained with skin stripping procedures [161]. Appropriate mathematical modelling of the data can give diffusion and partition coefficient values, enabling estimation of drug bioavailability [162, 163]. Combining tape stripping with spectroscopic methods enables drug assay in the skin [164]. Also skin state assessment can give an indirect measurement of bioavailability of the active, which has an influence on the skin properties. The skin state can be assessed with a whole array of non-invasive or minimally invasive techniques such as tape stripping combined with measurement of transepidermal water loss [165] or spectroscopic techniques such as ATR-FTIR [166, 167]. One of the non-invasive techniques to investigate skin conditions *in vivo* is Confocal Raman Spectroscopy [168, 169]. Evaluation of drug penetration can be done by observation of the pharmacological response such as vasodilatation or vasoconstriction [62, 70, 72, 170, 171]. These techniques

can also provide information on how excipients present in the formulation influence the permeation process of the drug and the state of the skin [172].

1.5.1 Tape stripping

Tape stripping is a method widely used in skin physiology, morphology and SC barrier research [172-178]. In this method adhesive tapes are pressed to the skin surface and thereafter the superficial layers of the SC are removed together with the tape [179, 180]. The tapes together with the removed tissue can be subject to further investigations such as assessment of the morphological and physiological properties of the stripped tissue [179, 181] or percutaneous penetration and retention of the drug applied topically [64, 103, 165]. Combining tape stripping with TEWL measurements can give information on skin resistance to damage [182, 183] and the depth of SC that is reached with the stripping procedure [184]. Various factors, such as the method of pressing the tape to the skin surface, pressure degree and duration, together with the type of tape used, can influence the outcome of tape stripping [173, 185]. Thus, it is crucial to establish all the experimental conditions involved in the stripping technique. This will enable the maintenance of the same conditions throughout the experiment and will allow for comparison between different studies [172].

1.5.2 Protein content

The analysis of the SC can involve quantification of the active component or the drug in the subsequent layers of the tissue which would result in the creation of a drug distribution depth profile [103, 163, 165]. The amount of SC removed with consecutive tapes from the skin surface can also be a good indicator of the tissue cohesion and thus gives information on the state of the skin [180, 186].

SC protein quantification on tape strips can be performed by a variety of analytical procedures, such as the gravimetric method [103, 187], or protein extraction and colorimetric quantification [174, 188]. These methods are usually difficult to perform, time consuming and often prevent the analysis of any other analyte on the same tape. Spectroscopic methods, which correlate the amount of protein with optical absorbance allow for quantification of the amount of the SC removed without damaging the sample [178, 189-191]. The results obtained with absorbance measurements are in agreement with traditional colorimetric measurements [174, 192].

Voegeli et al. [178] validated a method for SC protein quantification by infrared densitometry. Those workers derived calibration curves, which can be used for protein evaluation on tape strips by infrared absorbance measurement. This indirect method enables further analysis of

the tapes, because it does not destroy the SC samples. The optical absorption of SC tape strips proved to be linearly proportional to the protein content. The method is based on the determination of the absorption of the tapes after removing the SC layer at the infrared wavelength (850nm) according to:

$$A = I - \left(\frac{I_0}{I}\right)$$

Equation 1.32

where:

A - absorption [%],

I - intensity of the transmitted light,

I_0 - intensity of the incident light.

Data on the drug position in the SC (i.e. amount of drug quantified in the consecutive tapes at a given skin depth) can be normalized to the amount of the SC removed with the tape (i.e. the amount of protein on the tape). In this dermatopharmacokinetic approach, parameters describing drug transport in the SC (such as partition and diffusion coefficient) can be obtained [160, 165].

1.5.3 Transepidermal water loss

Transepidermal water loss (TEWL) measurements provide information on the SC barrier function against water loss [193]. TEWL indicates the extent of barrier improvement or disruption caused by various factors, such as experimental procedures [183, 194], dermatological products [195, 196] or diseases [197]. TEWL measures the quantity of water that passes from the inside to the outside of epidermal barrier by diffusion or evaporation (usually in [g/m^2/h]) [198, 199].

TEWL is used as an endogenous standard for *in vitro* permeation studies [200, 201] and as a skin water holding capacity indicator in the *in vivo* studies [199, 202, 203]. It has been shown that reduction in SC hydration correlates with increased values of TEWL, which indicated the barrier impairment [204]. Hence, TEWL can be considered as a valuable source of information in dry skin condition investigations [205].

TEWL reflects the hydration of the SC as described in terms of Fick's first law of diffusion in the following equation [148]:

$$TEWL = \frac{KD\Delta C}{h} = k_p \Delta C$$

Equation 1.33

where:

K - partition coefficient of water between the SC and the viable epidermis,

D - average apparent diffusion coefficient of water in the SC,

h - thickness of the SC,

ΔC - water concentration difference across the SC,

k_p - permeability coefficient of water across the SC.

It is worth noting that TEWL may be affected by environmental factors such as temperature, relative humidity and the season of the year [206, 207]. TEWL may also vary depending on the anatomic site [208, 209] and demographic parameters [209, 210]. Thus, it is crucial to control and take note of the experimental conditions during the TEWL measurement procedure [211].

The relationship between TEWL and skin hydration or dryness may not always be straightforward. Higher than normal levels of TEWL can be encountered not only in dry skin conditions, such as atopic dermatitis [186], but also in hyper hydrated skin [183, 194]. Usually the lowest values of TEWL are expected for healthy skin, but this may be also the case for skin, which appears to be dry in its outer layers and has the permeability barrier situated in the lower parts of the SC [212]. TEWL measurements are correlated with the thickness of the SC removed with tape stripping, i.e. the deeper stripped the tissue the higher the values of TEWL [165]. This is in agreement with the statement that SC cells and intercellular lipids are the main permeability barrier of the skin [172]. Thus, TEWL measurements after sequential removal of the SC with tape stripping procedure may provide information on skin integrity [192].

1.5.4 Stratum corneum protease activity

Several serine proteases are present in the epidermis and take part in various processes such as skin desquamation and maintenance of skin barrier function [30, 37-39]. Changes in their activity in the SC can give important information on the influence of the formulation on skin state, especially on the desquamation and inflammatory processes [213].

The key desquamatory enzymes are human tissue kallikreins (KLKs), which are a family of trypsin-like (SCTE) and chymotrypsin-like (SCCE) stratum corneum enzymes [30, 214, 215]. The skin KLKs function as an enzymatic cascade, i.e. a sequence of activation reactions where the

product of one reaction catalyses the next one [39, 216]. Cathepsins [217] and heparanase 1 [218] are also believed to be involved in epidermal desquamation.

50% of total serine protease activity in the SC comes from KLK5 (trypsin-like serine protease) and the major part of remaining activity is associated with KLK14 (which has a dual trypsin- and chymotrypsin-like activity, with significant predominance of the former). KLK7 constitutes almost all of the chymotrypsin-like KLK activity in SC [213]. The activity of SCTE and SCCE is higher in the outermost layers of the SC, where the desquamation occurs [39].

The SC protease activity was reported to vary depending on the anatomic site and the depth into the SC [214, 219, 220]. The protease activity is also influenced by external factors such as the season of the year [204], environmental conditions [221] and topically applied compounds [195, 222]. Reduced activity of KLK5 and KLK7 enzymes was found in dry skin [223-225]. On the other hand, elevated KLK7 expression was associated with inflammatory skin diseases such as atopic dermatitis [215, 226]. No differences in the SC protease activities were found between genders [177, 227]. Also KLK5 and KLK7 activity was found to be similar in Black and Caucasian ethnicities [177]. However, an effect of skin pigmentation on the SC protease activity was postulated by Gunathilake et al. [228].

Other SC enzymes important for the SC barrier function include: plasmin-, furin- and tryptase-like enzymes. These protease activities were found to be elevated in atopic skin suggesting their role in inflammatory skin diseases [213, 226]. Urokinase-type plasminogen activation was reported after SC barrier disruption [229].

1.5.5 Corneocyte size

The investigation of corneocyte size and maturity gives information on the skin cell proliferation and maturation process [230]. Also the permeation process of topically applied products depends on the size of the cells, especially when the intercellular route is considered as the major route of penetration [70, 231].

The surface area of corneocytes differs depending on the anatomic site [231-233], age and gender [234] ranging from approximately 0.5 to 1.2 mm^2. Hunter et al. [43] measured the area of corneocytes obtained by the skin stripping method from forearm, shoulder and thigh in different age and gender groups. An average value of 0.7-0.9 mm^2 was obtained, which was in agreement with theoretical calculations and more recent studies [235]. It was established that the size of corneocytes changes in the following order: axilla > thigh > upper arm > abdomen > scalp > lower arm > heel > hand > forehead [232]. Remarkably, larger corneocytes were found

on the eyelids, when compared with small corneocytes on cheek and nose [233]. Plewig [234] reported gender differences in corneocyte size, i.e. larger cells were obtained for female subjects. However, no statistical difference between men and women was found in other studies [177, 231, 236]. Also no differences in terms of corneocyte size were found between ethnicities [177, 235, 236].

1.5.6 Corneocyte maturity

As described in section 1.1.1.1, corneocytes in the SC have characteristic proteinaceous insoluble structures, called cell envelopes or cornified envelopes (CEs), which are formed through the crosslinking of several precursor proteins. Two main types of CE can be distinguished on the basis of their maturity and shape [29]. The more mature, polygonal, rigid cornified envelopes (CEr) can be found in the outermost layers of the SC, whereas irregular, less mature and less hydrophobic fragile cornified envelopes (CEf) are characteristic for the deeper layers of the SC and can be found in the outer layers of the SC of psoriatic and eczematous patients [237, 238] .

Since the CE is an insoluble structure, it can be separated from the other SC components by boiling of the SC samples (e.g. obtained by the tape stripping technique) in reducing agents and detergents. Then the CEr and CEf can be distinguished by the double staining method, described by Hirao et al., which uses Nile red and fluorescent anti-involucrin immunostaining [238]. Nile red is a fluorescent agent which is extensively used for detection of lipids [239]. Involucrin is one of the cornified envelope precursor proteins, which in the process of maturation are cross-linked by transglutaminases. These precursor proteins are present to some extent in the immature CEf, but not in CEr, which in addition acquire hydrophobicity by the attachment of lipids to its proteins. Because of their hydrophobicity CErs are Nile red-positive and stain little with anti-involucrin antibody. On the other hand CEfs are Nile red-negative and can be strongly stained with anti-involucrin allowing for the differentiation of CEs with the use of fluorescent microscopy [238].

1.6 Atopic dermatitis

Atopic dermatitis (AD) is a chronic skin condition, which is characterised by skin dryness, xerosis (cracking) and pruritus (itching), with excessive desquamation of the SC. It is inherently connected to the malfunction of the epidermal barrier and alterations in the barrier function are observed in both lesional and lesion-free skin of atopic patients [240-242].

The prevalence of AD is thought to have increased significantly over the past years, probably because of modern world lifestyle changes. The worldwide prevalence of AD in children ranges

from 1 to 20% and the United Kingdom is one of the countries with the highest prevalence rates [243]. The epidemiology of AD was reviewed by Daveiga [244].

1.6.1 Atopic dermatitis pathophysiology

In general, dermatitis is caused by disturbance of three basic skin responses:

- functional - impairment of function without morphologic changes,
- inflammatory - degenerative changes following cellular injury,
- proliferative - increase in number of different types of skin cells [1].

Atopy is a tendency to become sensitised and produce immunoglobulin E (IgE) antibodies in response to ordinary exposures to allergens [245]. However, 15 to 30% of AD patients do not show IgE-mediated sensitisation, manifesting intrinsic, non-IgE mediated AD [246, 247]. For many years AD was thought to be a consequence of internal inherent abnormal sensitisation ('inside-to-outside' hypothesis of AD pathophysiology). However, in the 1990s a new 'outside-inside' hypothesis became prominent [248]. Recently, some authors proposed an even more complex form of this view ('outside-inside-outside') on AD pathogenesis [249-252]. According to this hypothesis, a primary structural abnormality of SC (i.e. inherited defects in the SC structure and function such as gene mutation) can be activated by external agents. These abnormalities can further aggravate permeability barrier dysfunction. In other words, initial changes in barrier function might drive the disease activity.

One of the intrinsic factors responsible for AD may be mutation in the filaggrin encoding gene *FLG.* Filaggrin (FLG) is a protein of major importance for skin barrier function. A significant reduction in FLG expression was found in AD when compared with normal skin [253]. Studies in mice showed that *FLG* mutations can cause the primary structural abnormality of the SC encountered in AD [254, 255]. In humans, *FLG* mutations were associated with dry skin and higher TEWL values at 3 months of age [256]. Also, there are several variants of *FLG* associated with AD [257, 258]. Irvine [259] reviewed the prevalence of *FLG* mutations in the European population and their association with AD.

Impairment of the SC function in AD includes changes in:

- the permeability barrier [242, 260, 261],
- the antimicrobial barrier [262],
- the SC integrity (leading to excess scaling/abnormal desquamation) [213],
- the SC hydration [263].

The breakdown of epidermal barrier function in AD results from changes in such skin characteristics as: corneocyte size and maturation [230], desquamation [264], lipid profiles [265, 266] and some protease activities [213, 226]. A modulation in sensory nerves (dendritic cell signalling) is responsible for the itch-scratch vicious cycle [267].

1.6.1.1 Biophysical changes

Barrier abnormalities in AD can be reflected in the changes in biophysical properties of the skin: increase in TEWL and decrease in skin capacitance (i.e. skin ability to conduct electrical current, which reflects skin hydration) [194, 212, 242, 256, 268-270].

Higher TEWL and decreased capacitance values were observed for the eczematous skin of AD patients when compared with lesion-free skin sites and to the skin of healthy volunteers. What is more, these parameters differed significantly between uninvolved atopic skin and the skin of healthy subjects [242, 271]. Also increased TEWL and decreased capacitance values were observed after treatment of atopic skin with detergent and non-detergent cleansers [269].

Lee at al. [268] correlated the increase in TEWL with high IgE and beta-endorphin serum levels in AD patients. The authors concluded that TEWL may be a good biomarker for the assessment of itch intensity. TEWL was also correlated with the severity of the disease described by the SCORAD index [272, 273]. A correlation between capacitance measurements and severity of skin dryness [274] or disease activity [270] was also established. Voegeli at al. [226] reported elevated basal TEWL, reduced skin hydration and increased irritation in lesional skin of AD patients, which indicated barrier dysfunction. The effects of topical treatment of AD with glycerol were also evaluated with TEWL and capacitance instrumental measurements [212]. The authors found a significant ($r=0.33$, $p=0.0004$) correlation between the TEWL values and the skin dryness assessed by a dermatologist postulating that TEWL measurements may be a helpful tool to determine AD severity.

1.6.1.2 Serine proteases

Symptoms of AD are similar to one of the symptoms of Netherton syndrome, which is associated with loss-of-function mutations in the *SPINK5* gene. *SPINK5* encodes the serine protease inhibitor LEKTI 5 - lympho-epithelial Kazal-type inhibitor 5 [275]. This similarity leads to an assumption that one of the probable pathomechanisms of AD lies in an increased activity of serine proteases (SPs) [276]. This increase may explain the global decrease in extracellular lipids, decrease in ceramide levels, immunologic abnormalities and disrupted cell proliferation and desquamation encountered in AD [266, 276-279].

The increase of KLK5 activity alone induces abnormalities similar to the characteristics of AD [277]. Also mutations in the KLK7 encoding genes (*KLK7*) were found to be associated with AD [280]. *KLK7* deregulation was detected in AD patients who showed no IgE level elevation. Thus, SP expression could be an independent AD factor from atopy or IgE-mediated sensitization. KLKs may also be contributing to the regional inflammatory reaction in AD, by triggering the protease-activated receptor 2 (PAR2) signalling, which modulates inflammatory responses [213, 281, 282].

Voegeli et al. [226] reported increased SP activities in acute eczematous AD, especially in deeper layers of the SC. This increase was associated with impaired barrier function, irritation, reduced skin capacitance, elevated TEWL and increased skin cohesion.

Not all SP activities are elevated in AD. Abnormally high proteolytic activity of plasmin was observed by Komatsu and co-workers in AD and psoriasis. In this study, elevation of KLK7 activity was predominant, compared with trypsin-like KLKs, implying that an increase in KLK7 may be crucial in AD pathogenesis or manifestations. The alterations of KLKs levels in the SC were more evident than in serum, suggesting that KLKs could be involved in a regional inflammatory reaction [213, 283]. It was shown that in lesional AD skin SC tryptase-like enzyme, plasmin, urokinase, trypsin-like KLKs and chymotrypsin-like KLK activity values are significantly increased compared with healthy or non-lesional skin. The enzymatic activity increased towards the surface of the SC in healthy and non-lesional skin. On the contrary, their elevated activity was maintained across lesional SC, indicating KLKs function in manifestation of AD symptoms [226].

1.6.1.3 Epidermal differentiation

A shorter epidermal turnover time was observed in AD when compared with healthy skin [284]. Also smaller corneocytes with less water-soluble amino acids were found in the skin of atopic patients [284, 285]. Uninvolved atopic skin was characterised by thinner SC when compared with healthy skin. However, the overall thickness of the epidermis was increased [285]. Corneocytes in AD were shed as aggregates rather than individual cells [284, 285].

At the final stage of differentiation, keratinocytes form a cornified envelope (CE) consisting of lipid and protein components [50, 286]. This structure is responsible for the SC cohesion [287] thanks to covalent bonds between the extracellular lipids and proteins (mainly involucrin) [288]. Ceramides are the main protein-bound lipids in healthy skin (~50%). However, a decrease in this percentage was observed in atopic patients, both in lesion-free and affected skin [25].

1.6.2 Treatment of atopic dermatitis

In accordance with the inflammatory theory of AD pathogenesis, treatment of this condition is mainly focused on diminishing immunological response. Nevertheless, there is increasing evidence of barrier impairment, rather than improvement, caused by topical glucocorticosteroids [289] and calcineurin inhibitors [290] used in AD therapy. Among other side effects, the treatment with topical corticosteroids caused skin thinning and disruption in the intercellular lipids, reflected in elevated TEWL [291]. Thus, other AD therapy options should be considered.

According to the 'outside-inside-outside' theory, which states that inflammation is a result of inherited and acquired barrier impairment, restoring barrier function of the epidermis should be an effective treatment of AD [251]. Because AD is inherently bound to dry and scaly skin, topical moisturisers are widely used to alleviate these symptoms [212] and decrease the use of steroids [292]. However, care must be taken in terms of the composition of moisturisers. Depending on the vehicle and actives present in the formulation, the topical preparations may improve or deteriorate skin barrier properties and symptoms of AD [212, 293-298].

The use of lipids and lipophilic substances in the treatment of AD is popular. Petrolatum accelerated barrier recovery after acetone-induced disruption. It was found in the intercellular space in the SC, suggesting that its effect is because of skin lipid replacement, rather than an occlusive effect [299]. Specific lipid-replacement therapy of AD gave comparable effects to mid-strength steroid treatment [300]. Also ceramide treatment of AD proved effective in alleviating the disease symptoms [297].

Based on the elevated levels of SP activities in the SC of AD patients, it is postulated that the use of SP inhibitors might be a potential treatment of AD [226]. Use of topical nicotinamide has been shown to accelerate barrier recovery in experimentally damaged skin barrier models [301] and in patients with dry skin disorders [302]. However, its influence on the SP activity in the SC has not yet been fully documented.

1.7 Nicotinamide

Nicotinamide (NA) is a component of the vitamin B complex. Together with nicotinic acid, NA is a constituent of vitamin B_3. NA is involved in over 200 enzymatic reactions as NAD (Nicotinamide Adenine Dinucleotide) and NADP (Nicotinamide Adenine Dinucleotide Phosphate) - enzyme cofactors, which serve as electron transporters in cell metabolism. Humans are able to synthesise nicotinic acid from tryptophan. Also intestinal bacterial flora is a source of nicotinic acid. Nevertheless, it is necessary to deliver vitamin B_3 in products such as

grains, meat and milk. The deficiency of this vitamin initially induces non-specific symptoms: fatigue, anorexia and irritability. Subsequently, shortage of vitamin B_3 can lead to more severe condition called pellagra, which is associated with dermatitis, diarrhoea and dementia [303].

Oral NA is used in vitamin B_3 deficiency. It can be found in tablets or capsules of multivitamin preparations used as dietary supplements [304]. Also bread, flour and other grain-derived products are in some countries enriched in NA [303]. NA is an ingredient of numerous cosmetics, where it serves as a hair and skin conditioning agent [305]. Currently in the UK, there are two topical pharmaceutical formulations of NA (4%) registered for the treatment of acne vulgaris [304, 306]

1.7.1 Physicochemical properties

The physicochemical properties of NA are listed in Table 1.1 and the structural formula is shown in Figure 1.9.

Table 1.1 Physicochemical properties of nicotinamide

Chemical name	3-pyridinecarboxamide; pyridine-3-carboxamide [307]
Molecular formula	$C_6H_6N_2O$ [307]
Molecular weight	122.13g/mol [307]
Appearance	White crystalline powder or colourless crystals [307]
Water solution stability	Stable in pH 3.5 - 7 [303]
Dissociation Constant	pK_a 3,35 (20˚C) [307]
Partition coefficient	$logK_{o/w}$ -0,38 at 22˚C [307]
Melting point	128-131˚C [307]
Water solubility	Freely soluble 1:1 [307]
Ethanol solubility	Freely soluble 1:1.5 [307]
Glycerol solubility	Freely soluble (1:10) [307]
Maximum UV absorption	255nm; 262.5nm [303, 307]
UV detection wavelength	260-264nm [303, 307]

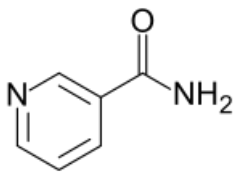

Figure 1.9 Structural formula of nicotinamide

1.7.2 Absorption, distribution and excretion

NA is readily absorbed from the intestinal tract and from parenteral sites of administration (e.g. skin). It is widely distributed in all tissues throughout the body [308]. Of the constituents of vitamin B_3, it is NA that is transported between different tissues as a precursor of NAD [303].

NA is a hydrophilic compound and thus it is not assumed that it penetrates through lipid lamellar structures between corneocytes easily. However, *in vitro* studies using human cadaver skin confirmed that NA penetrates the skin after topical application of various products containing this compound [305]. NA permeation across pig and rabbit skin was also investigated *in vitro* [309-311].

1.7.3 Nicotinamide in the therapy of inflammatory and dry skin disorders

The symptoms of vitamin B_3 deficiency, pellagra, include skin xerosis and pruritus. Hence, NA has found use in alleviating dry and itchy skin conditions. However, it is only recently that the probable mechanisms of NA action in dry skin have been hypothesised [312].

NA is a precursor of endogenous enzyme cofactors: NAD, its phosphorylated form (NADP), and their reduced forms NADH and NADPH. These cofactors may influence skin properties by participating in various skin enzymatic reactions [301]. Namazi [313] suggested that because of its anti-inflammatory and antioxidant properties, together with the ability to inhibit and suppress various enzymes and reactions in the skin (such as nitric oxide synthase and antigen-induced lymphocyte transformation), NA might be useful in the topical treatment of AD.

NA is in general regarded as safe at pharmacologic doses and thus can be used in long-term topical therapy [93, 302, 314-316].

1.7.3.1 Nicotinamide influence on stratum corneum lipids

Tanno et al. [316] postulated that NA increases the levels of SC ceramides. *In vitro* studies with cultured normal human keratinocytes incubated for 6 days with 1-30µmol/L NA showed that it increases intercellular lipid synthesis: glucosylceramides, sphingomyelin, cholesterol and fatty acids. The mechanism of NA action is the augmentation of serine palmitoyltransferase (SPT) activity, by increasing the levels of human LCB1 and LCB2 mRNA, which both encode subunits of SPT – the rate-limiting enzyme in sphingolipid synthesis [317].

In vivo studies on volunteers with dry skin conducted for 4 weeks confirmed that NA influences skin lipid composition. Topical application of 2% NA increased ceramide, cholesterol and free fatty acids levels in SC (via an increase in acetyl coenzyme A activity) and decreased TEWL [316].

1.7.3.2 Nicotinamide influence on epidermal differentiation

An increase in the SC thickness and the total skin water content was observed in a study conducted by Crowther et al. in 2008 [93]. After 2 weeks of treatment with a formulation containing NA and 1-week regression, compared with 2 formulations without NA, NA was found to improve the SC barrier function and decrease desquamation. The formulation containing NA induced a statistically significant ($p<0.05$) increase in the SC thickness after 2 weeks (mean 2µm, i.e. 10% increase). It also increased the SC water content and improved the SC water profile. The proposed mechanism of these changes was an improvement in keratinocyte differentiation and an increase in ceramide synthesis.

Kitamura et al. [318] investigated the influence of NA on the cornified envelope formation and the presence of differentiated keratin K1. Both of these markers of keratinocyte maturation were highly expressed in NA treated cells. Also TGase, the enzyme crucial for CE formation, showed high activity. The authors concluded that NA promotes differentiation of human keratinocytes.

1.7.3.3 Anti-inflammatory activity of nicotinamide

NA is an inhibitor of Poly-ADP-ribose polymerase 1 (PARP-1), an enzyme which plays an important role in the expression of adhesion molecules and inflammatory mediators [319]. It also inhibits cAMP phosphodiesterase and stabilises mast cells and leukocytes, thus decreasing the release of histamine and IgE [320, 321].

The potent anti-inflammatory activity of NA has been used in the treatment of acne vulgaris. A 4% NA gel reduced acne lesions (papules and pustules) and acne severity at a rate of 60% and 52% respectively. In *in vivo* studies, comparable results to the effects of 1% clindamycin were obtained after NA formulations were applied [315]. Fiveson [314] proposed a variety of potential mechanisms of action of the combination of NA and zinc used in the treatment of acne, mainly related to the anti-inflammatory potential of this combination. NA was also found to inhibit production of IL-8 induced by *Propionibacterium acnes* [322].

A topical moisturiser containing 2% NA was shown to be beneficial in patients with rosacea. Following a 4 weeks twice-daily application period, the skin of 50 patients participating in the study exhibited higher resistance to damage (DMSO test) and better hydration, together with lower TEWL values and erythema scores [323].

1.7.3.4 Nicotinamide influence on barrier enhancement and hydration

NA significantly improved SC barrier function in a vehicle-controlled study with a sodium lauryl sulphate (SLS) and a dimethylsulfoxide (DMSO) challenge model [301]. Significantly lower TEWL values ($p<0.01$), together with improved redness ($p<0.0005$) and wheal ($p<0.05$) scores were observed in the challenge tests after 24 days treatment with 2% NA applied twice daily. Baseline TEWL values were also significantly ($p<0.02$) lower for the NA treated group when compared with control.

In a study by Crowther et al. [93], an increase in skin thickness brought about by formulations containing NA was accompanied by changes in epidermal water gradients and increased skin hydration.

In conclusion, because of its anti-inflammatory and skin barrier enhancing properties, NA has potential as an active substance to be used in the topical treatment of AD.

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2 Aims and objectives

Chapter 1 provided general information about the skin, percutaneous absorption and methods used in skin research. It also summarised the pathophysiology and the treatment of atopic dermatitis and highlighted the properties of nicotinamide as a potential active compound for the treatment of inflammatory skin diseases.

Taking into account the information provided in the introductory chapter, the following aims and objectives of this work were identified:

- to optimise the skin delivery of nicotinamide with the use of appropriate excipients,
- to evaluate nicotinamide skin delivery with *in vitro* and *in vivo* techniques,
- to investigate the efficacy of nicotinamide formulations in improving the skin state *in vivo*.

In Chapter 3, pre-formulation studies, i.e. solubility parameter calculation, miscibility, solubility and NA stability studies, are described. Based on these studies, the optimal excipients for prototype nicotinamide formulations were chosen and further studied in the following chapters. Chapter 4 encompasses *in vitro* uptake and permeation studies in silicone membrane and pig skin. Evaluation of prototype nicotinamide formulations *in vivo* was described in Chapter 5. The performance of formulations in terms of nicotinamide percutaneous absorption was compared with their influence on the skin condition.

Conclusions regarding the *in vitro* and *in vivo* studies performed within the boundaries of this thesis are given in Chapter 6 and possible future developments discussed in Chapter 7. Additional information regarding the methodology used in this research can be found in the appendices.

This work looks at nicotinamide from a new perspective of its potential use in atopic dermatitis treatment. Also, a practical approach to topical formulation development is attempted, i.e. theoretical calculations of solubility parameter are used to choose the optimal vehicle components for a successful topical delivery of the model drug.

3 Pre-formulation studies

Pre-formulation studies are the preliminary experiments performed during the development of any formulation. In the case of topical formulations, it is crucial to determine the solubility and stability of the drug in the chosen excipients and the mutual miscibility of the formulation components. These parameters influence the performance of the formulation in terms of delivery of the drug and the achievement of the required pharmacological effect.

Investigation of the drug solubility in the chosen solvents gives information on the drug thermodynamic activity, which is important in the diffusion process [1, 2].

Knowledge of the mutual miscibility of the formulation components is essential, because not only simple solvents but also their combinations must be taken into account in the choice of the final formulation. Synergistic effects of solvents on percutaneous absorption have been reported in the literature [3-8] and are explored in other sections of the present work.

The physicochemical properties of the drug, the vehicle and the skin are of major importance for percutaneous absorption. The structural complexity of the skin further complicates this process. Thus, theoretical calculations of solubility parameter could be helpful in the determination of solubility and miscibility, providing guidelines for choosing the best excipients in the formulation development process. Predicting the interactions between the drug, the vehicle and the model membrane from theoretical solubility parameter values has been attempted previously [9-13] and was investigated further in the present work.

3.1 Materials

Table 3.1 Materials used in the pre-formulation studies

Material	Supplier
Nicotinamide (NA)	Dermal Laboratories Ltd., UK
Glycerol (GLY)	Dermal Laboratories Ltd., UK
Mineral oil (MO)	Dermal Laboratories Ltd., UK
Isopropyl myristate (IPM)	Dermal Laboratories Ltd., UK
Propylene glycol monolaurate type II (PGML)	Gattefossé, France
Propylene glycol monocaprylate type II (PGMC)	Gattefossé, France
Propylene glycol dipelargonate (DPPG)	Gattefossé, France
Propylene glycol (PG)	Sigma Aldrich, UK
Laboratory water purifier (OPTION 3, 75 litre volume)	USF Elga, UK
Phosphate Buffered Saline (PBS) tablets (DulbeccoA, pH 7.3±0.2)	Oxoid, UK
Nuova II stirrer	Thermolyne, UK
Dow Corning 7-4107 Silicone Elastomer Membrane	Seneffe, Belgium
High Vacuum Silicone Grease	Dow Corning, UK
Water bath type JB5	Grant Instruments Ltd., UK
Variomag® HP16 Stirring Drive Unit	H+P Labortechnik GmbH, Germany
Teflon coated magnetic stirrers (7, 12, 25 and 35mm length)	Fisher Scientific, UK
HPLC grade solvents: Acetonitrile, Methanol and Water	Fisher Scientific, UK
HPLC vials with flat bottom inserts and caps with rubber seal	Fisher Scientific, UK
Sudan III	Sigma Aldrich, UK
Polyoxyethylene (20) oleyl ether	Sigma Aldrich, UK
AnalaR Normapur Perchloric acid 70%	WVR International, UK
Digitron™-22 Differential Digital Thermometer with probe	RS Components, UK
Eppendorf Research® pipettes	Eppendorf AG, Germany
Analytical balances (accuracy 0.0001g and 0.0001mg)	Sartorius GmbH, Germany
HPLC Column Primesep A 250x4.6mm, Particle 5µm, 100Å	SIELC Technologies Inc., USA
SecurityGuard Cartridge with C_{18} 4mmLx3mm filter	Phenomenex, USA
Stuart® Rotator SB3	Bibby Scientific Ltd., UK
Laboratory glassware and containers	UCL School of Pharmacy
Metallic membrane cutter	UCL School of Pharmacy
Parafilm®M	Bemis Flexible Packaging, USA
Orbital incubator S150	Stuart Scientific, UK

Traceable® Extra - Extra Loud Timer	Fisher Scientific, UK
Molecular Modelling PRO software Version 6.3.3.	Norgwyn Montgomery Software Inc., USA
IBM SPSS Statistics software Version 19	SPSS Inc., an IBM Company

3.2 Methods

3.2.1 Theoretical calculations of solubility parameter

Van Krevelen and Hoftyzer three-dimensional solubility parameter (δ) [$cal^{1/2}/cm^{3/2}$] values were determined for the chosen molecules with Molecular Modelling PRO software Version 6.3.3. (Norgwyn Montgomery Software Inc., USA).

The δ values for the mixtures of solvents were calculated according to the following equation [14, 15]:

$$\delta_{mixture} = \sum(\varphi_1 \times \delta_1) + \cdots + (\varphi_n \times \delta_n)$$

Equation 3.1

where:

$\delta_{mixture}$ - solubility parameter of the solvent mixture,

φ_n - volume fraction of the n^{th} solvent,

δ_n - solubility parameter of the n^{th} solvent.

3.2.2 Miscibility studies

Miscibility studies were performed at room temperature for the binary mixtures of the chosen solvents within a range of proportions in 10% steps. Miscibility was determined by observation of the point (ratio of the solvents) where the mixtures stopped being clear or phase separation could be seen. Sudan III (Sigma Aldrich, UK) was used to dye the lipophilic phase in cases where it was difficult to determine whether the separation occurred. After mixing, the samples were left at 32°C overnight to check if any changes in miscibility might occur in contact with the skin surface which temperature is believed to be 32°C [16-19].

3.2.3 Solubility studies

Excess drug was added to each solvent system and mixed. Throughout the study the samples were maintained at 32 ± 1°C and stirred continuously at 40rpm. Solubility was determined after 48 hours. This equilibration time was validated in the preliminary studies where solubility was measured after 24, 48 and 72h equilibration. No statistically significant differences (one-way ANOVA, $p>0.05$) were found between the results obtained at each time point. In the

literature, a 48h equilibration period was used for NA solubility determination [20, 21]. Thus, a 48h equilibration time was chosen for further experiments.

At the end of the equilibration period, suspensions of the drug in the solvents were centrifuged for 10min at 12000rpm and the temperature of the centrifuge (Eppendorf, UK) was maintained at 32 ± 1°C throughout the process. The samples of supernatant were analysed with high performance liquid chromatography (HPLC) after appropriate dilutions. The experiment was performed in triplicate for each solvent.

3.2.4 Nicotinamide stability in chosen solvents

NA stability in various solvents was tested at 32 ± 1°C for 48h (taking into account the permeation study temperature and the length of the testing period). Solutions of a known concentration of NA in the solvent were prepared and mixed with magnetic stirrer bars. At designated time points, aliquots of NA solutions were collected and, after appropriate dilutions, analysed for NA concentration with HPLC. The experiment was performed in triplicate.

3.2.5 Sample analysis

HPLC with UV detection was used for NA quantification in the permeation samples. The method was validated according to the guidelines and met the required criteria (coefficient of variance≤10-15%; r^2≥0.999) [22, 23]. The parameters of the HPLC method used for NA quantification are listed in Table 3.2.

Table 3.2 Parameters of the HPLC method for nicotinamide quantification

Column	**Primesep A 250x4.6mm, Particle 5µm, 100Å** (SIELC Technologies, USA)
Guard column	**SecurityGuard Cartridge System with C_{18} (ODS, Octadecyl) 4mmLx3mm filter** (Phenomenex, USA)
HPLC System	**Agilent Technologies 1100 Series HPLC system**
Software	**ChemStation for LC Systems software** Copyright© Agilent Technologies
Mobile phase	**Mobile phase A (80%):** 0.07% Perchloric Acid in Purified Water (1mL of 70% Perchloric Acid in 1L), pH 2 **Mobile phase B (20%)**: Acetonitrile (HPLC grade)
Injection volume	10µL
Flow rate	2 mL/min
Temperature control	40°C
UV detection wavelength	263nm
Retention time of nicotinamide	~9min
Analysis time	~11min
Calibration curve range	2-200µg/mL (standard concentrations: 2; 10; 20; 50; 100; 200µg/mL prepared from a 1000µg/mL stock solution by dilution with PBS)

3.2.6 Data analysis

Statistical significance was determined using one-way ANOVA with the Tukey post-hoc test or the independent samples 2-tailed t-test performed with IBM SPSS Statistics Version 19 software (SPSS Inc., an IBM Company). A probability of $p<0.05$ was considered statistically significant. All results are presented as mean ± SD (n=3) unless otherwise stated.

3.3 Results

3.3.1 Solubility parameter

Table 3.3 summarises the δ values of the chosen solvents calculated with Molecular Modeling PRO software (Norgwyn Montgomery Software Inc., USA) and the literature values for skin [12] and silicone membrane [24]. The δ values summarised in the table are the van Krevelen and Hoftyzer type 3-D solubility parameters.

Table 3.3 Solubility parameters of chosen solvents

Compound/membrane	Solubility parameter $[cal^{1/2}/cm^{3/}]^2$
Silicone	7.50
MO	7.74
IPM	8.21
DPPG	8.66
PGML	9.44
PGMC	9.85
Skin	10.00
NA	13.85
PG	14.07
GLY	17.14
Water	22.97

3.3.2 Miscibility studies

Table 3.4 shows the results of binary solvent system miscibility studies over the whole range of proportions tested. After mixing, the samples were left at 32°C overnight and changes in miscibility were noted. In general, no changes in miscibility were observed the following day. Solvents with δ values close to each other proved to be miscible, which is in line with the approach to use this parameter to predict miscibility [14].

Table 3.4 Miscibility of binary solvent systems (M-miscible, IM-immiscible)

	MO	IPM	DPPG	PGMC	PGML	PG	GLY	Water
MO		M	M	M	M	IM	IM	IM
IPM	M		M	M	M	IM	IM	IM
DPPG	M	M		M	M	IM	IM	IM
PGMC	M	M	M		M	M	IM	IM
PGML	M	M	M	M		M	IM	IM
PG	IM	IM	IM	M	M		M	M
GLY	IM	IM	IM	IM	IM	M		M
Water	IM	IM	IM	IM	IM	M	M	

3.3.3 Solubility studies

The saturated solubility of NA in single solvents and in binary solvent systems was determined and the results are summarised in Table 3.5 and Table 3.6.

Table 3.5 Solubility of NA in chosen single solvents at 32 ± 1°C (mean ± SD, n=3)

Solvent	Solubility 48h ± SD [mg/mL]	Solubility parameter [$cal^{1/2}/cm^{3/2}$]
MO	0.006 ± 0.001	7.74
IPM	0.68 ± 0.04	8.22
DPPG	1.63 ± 0.01	8.66
PGML	22.15 ± 0.35	9.44
PGMC	38.17 ± 1.25	9.85
PG	243.37 ± 8.76	14.07
Water	552.70 ± 11.49	22.97

Table 3.6 Solubility of NA in binary (1:1) solvent systems at 32 ± 1°C (mean ± SD, n=3)

Solvent	Solubility 48h ± SD [mg/mL]	Solubility parameter [$cal^{1/2}/cm^{3/2}$]
IPM:DPPG	1.12 ± 0.02	8.44
IPM:PGML	8.29 ± 0.09	8.83
DPPG:PGML	9.93 ± 0.23	9.05
IPM:PGMC	12.70 ± 0.37	9.03
DPPG :PGMC	15.74 ± 0.24	9.25
PGML:PGMC	31.35 ± 1.38	9.64
PG:PGML	128.54 ± 4.24	11.75
PG:PGMC	143.75 ± 3.12	11.96
PG:Water	471.96 ± 6.67	18.52

The solubility of NA in the chosen solvents ranged from very low values for the most lipophilic solvents, MO and IPM, to extremely high solubility obtained in water. The solubility of NA increased with increasing δ value of the solvent system. The more hydrophilic the solvent, the higher its potential to dissolve NA. A good linear correlation was established between NA solubility and the δ value for the solvent systems tested (r^2=0.98) and is illustrated in Figure 3.1.

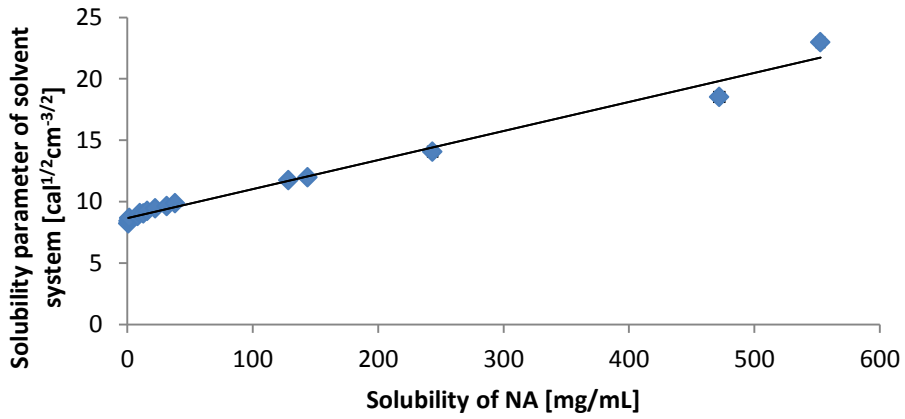

Figure 3.1 Correlation between solubility parameter and experimental solubility of NA in chosen solvent systems (mean ± SD, n=3)

3.3.4 Nicotinamide stability in chosen solvents

Table 3.7 summarises the results of the stability test.

Table 3.7 Stability of NA in chosen solvents after 48h equilibration at 32 ± 1°C (results shown as mean ± SD, n=3)

Solvent	Recovery [%]
IPM	105.04 ± 2.71
DPPG	100.87 ± 3.32
PGML	106.58 ± 5.54
PGMC	112.29 ± 8.67
PG	97.93 ± 1.62
PBS	101.98 ± 1.76

3.4 Identification of optimal solvents and formulations

Investigated solvents encompass a wide range of solubility parameters: from 7.74 $cal^{1/2}/cm^{3/2}$ for MO to 22.97 $cal^{1/2}/cm^{3/2}$ for water. PGMC, PGML, DPPG and IPM have a δ value close to that of skin (approximately 10 $cal^{1/2}/cm^{3/2}$). Moreover, close values of δ for NA and PG implicate their strong interaction and possible positive effect of this solvent on NA percutaneous absorption. These solvents were further investigated in the rational development process of topical NA formulations. The application of δ calculation in prediction of topical delivery was tested and will be described in other chapters in this thesis.

Based on the results of miscibility studies, simple miscible solvent systems were chosen to be investigated further. NA permeation from the miscible solvent systems was investigated *in vitro.* Miscible solvent systems were used to simplify the experimental conditions and the analysis of the data. In the case of miscible solvent systems, NA partitioning occurs between the solvent system and the skin, as opposed to a more complex formulation where, for example, the drug will distribute between the immiscible phases of the vehicle e.g. a cream.

Knowledge of the saturated solubility allows determination of the maximal concentration of the drug which can be incorporated into the vehicle. Also, application of the saturated solution on the skin will result in the maximal thermodynamic activity of the drug in the vehicle, and thus the maximal flux across the skin can be achieved [2]. Further *in vitro* permeation studies were performed with saturated solutions of NA, to ensure the maximal thermodynamic activity of the drug in the vehicle.

The stability of the drug is crucial for the development of a safe formulation. NA hydrolysis to nicotinic acid, which can induce skin reddening [25], should be avoided. In general, NA proved to be stable for 48h at 32 ± 1°C in the range of solvents tested. This means that a stable NA formulation can be developed with the chosen solvents.

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4 *In vitro* studies in silicone membrane and pig skin

The rate and the extent of drug diffusion and absorption from a topical product depends on the physicochemical properties of the drug, the amount of the formulation applied and the thermodynamic activity of the drug in the vehicle [1]. It is also known that excipients influence permeation of the drug, although the mechanisms of their action are not yet fully elucidated. Thus, optimisation of the formulation composition is an important step in the development of a topical preparation and investigating the role of excipients in the drug permeation process is crucial for appropriate execution of this task [2].

Two prototype formulations containing 4% NA were prepared by Dermal Laboratories Ltd., UK. The strategy was to investigate NA performance in terms of improving skin state separately from other excipients present in the formulation. Thus, one of the formulations (DENI) contained several components, including IPM, MO and GLY, while the other (NIAD) contained approximately 93% of water.

Before the formulation reaches the stage of *in vivo* investigations, i*n vitro* studies should be performed to obtain information about the interactions between the drug, skin and the vehicle. Understanding these interactions is important for achievement of an optimal topical delivery as it provides the basis for the choice of an appropriate vehicle for the given drug.

Uptake studies, using silicone as a model membrane, have shown that high solvent sorption into the membrane enhances drug permeation [3]. The authors suggested that increased drug flux was because of higher drug solubility in the membrane caused by the presence of solvent. This would indicate that drug partitioning into the membrane was increased when an appropriate solvent was used. The drug diffusivity was considered to be also influenced by the vehicle, although to a lesser extent.

In vitro permeation studies with human skin as a barrier membrane are the closest representation of the conditions *in vivo*. However, the complexity and variability of human skin, together with its limited availability causes the need for alternative membranes. Animal skin and artificial model membranes can be used instead, as they are readily available and less variable than human skin. Silicone membrane has been used by many authors to study drug permeation and to help to elucidate more complex skin-drug-vehicle interactions [4-14]. Also, pig skin is considered to be one of the best substitutes of human skin [15-18]. Although human

skin usually is less permeable many authors support the use of pig skin, especially from the outer region of the ear, for *in vitro* permeation studies [19-24].

Since the vehicle components can influence the permeation of an active, knowing the solvent fate in the permeation process is equally as important as monitoring the transport of the drug. To investigate how solvents with different physicochemical properties influence nicotinamide (NA) transport in model membranes, solvent uptake, NA partitioning and *in vitro* permeation studies were performed. Permeation of both NA and specific solvents were investigated, to see if any relationship between these two could be established. For a better understanding of NA permeation and retention in the membrane after application of prototype NA formulations and simple solvent systems containing NA, both infinite (for prototype formulations) and finite dose studies across silicone membrane were performed. Silicone membrane was chosen for these preliminary studies to simplify experimental conditions, particularly to reduce the impact of biological variability. Permeation was further studied using pig ear skin, bringing the conditions closer to the real life situation when a finite amount of formulation is applied to the skin. Washing and extraction of the skin and the silicone membrane was included in the finite dose studies to enable assessment of the retention of NA in these model membranes.

4.1 Materials

Table 4.1 Materials used in the *in vitro* studies in silicone membrane and pig skin

Material	Supplier
Nicotinamide (NA)	Dermal Laboratories Ltd., UK
Glycerol (GLY)	Dermal Laboratories Ltd., UK
Mineral oil (MO)	Dermal Laboratories Ltd., UK
Isopropyl myristate (IPM)	Dermal Laboratories Ltd., UK
Propylene glycol monolaurate type II (PGML)	Gattefossé, France
Propylene glycol monocaprylate type II (PGMC)	Gattefossé, France
Propylene glycol dipelargonate (DPPG)	Gattefossé, France
Propylene glycol (PG)	Sigma Aldrich, UK
Laboratory water purifier (OPTION 3, 75 litre volume)	USF Elga, UK
Phosphate Buffered Saline (PBS) tablets (DulbeccoA, pH 7.3±0.2)	Oxoid, UK
Nuova II stirrer	Thermolyne, UK
Dow Corning 7-4107 Silicone Elastomer Membrane	Seneffe, Belgium
Franz-type diffusion cells	UCL School of Pharmacy
Metallic clamps	UCL School of Pharmacy

Electronic Outside Micrometer, 0-25mm, min 0.001mm	RS Components, UK
High Vacuum Silicone Grease	Dow Corning, UK
Water bath type JB5	Grant Instruments Ltd., UK
Variomag® HP16 Stirring Drive Unit	H+P Labortechnik GmbH, Germany
Impedance meter	UCL School of Pharmacy
Stainless steel hardened electronic callipers	Fisher Scientific, UK
Teflon coated magnetic stirrers (7, 12, 25 and 35mm length)	Fisher Scientific, UK
Silicone tubing	Nalge Nunc Corp., USA
HPLC grade solvents: Acetonitrile, methanol and water	Fisher Scientific, UK
HPLC vials with flat bottom inserts and caps with rubber seal	Fisher Scientific, UK
Plastic syringes (1, 2 and 5mL) and needles	Fisher Scientific, UK
Microscope cover glasses	Fisher Scientific, UK
Surgical scalpels, scissors and tweezers	Fisher Scientific, UK
Polyoxyethylene (20) oleyl ether	Sigma Aldrich, UK
AnalaR Normapur Perchloric acid 70%	WVR International, UK
Essential Waitrose cotton buds	Waitrose, UK
Digitron™-22 Differential Digital Thermometer with probe	RS Components, UK
Eppendorf Research® pipettes	Eppendorf AG, Germany
Analytical balances (accuracy 0.0001g and 0.0001mg)	Sartorius GmbH, Germany
HPLC Column Primesep A 250x4.6mm, Particle 5µm, 100Å	SIELC Technologies Inc., USA
SecurityGuard Cartridge with C_{18} 4mmLx3mm filter	Phenomenex, USA
Stuart® Rotator SB3	Bibby Scientific Ltd., UK
Laboratory glassware and containers	UCL School of Pharmacy
Metallic membrane cutter	UCL School of Pharmacy
Parafilm®M	Bemis Flexible Packaging, USA
Orbital incubator S150	Stuart Scientific, UK
Traceable® Extra - Extra Loud Timer	Fisher Scientific, UK
Prototype formulations (DENI and NIAD)	Dermal Laboratories Ltd., UK

Table 4.2 Composition of DENI formulation

Material	% w/w
Nicotinamide EP	4.0
Isopropyl Myristate EP	
Paraffin Liquid EP	
Glycerol EP	
Modified acrylic polymer (Pemulen^{TM} HSE TR1)	
Sorbitan Laurate EP	
Triethanolamine BP 2000	
Phenoxyethanol EP	
Water Purified EP	

Table 4.3 Composition of NIAD formulation

Material	% w/w
Nicotinamide EP	4.0
Modified acrylic polymer (Pemulen^{TM} HSE TR1)	
Sorbitan Laurate EP	
Citric Acid Anhydrous EP	
Phenoxyethanol EP	
Triethanolamine BP 2000	
Water Purified EP	

4.2 Methods

4.2.1 Solvent uptake into the silicone membrane

The silicone membrane was cut to size with a metallic membrane cutter and weighed on an analytical balance (Sartorius GmbH, Germany, accuracy 0.0001mg). The membranes were immersed in single solvents (water, GLY, PG, PGML, MO, PGMC, DPPG and IPM) and allowed to equilibrate for 48h at 32 ± 1°C. Following the equilibration period, the membranes were taken out of the solvent, patted dry with a tissue paper and weighed again. The % increase in the mass of the membrane was determined. The experiment was performed in triplicate for each solvent.

4.2.2 Solvent uptake into the stratum corneum

The stratum corneum (SC) was isolated from full thickness pig ear skin using the heat separation method followed by trypsinisation [25]. The full thickness skin was immersed in distilled water heated to 60°C for 45 seconds and the epidermis was gently removed. Isolated epidermal membranes were stored at -20°C until used. SC was obtained by 12 hours incubation with 0.1% trypsin solution in phosphate buffered saline (PBS) at 37°C. The isolated SC was washed with distilled water and dried over silica gel for at least 2 days. Dry SC was cut into pieces of similar size, weighed on an analytical balance (Sartorius GmbH, Germany, accuracy 0.0001mg) and hydrated over a saturated solution of sodium chloride (relative humidity 75%) at 25°C to approximately 5% hydration. Hydration was calculated according to Equation 4.1.

$$\%\ hydration = \frac{hydrated\ SC\ mass - dry\ SC\ mass}{dry\ SC\ mass} \times 100\%$$

Equation 4.1

The SC was placed into glass sample vials, immersed in a single solvent (PG, PGML, PGMC, DPPG and IPM) and allowed to equilibrate for 24h at 32 ± 1°C. Following the equilibration period, the SC was taken out of the solvent, patted dry with a tissue paper and weighed again. The per cent increase in the dry mass of the membrane was determined. The experiment was performed in triplicate for each solvent.

4.2.3 Nicotinamide partitioning into model membranes

The silicone membrane was cut to size with a metallic membrane cutter, weighed and placed into glass sample vials. Saturated solutions of NA in the single solvents were prepared by 48h equilibration at 32 ± 1°C. The suspensions of the drug in the solvents were centrifuged (10min, 12000rpm, 32 ± 1°C). The concentration of NA in the solvents was determined before uptake with HPLC, after appropriate dilutions. The membrane was immersed in the supernatant and allowed to equilibrate for 24h at 32 ± 1°C. Following the equilibration period, membranes were taken out of the solvent, patted dry with a tissue paper and weighed again. The percentage increase in the mass of the membrane was determined. The membranes were extracted with 1mL of methanol for 1h at 45°C and the amount of NA extracted and present in the solvent was quantified, after appropriate dilutions, with HPLC. The extraction method was validated prior to the experiment, as described in appendices. The experiment was performed in triplicate for each solvent. The partition coefficient (K) of NA was calculated according to Equation 4.2.

$$K = \frac{Concentration\ of\ NA\ in\ the\ membrane}{Concentration\ of\ NA\ in\ the\ solvent}$$

Equation 4.2

4.2.4 Equipment preparation for *in vitro* permeation studies

PBS was prepared by dissolving 10 tablets (Dulbecco A, pH 7.3±0.2 at 25°C, Oxoid, UK) in 1 litre of purified water and was used as a receptor solution in the nicotinamide permeation studies. For the solvent permeation studies 6% polyoxyethylene (20) oleyl ether (PE(20)OE) in PBS was used as a receptor solution. The receptor solution was degassed by high speed stirring under vacuum (Nuova II stirrer, Thermolyne, UK) for at least 25 minutes to prevent the formation of air bubbles below the membrane.

The diameter of each Franz cell receptor compartment was accurately measured with electronic callipers (Fisher Scientific, UK). The permeation data were corrected for the exact diffusion area of each cell. The diffusional area of the Franz cells used in the experiments was approximately 1cm^2.

Donor and receptor compartments of Franz cells were smeared at the adjoining surfaces with silicone grease (Dow Corning, UK). The model membrane was aligned on the receptor compartment of the Franz cell. The donor compartment was then fitted on top of the membrane and the compartments were secured together with a metallic clamp.

The receptor compartments were filled with a known amount of degassed receptor solution, which was determined gravimetrically by weight difference between the Franz cell before and after adding the receptor solution. The volume of the receptor solution varied between 3 to 4 cm^3 depending on the size of the cell.

The micro magnetic stirrer bars (7mm) were placed inside the receptor compartments and the receptor phase was continuously stirred throughout the experiment to ensure sink conditions. The sampling ports of the receptor compartments were sealed with Parafilm®M (Bemis Flexible Packaging, USA) to prevent receptor phase evaporation. The assembled Franz cells were placed in a temperature controlled water bath (Grant Instruments Ltd., UK) and the temperature was allowed to equilibrate for 30 minutes. The temperature of the water bath was maintained at a level sufficient to achieve 32 ± 0.5°C at the membrane surface. The temperature of the membrane in each cell was measured with a Digitron™-22 digital thermometer (RS Components, UK) prior to the experiment. The temperature of the water

bath was 34.0±0.5°C and 37.0±0.5°C throughout the study, for silicone and pig skin studies respectively.

Before application of the formulations, the t_0 sample was taken from the receptor compartment and replaced with fresh PBS solution, which was maintained at the experimental temperature throughout the study. The time of the application of a formulation to the membrane was considered as the beginning of the experiment. At designated time points 200 µL of the receptor solution was sampled from each cell and replaced by the fresh PBS solution to maintain a constant volume of the receptor phase.

4.2.5 Silicone membrane preparation for *in vitro* permeation studies

The silicone membrane was cut to size with a metallic membrane cutter and soaked overnight in the PBS solution at the experimental temperature. The pre-treated membranes were taken out of the PBS solution and carefully blotted with an absorbent tissue paper to remove the excess moisture before alignment on the receptor compartments of Franz cells. The thickness of the membrane was measured at the end of the experiment (Electronic Outside Micrometer, RS Components, UK) and averaged (n=5 for each formulation).

4.2.6 Pig skin preparation for *in vitro* permeation studies

Pig ear skin preparation was based on the literature [21, 24, 26-34]. Pig ears obtained from a local abattoir were kept at -20°C before the membrane preparation. The ears were defrosted prior to preparation and cleaned under cold running tap water. Full thickness skin was removed from the underlying cartilage with a scalpel. The skin was further cleaned by floating in purified water in a metal basin. Circles of a similar size, large enough to cover the opening of a Franz cell receptor, were marked with a metallic membrane cutter and cut to size with surgical scissors. Hair was trimmed with surgical scissors. Isolated skin samples were cleaned with purified water and stored on aluminium foil at -20°C until use. The integrity of skin samples was tested prior to the experiment by impedance measurements. In general, for pig ear skin experiments, impedance values were above 4kΩ, which is in line with recommendations [19]. The donor compartments of the cells were dried with an absorbent paper tissue prior to the application of formulations.

4.2.7 Infinite dose permeation studies

In the case of infinite dose permeation studies, the donor compartment of each Franz cell was loaded with approximately 1g of a prototype formulation (1.08±0.08g/cm^2, n=5 for each formulation). Because of the high viscosity of the prototype formulations, 2mL disposable

plastic syringes (Fisher Scientific, UK) were used to apply the formulations. The dosing, i.e. the amount of formulation added to each cell, was determined gravimetrically.

For the simple solvent systems, 1mL of saturated NA solution in the solvent system was applied to the model membrane with an Eppendorf Research® pipette (Eppendorf AG, Germany). To avoid drug depletion, the solvent system applied into the donor compartment contained excess crystals of the drug.

The permeation study was conducted for 6 to 10 hours, which allowed for observation of the expected permeation profile. The collection time points were: 0, 1, 2, 4, 6, 8 and 10 hours. Each formulation was tested in five Franz cells. The donor compartment was sealed with Parafilm®M (Bemis Flexible Packaging, USA) to prevent evaporation of formulation components.

4.2.8 Finite dose silicone permeation studies

In the case of finite dose silicone permeation studies, the donor compartment of each Franz cell was loaded with approximately 10mg of a prototype formulation (11.01±1.78mg/cm^2, n=5 for each formulation). Formulations were applied to, and spread uniformly on the membrane using a glass rod. The dosing was determined by gravimetric measurements of the rod before and after application.

For the simple solvent systems, 10µL of saturated NA solution in the solvent system was applied to the model membrane with an Eppendorf Research® pipette (Eppendorf AG, Germany).

To mimic i*n vivo* conditions, the donor compartment was not sealed, to allow for formulation evaporation. The permeation study was conducted for 24 hours to enable observation of the plateau phase. The collection time points were: 0, 2, 5, 8, 18, 21 and 24 hours. Each formulation was tested in five Franz cells. At the end of the study the membrane was washed and extracted according to the protocol described below.

4.2.8.1 Mass balance protocol for finite dose silicone permeation study

Prior to cell disassembly, the membrane was washed with 1mL of 6% polyoxyethylene (20) oleyl ether (PE(20)OE) and rubbed thoroughly with a cotton bud dipped in the washing solution. The cotton bud was then added into the washing fraction and extracted for 1h at 45°C. Thereafter, the cells were disassembled. The membrane was placed in the Eppendorf vial and extracted with 1mL of purified water or methanol (for solvent analysis) for 1h at 45°C in an

orbital incubator (Stuart Scientific, UK). Appropriate aliquots of the washing and extraction samples were taken for dilution and further HPLC analysis.

4.2.9 Finite dose permeation studies in pig ear skin

In the case of finite dose pig ear permeation studies, the donor compartment of each Franz cell was loaded with approximately 10mg (11.17±1.80mg/cm^2, n=10, i.e. 5 cells for each formulation) of a prototype formulation.

For the simple solvent systems, excess drug was added to each solvent system and equilibrated for 48h at 32 ± 1°C. The suspensions of the drug in the solvents were centrifuged (10min, 12000rpm, 32 ± 1°C). The donor compartment of each Franz cell (n=5 per solvent system) was loaded with 10µL of supernatant (i.e. saturated solution of NA in a solvent system).

The donor compartment was not sealed to mimic *in vivo* conditions. The permeation study was conducted for 8 hours, which allowed for observation of an expected permeation profile. The collection time points were: 0, 1, 2, 4, 6 and 8 hours. Each of the formulations was tested in five Franz cells.

4.2.9.1 Mass balance protocol for finite dose pig ear permeation study

The membrane washing and extraction procedures were performed using a protocol similar to the mass balance study described in section 4.2.8.1. The membrane was washed with 1mL of 6% PE(20)OE and wiped thoroughly with a cotton bud dipped in the washing solution. The cotton bud was added to the washing solution and extracted for 1h at 45°C. Cells were disassembled and the full thickness pig ear skin was separated into epidermis and dermis prior to the extraction procedure. This enabled assessment of NA retention in different skin layers. The epidermis was separated from the dermis using a dry heat separation method described by previous authors [31, 33, 35]. Skin samples were subject to the hot air treatment at a temperature of 50°C for 30 seconds followed by the removal of the epidermis with a surgical scalpel. The temperature and the time of the hot air treatment were controlled (Digitron™-22 Thermometer, RS Components, UK and Traceable® Extra - Extra Loud Timer, Fisher Scientific, UK). The epidermis and the dermis were placed in pre-weighed Eppendorf vials and the mass of the membrane was determined. This was followed by the epidermis and the dermis extraction with 1mL of purified water or methanol for 1h at 45°C. Appropriate aliquots of the washing and extraction samples were taken for dilution and further HPLC analysis.

4.2.10 Sample analysis

HPLC with UV detection was used for NA quantification as described in section 3.2.5.

4.2.11 Data analysis

The permeation was evaluated by plotting the cumulative amount (Q_A) of the compound permeated per unit surface area of the membrane against the collection time for each diffusion cell.

For the infinite dose studies with silicone as a model membrane, J_{ss} was calculated by linear regression using Microsoft Excel software within the interval of 2 to 6 hours. The lag time (t_{lag}) was determined by the visual inspection of the permeation profiles. Knowing the thickness (h) of the membrane (0.00836cm), the diffusion coefficient (D) was calculated according to Equation 4.3.

$$t_{lag} = \frac{h^2}{6D}$$

Equation 4.3

For the prototype formulations, the concentration of the drug in the donor compartment (C_v) was 40000µg/g. In the case of simple solvent systems, the concentration of the drug was equal to its saturated solubility in the solvent system. Thus, the partition coefficient (K) could be obtained from Equation 4.4.

$$J = ADK_{m/v}\frac{C_v}{h}$$

Equation 4.4

The permeability coefficient (k_p) was calculated according to Equation 4.5.

$$k_p = \frac{KD}{h}$$

Equation 4.5

The modelling of both non-steady and steady state data using Scientist® Version 3.0 software (MicroMath Scientific, USA) was performed using Equation 4.6.

$$\overline{Q_A} = \frac{\overline{\bar{J}(s)}}{s} = \frac{AKC_v}{s\sqrt{\frac{s}{D}}\sinh\left(\sqrt{\frac{sh^2}{D}}\right)}$$

Equation 4.6

K and D parameters obtained from the fitting were used to calculate J_{ss} and k_p. J_{ss} calculated with both methods (Excel and Scientist®) was compared.

In the finite dose studies, only Scientist® was used to fit diffusion equations to experimental data, because of the limitations of the lag time method [36-38]. The modelling was performed according to Equation 4.7 (for silicone membrane)

$$\overline{Q_A} = \frac{KC_v V\sqrt{\frac{D}{s}}}{s^2\left(AKcosh\sqrt{\frac{sh^2}{D}} + Vsinh\sqrt{\frac{sh^2}{D}}\right)}$$

Equation 4.7

and Equation 4.8 (for the skin)

$$\overline{Q_A} = \frac{AP_1Q_0}{s\left(V\sqrt{\frac{s}{P_2}}sinh\sqrt{\frac{s}{P_2}} + P_1Acosh\sqrt{\frac{s}{P_2}}\right)}$$

Equation 4.8

The parameters K and D or P_1 and P_2 were calculated for silicone and pig ear skin studies respectively and k_p was calculated according to Equation 4.5 and Equation 4.9.

$$k_p = P_1P_2$$

Equation 4.9

The percentage of the applied dose permeated, washed and extracted from the membrane was compared for the formulations, in both silicone and pig ear skin finite dose studies. Also the retention of NA in the epidermis and the dermis [μg/g] was calculated according to Equation 4.10.

$$retention = \frac{amount\ of\ NA\ found\ in\ the\ skin}{weight\ of\ the\ skin}$$

Equation 4.10

For prototype formulations, statistical significance was determined using an independent sample 2-tailed t-test performed with IBM SPSS Statistics Version 19 software (SPSS Inc., an IBM Company). A probability of $p<0.05$ was considered statistically significant. All results are presented as the mean ± SD (n=5) unless otherwise stated.

For simple solvent systems, the percentage of the applied dose permeated, washed and extracted from the membrane was compared for the formulations, in both silicone and pig ear skin finite dose studies. Statistical significance was determined using one-way ANOVA with Tukey post-hoc test performed with IBM SPSS Statistics Version 19 software (SPSS Inc., an IBM Company). A probability of $p<0.05$ was considered statistically significant. All results are presented as mean ± SD (n=5) unless otherwise stated.

4.3 Results - silicone membrane studies

4.3.1 Solvent uptake studies

The results of solvent uptake studies are shown in Table 4.4.

Table 4.4 Solvent uptake into model membranes, mean ± SD, n=3

Solvent	Solubility parameter [$cal^{1/2}/cm^{3/2}$]	Molecular weight [g/mol]	Solvent uptake into silicone membrane [%]
MO	7.44	>500	1.74 ± 0.14
IPM	8.22	270.45	58.18 ± 8.87
DPPG	8.66	356.54	15.74 ± 0.48
PGML	9.44	258.40	1.13 ± 0.98
PGMC	9.85	230.31	1.57 ± 0.10
PG	14.07	76.09	0.19 ± 0.38
Water	22.97	18.02	0.83 ± 0.37

The highest solvent uptake into the silicone membrane was obtained for IPM, which is one of the most lipophilic of the chosen solvents (with the exception of MO) with a solubility parameter (δ) value closest to the solubility parameter postulated for the silicone membrane, i.e. 7.5 $cal^{1/2}/cm^{3/2}$ [39].

4.3.2 Nicotinamide partitioning

The investigation of the influence of solvents on NA partitioning into the silicone membrane was performed to provide insight into the drug affinity for the membrane. The partition coefficient of NA was calculated according to Equation 4.2. The results are shown in Table 4.5.

Table 4.5 Nicotinamide partitioning into silicone membrane, mean ± SD, n=3

Solvent	Concentration of NA in the solvent before uptake [mg/g]	Concentration of NA in the membrane [mg/g]	Concentration of NA in the solvent after uptake [mg/g]	Partition coefficient	Increase in the mass of the membrane [%]
IPM	3.10	0.19 ± 0.01	3.05 ± 0.01	0.0626 ± 0.0040	52.92 ± 1.67
DPPG	4.30	0.27 ± 0.01	4.26 ± 0.02	0.0626 ± 0.0020	14.76 ± 0.87
PGML	40.56	0.28 ± 0.02	41.50 ± 2.00	0.0067 ± 0.0008	1.36 ± 0.32
PGMC	56.34	0.23 ± 0.03	49.70 ± 15.53	0.0049 ± 0.0015	1.82 ± 0.06
PG	300.37	0.21 ± 0.01	290.99 ± 9.65	0.0009 ± 0.0003	0.02 ± 0.03

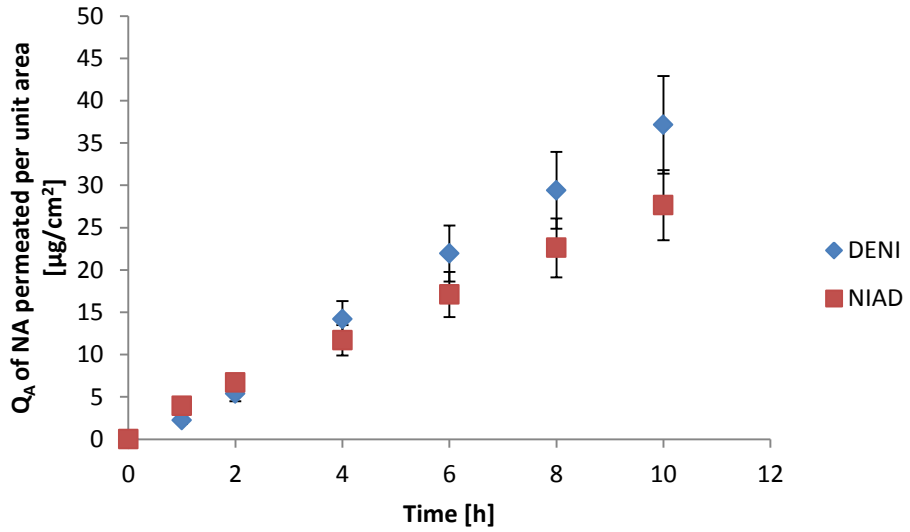
The highest partition coefficient ($p<0.05$, one-way ANOVA) was obtained for IPM and DPPG. The lowest partition coefficient ($p<0.05$, one-way ANOVA), was obtained for PG.

4.3.3 Nicotinamide permeation from prototype formulations

4.3.3.1 Infinite dose permeation studies across silicone membrane

The infinite dose permeation profiles of prototype DENI and NIAD formulations obtained in the silicone permeation study are shown in Figure 4.1.

a)



b)

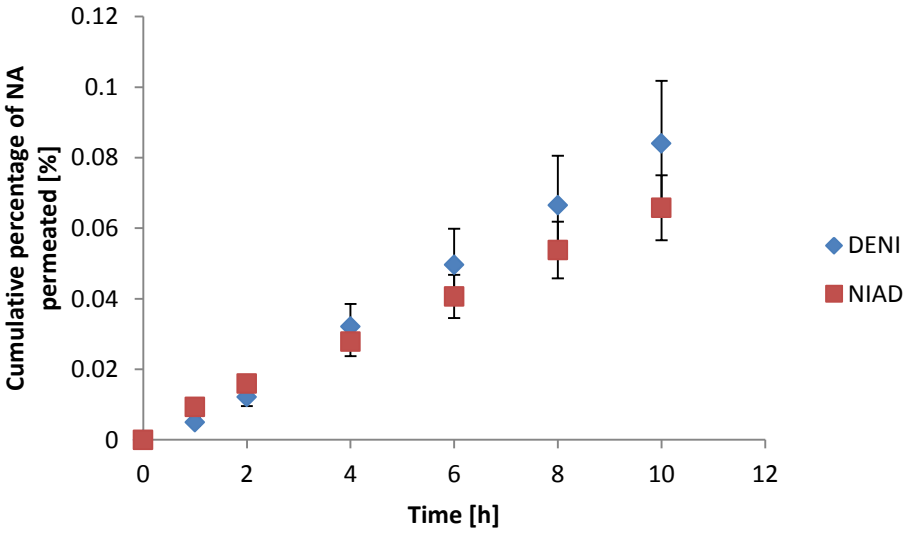


Figure 4.1 Infinite dose permeation profiles of prototype DENI and NIAD formulations across silicone membrane: a) cumulative amount of nicotinamide, b) percentage of nicotinamide permeated (data shown as mean ± SD, n=5)

The prototype NA formulations are different in terms of cumulative amounts (Q_A) of NA permeated across silicone membrane in the infinite dose studies. A statistically significant

difference (t-test, p<0.05) in Q_A was found after 6, 8 and 10 hours, with higher values for the DENI formulation.

Statistically significant differences (t-test) were observed for J_{ss} (p<0.005), K (p<0.05) and k_p (p<0.005) calculated with the lag time method and for all compared permeation parameters obtained by Scientist® modelling: J_{ss}, k_p (p<0.05) and K, D (p<0.0005). The permeation parameters are shown in Table 4.6.

Table 4.6 Permeation parameters obtained in silicone infinite dose studies for DENI and NIAD formulations (data shown as mean ± SD (n=5), *p<0.05, **p<0.005, ***p<0.0005)

	Parameter	[unit]	DENI	NIAD
	Q_A* (after 10h of experiment)	**[µg/cm^2]**	37.15 ± 5.76	27.65 ± 4.13
Lag time	**J_{ss}****	**[µg/cm^2/h]**	3.99 ± 0.61	2.65 ± 0.43
	D	**[cm^2/h]**	2.33×10^{-5} ± 0.00	2.33×10^{-5} ± 0.00
	K*		0.036 ± 0.006	0.024 ±0.004
	k_p**	**[cm/h]**	$9.97\times10^{-5} \pm 1.53\times10^{-5}$	$6.64\times10^{-5} \pm 1.08\times10^{-5}$
Scientist	**J_{ss}***	**[µg/cm^2/h]**	3.93 ± 0.60	2.88 ± 0.43
	D***	**[cm^2/h]**	$2.36\times10^{-5} \pm 0.04\times10^{-5}$	$2.37\times10^{-3} \pm 0.11\times10^{-3}$
	K***		0.036 ± 0.007	$2.55\times10^{-4} \pm 0.04\times10^{-4}$
	k_p*	**[cm/h]**	$9.84\times10^{-5} \pm 1.50\times10^{-5}$	$7.2\times10^{-5} \pm 1.08\times10^{-5}$
	t_{lag}	**[h]**	$2.74\times10^{-10} \pm 4.69\times10^{-11}$	$2.76\times10^{-8} \pm 1.33\times10^{-9}$

There was no significant difference (t-test, p<0.05) between flux and permeability coefficient calculated from steady state data (lag time method) and from non-steady and steady state data (Scientist®). Thus, modelling of the permeation data with Scientist® software may be helpful in determination of the permeation parameters.

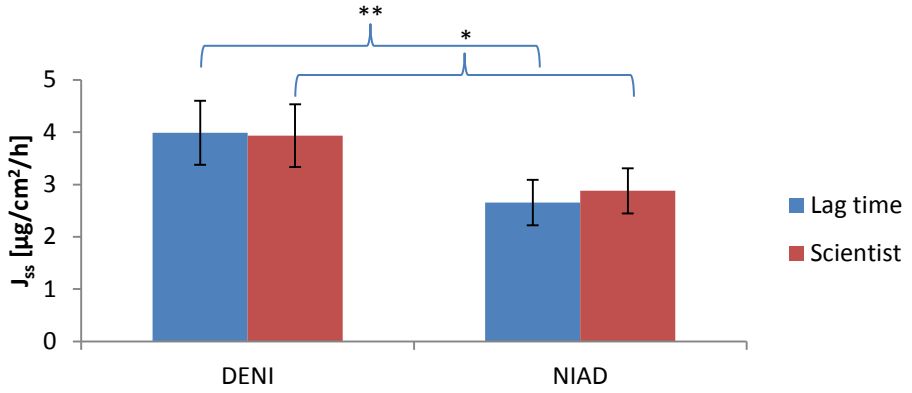


Figure 4.2 Steady state flux for infinite dose NA permeation across silicone membrane from prototype DENI and NIAD formulations calculated with two different methods (mean ± SD, n=5, *p<0.05, **p<0.005)

4.3.3.2 Finite dose permeation studies across silicone membrane

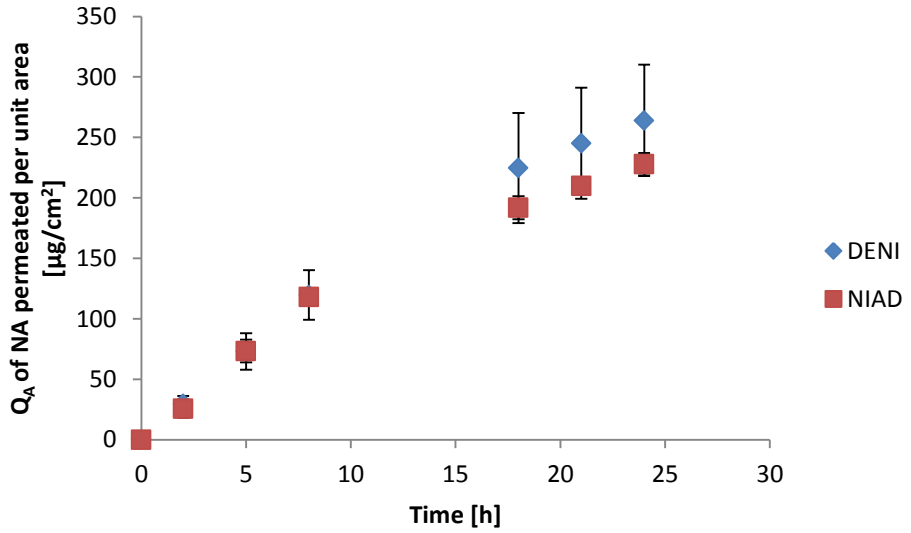
The finite dose permeation profiles of prototype DENI and NIAD formulations obtained in the finite dose silicone permeation studies are shown in Figure 4.3.

No significant differences (t-test, p>0.05) were found for the permeation parameters (Q_A, D, K and k_p). Also no differences (t-test, p>0.05) in the mass balance of NA between the membrane and the cell compartments were found for the two formulations. The summary of the results is shown in Table 4.7. Presence of several excipients in the tested formulations might have complicated the experimental conditions causing a high standard deviation.

Table 4.7 Permeation parameters obtained in silicone finite dose studies for DENI and NIAD formulations (data shown as mean ± SD, n=5)

Parameter [unit]	DENI	NIAD
Q_A [µg/cm^2] after 24h	264.08±46.18	227.93±9.26
% of NA permeated	59.07±15.97	55.24±6.30
% washed from the membrane	43.12±19.09	39.31±7.81
% extracted from the membrane	0.00±0.00	0.00±0.00
Total recovery [%]	102.19±4.63	94.55±2.23
D [cm^2/h]	$4.20\times10^{-5}\pm1.84*10^{-5}$	$7.50\times10^{-5}\pm7.11*10^{-5}$
K	131.12 ± 138.01	90.16 ± 135.41
k_p	0.43 ± 0.20	0.39 ± 0.30
t_{lag}	$4.86\times10^{-10}\pm2.15\times10^{-11}$	$8.73\times10^{-9}\pm8.28\times10^{-9}$

a)



b)

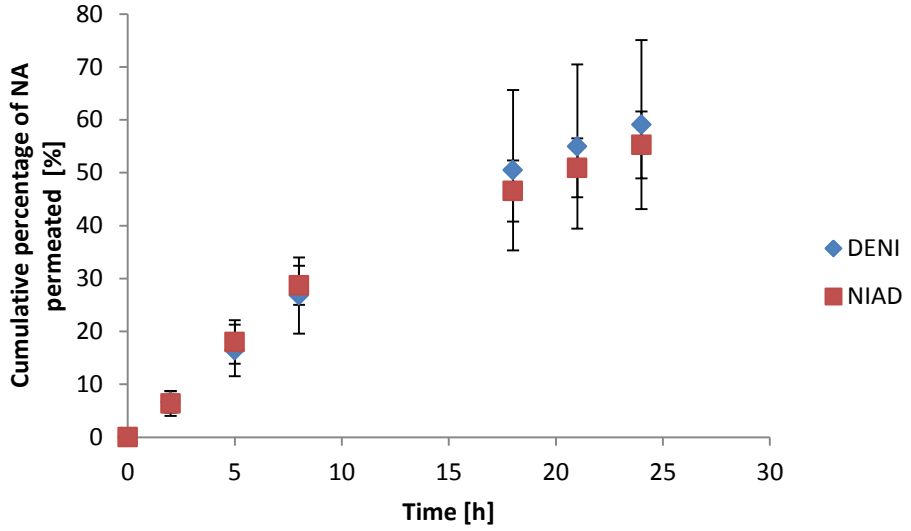


Figure 4.3 Finite dose permeation profiles of prototype DENI and NIAD formulations across silicone membrane: a) cumulative amount of nicotinamide, b) percentage of nicotinamide permeated (data shown as mean ± SD, n=5)

4.3.4 Nicotinamide permeation from simple solvent systems - infinite dose silicone studies

In vitro infinite dose Franz-cell permeation studies with silicone as a model membrane were conducted to evaluate the single solvents and simple solvent systems in terms of influence on NA diffusion across the membrane. The solubility of NA in the chosen solvent systems and the solubility parameter difference between the vehicle (solvent) and the membrane (δ_v - δ_m) is shown below to allow for comparison between the systems tested.

The permeation of NA from the following single solvent systems was studied:

Table 4.8 Single solvents used in the infinite dose silicone studies

Solvent	Solubility 48h ± SD [mg/mL]	$\delta_v - \delta_m$ [$cal^{1/2}/cm^{3/2}$]
IPM	0.68 ± 0.04	0.72
DPPG	1.63 ± 0.01	1.16
PGML	22.15 ± 0.35	1.94
PGMC	38.17 ± 1.25	2.35
PG	243.37 ± 8.76	6.57
Water	552.70 ± 11.49	15.47

The cumulative amount of NA permeated per unit area (Q_A) [µg/cm^2] from each solvent system was plotted against time [h]. The permeation profiles obtained for the single solvent systems are shown in Figure 4.4.

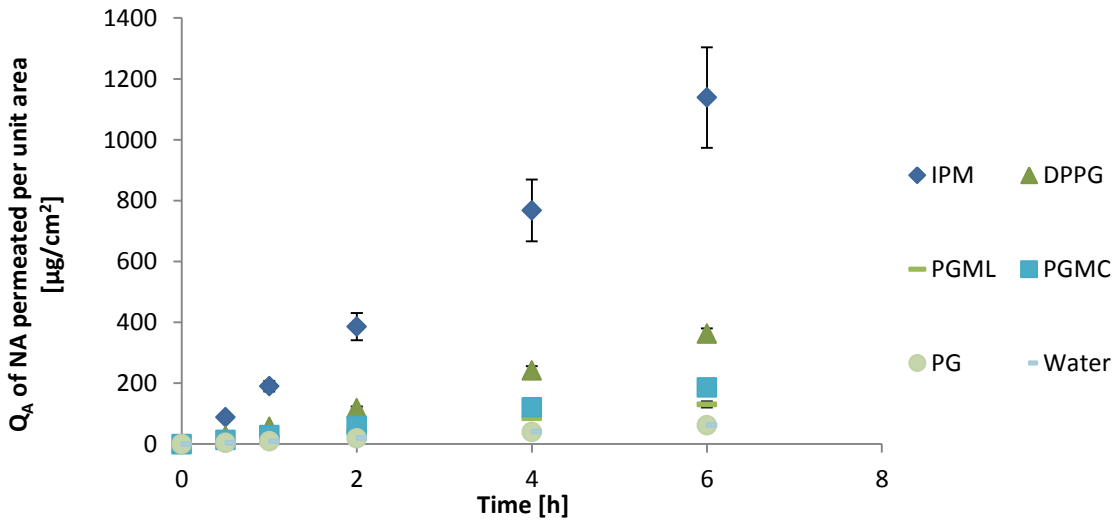


Figure 4.4 Infinite dose permeation profiles of NA applied in single solvents to the silicone membrane at 32 ± 0.5°C (mean ± SD, n=5)

The Q_A of NA permeated after 6 h from single solvents is shown in Figure 4.5.

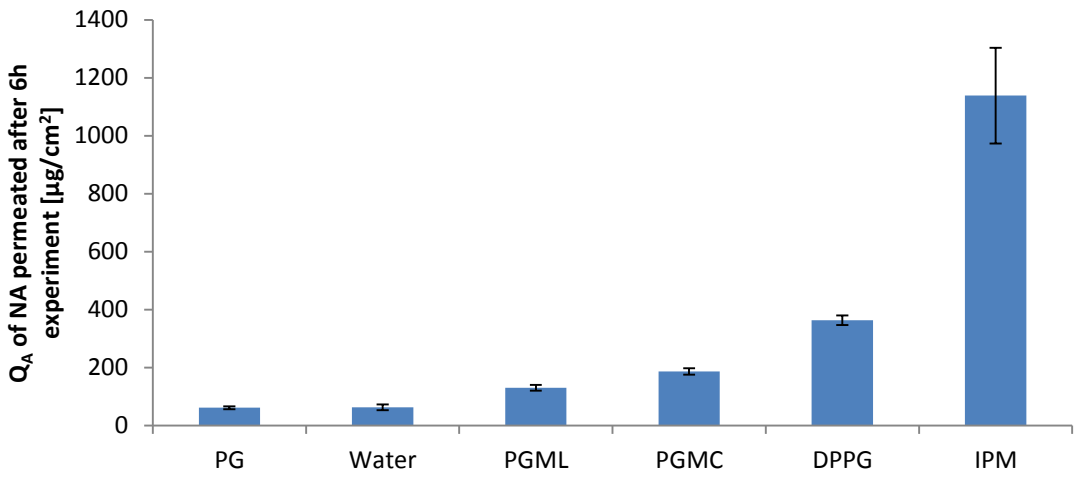


Figure 4.5 Cumulative amount of NA permeated after 6 h from single solvents in the infinite dose permeation studies across silicone membrane at 32 ± 0.5°C (mean ± SD, n=5)

Because synergistic effects on permeation were observed for combinations of PG with fatty acids and their esters by previous authors [40-47], the 50:50 mixtures of the chosen single solvents were further investigated in the infinite dose silicone permeation studies.

The following binary solvent systems were investigated:

Table 4.9 Binary solvent systems used in the infinite dose silicone studies

Solvent system	Ratio of solvents used	Solubility 48h ± SD [mg/mL]	$\delta_v - \delta_m$ [$cal^{1/2}/cm^{3/2}$]
IPM:DPPG (ID)	50:50	1.12 ± 0.02	0.94
IPM:PGML (IL)	50:50	8.29 ± 0.09	1.33
DPPG:PGML (DL)	50:50	9.93 ± 0.23	1.53
IPM:PGMC (IC)	50:50	12.70 ± 0.37	1.55
DPPG:PGMC (DC)	50:50	15.74 ± 0.24	1.75
PGML:PGMC (LC)	50:50	31.35 ± 1.38	2.14
PG:PGML (PL)	50:50	128.54 ± 4.24	4.25
PG:PGMC (PC)	50:50	143.75 ± 3.12	4.46
PG:H2O (PH)	50:50	471.96 ± 6.67	11.02

The permeation profiles obtained for the binary solvent systems are shown in Figure 4.6.

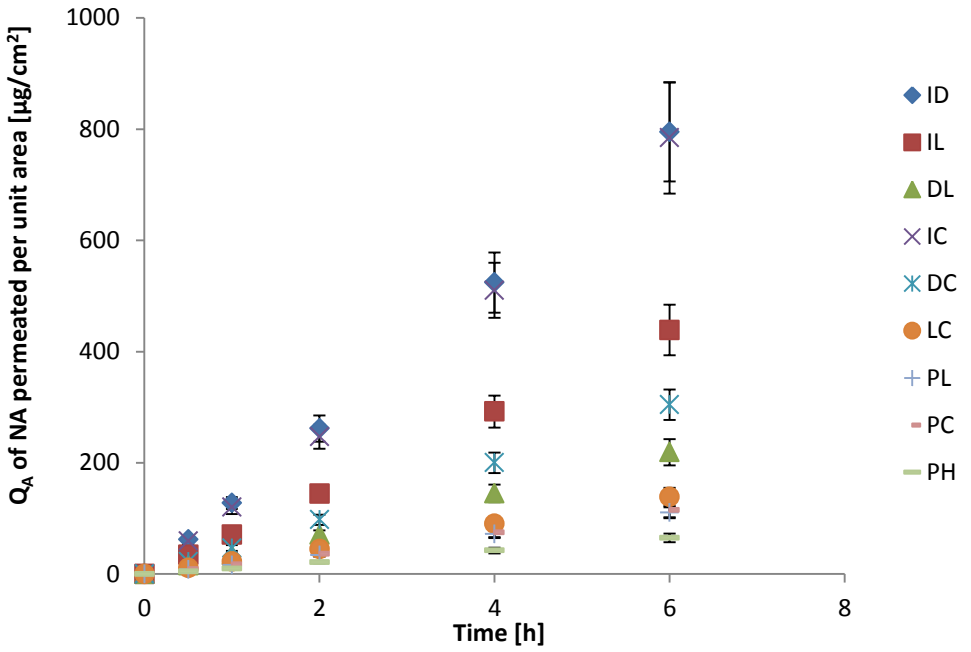


Figure 4.6 Infinite dose permeation profiles of NA applied in binary solvent systems to the silicone membrane at 32 ± 0.5°C (mean ± SD, n=5)

The Q_A of NA permeated after 6 h from each of the binary solvent systems tested is shown in Figure 4.5.

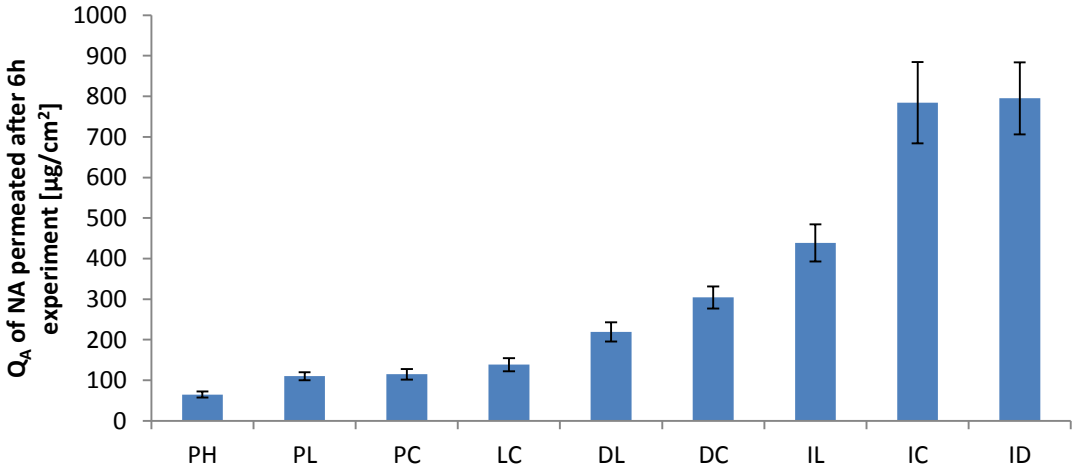


Figure 4.7 Cumulative amount of NA permeated after 6 h from binary solvent systems in the infinite dose permeation studies across silicone membrane at 32 ± 0.5°C (mean ± SD, n=5)

Steady state data (in general, the time points from 2 to 6 hours) were taken into account to calculate J_{ss} using the lag time method. Steady state data and J_{ss} obtained from these data were used to determine k_p. Also, J_{ss} and k_p were calculated from the results obtained with Scientist® software, by fitting both non-steady and steady state data to the relevant equations

(see section 4.2.7). J_{ss} and k_p values obtained with both methods ware not significantly different (t-test, p>0.05). On the contrary, there was a strong correlation between J_{ss} and k_p values ($r^2 \approx 1$ both for J_{ss} and k_p) once again confirming the utility of Scientist® software modelling for the permeation data. The correlation plots are shown in Figure 4.8.

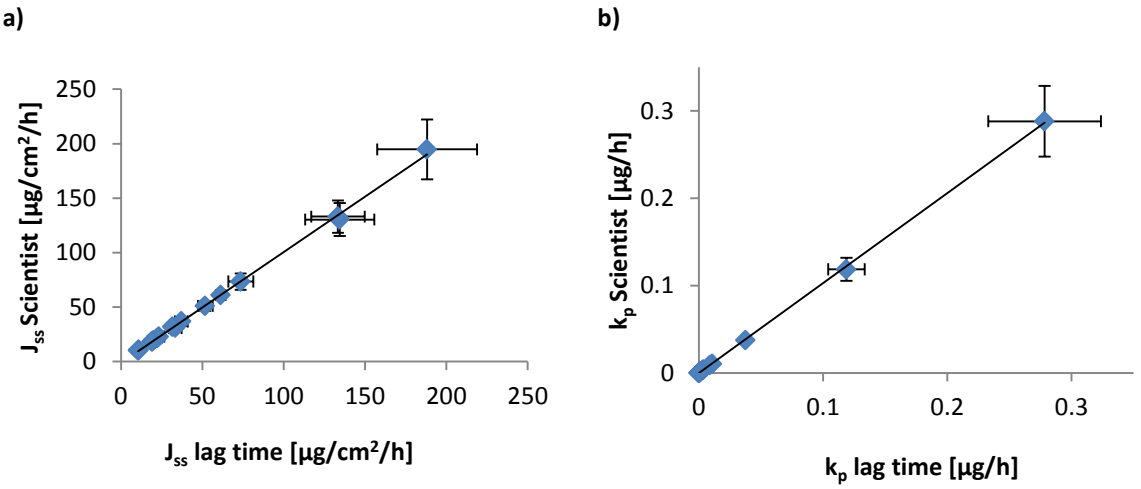


Figure 4.8 Correlation between lag time method and Scientist® calculations: a) steady state flux, b) permeability coefficient (mean ± SD, n=5)

The J_{ss} and k_p values obtained from the NA silicone permeation studies for simple solvent systems are shown in Figure 4.9 and Figure 4.10.

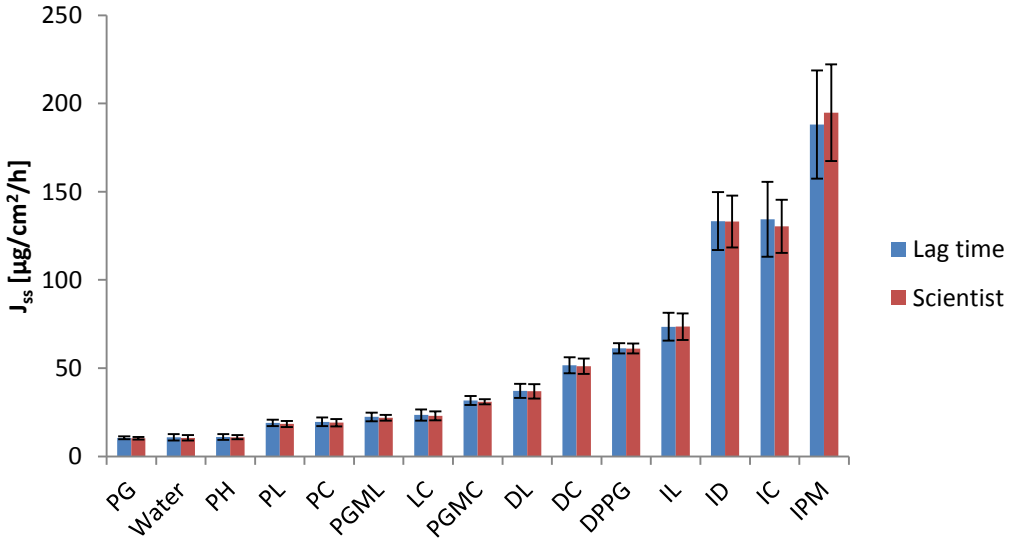


Figure 4.9 Steady state flux for infinite dose NA permeation across silicone membrane from simple solvent systems at 32 ± 0.5°C (mean ± SD, n=5)

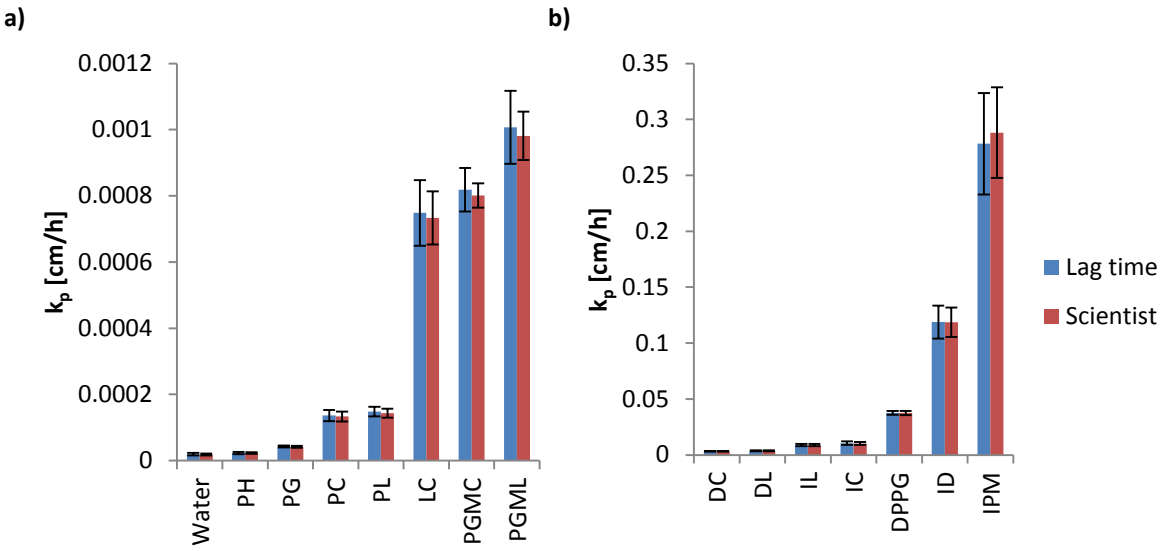


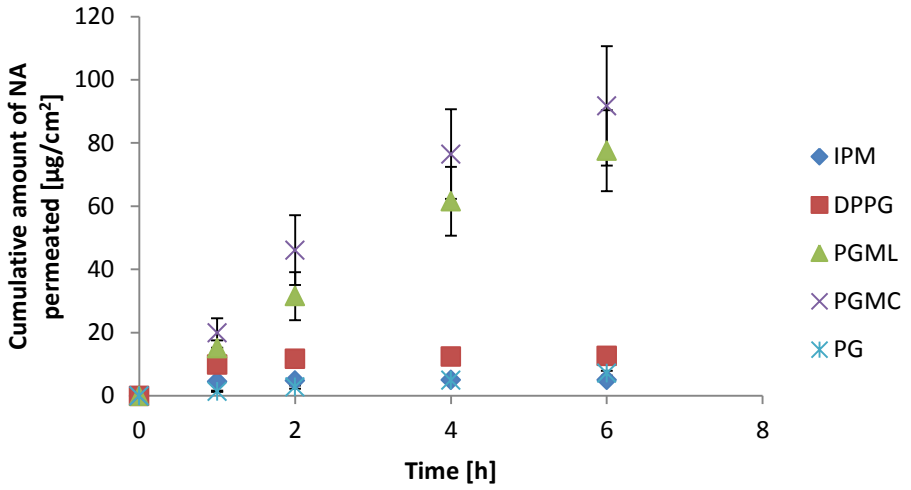
Figure 4.10 Permeability coefficients for infinite dose permeation across silicone membrane from simple solvent systems at 32 ± 0.5°C (mean ± SD, n=5); a) formulations with low permeability coefficients, b) formulations with high permeability coefficients

From the data shown above, it can be seen that NA permeation across silicone membrane depends strongly on the solvent in which it is applied. From the solvent systems tested, the highest J_{ss} and k_p were obtained for IPM (one-way ANOVA, p<0.05). The lowest J_{ss} and k_p values were found for water, PG and their 50:50 mixture (PH). These three systems were not significantly different from each other in terms of J_{ss} and k_p (one-way ANOVA, p>0.05). For the solvent systems with the highest J_{ss} across silicone membrane, the flux decreased in the following order: IPM > IC ≈ ID > IL > DPPG. These systems have close δ values to the δ of silicone membrane (7.5 $cal^{1/2}/cm^{3/2}$) [39]. The differences (δ_v - δ_m) between the solubility parameter of the solvent system (δ_v) and the solubility parameter of the membrane (δ_m) were calculated and are shown in Table 4.8 and Table 4.9. Also high J_{ss} and k_p values were observed for the solvents with low solubility of the drug in the solvent system and high solvent uptake into the membrane.

4.3.5 Solvent and nicotinamide permeation - finite dose silicone studies

Finite dose permeation studies across silicone membrane were performed. NA and solvent permeation profiles are shown in Figure 4.11 and Figure 4.12, respectively.

a)



b)

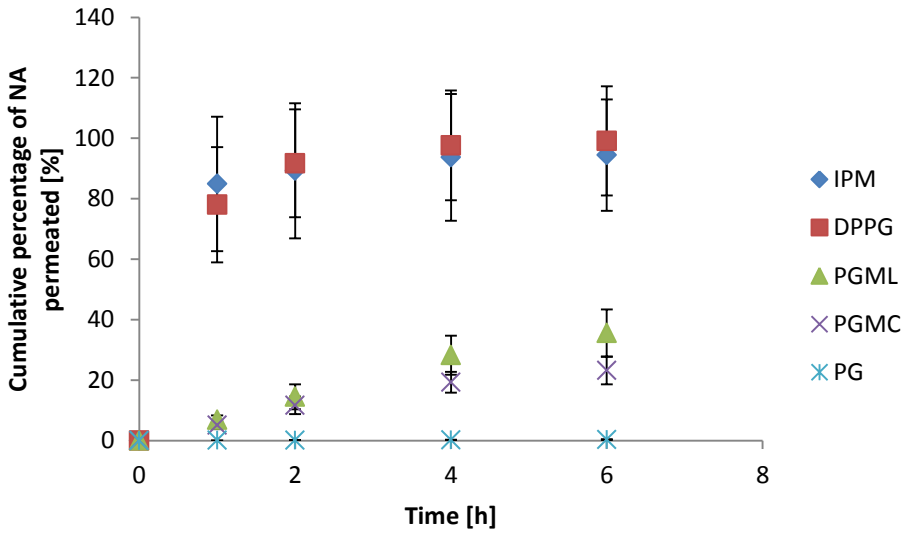
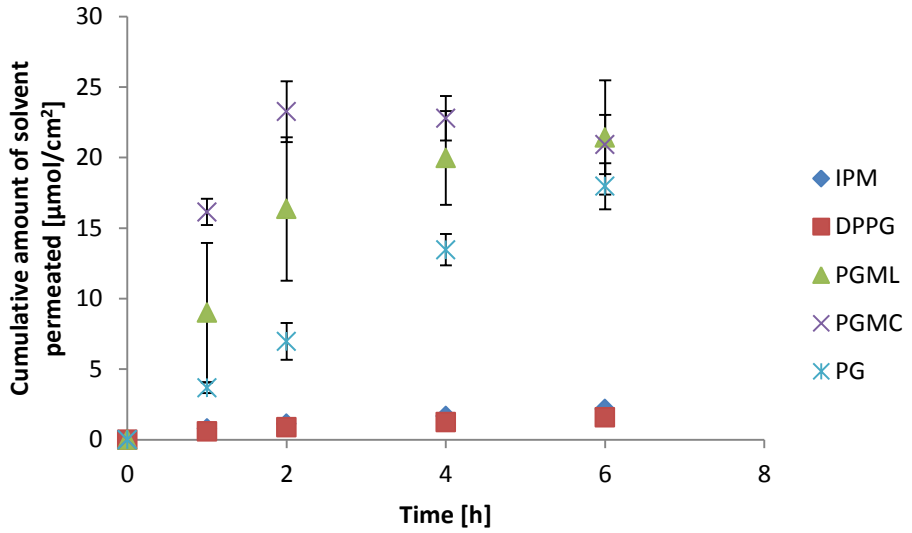


Figure 4.11 Finite dose silicone permeation profiles for nicotinamide: a) cumulative amonut of nicotinamide permeated, b) cumulative percentage of nicotinamide permeated at 32 ± 0.5°C (mean ± SD, n=5)

a)



b)

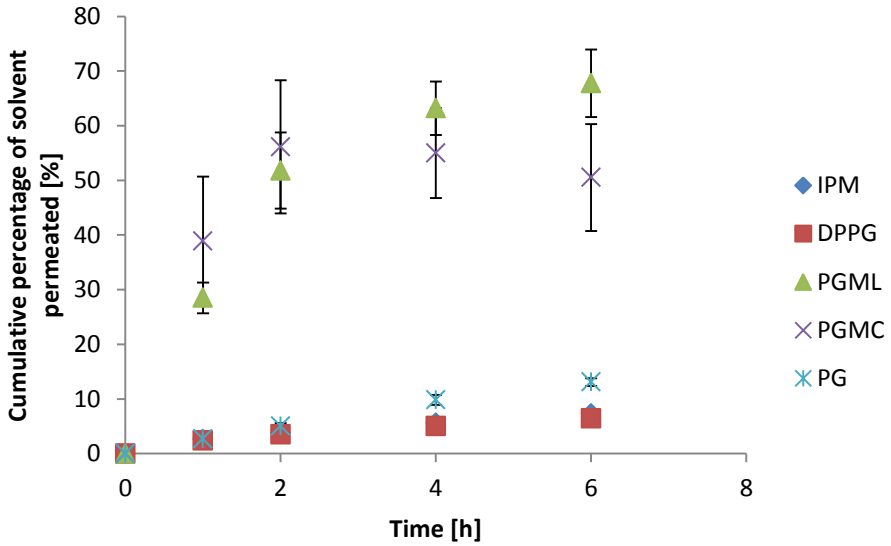


Figure 4.12 Finite dose silicone permeation profiles for solvents: a) cumulative amount of solvent permeated, b) cumulative percentage of solvent permeated at 32 ± 0.5°C (mean ± SD, n=5)

The highest cumulative amount of NA ($p<0.05$, one-way ANOVA) permeated from PGML and PGMC, which is in line with the highest cumulative amount of these solvents permeated ($p<0.05$, one-way ANOVA) when compared with IPM and DPPG. The lowest cumulative amount of NA ($p<0.05$, one-way ANOVA) was obtained for IPM, DPPG and PG. In the case of IPM and DPPG, this is because of the low solubility of NA in these solvents and thus, low dose of NA applied. For PG, the cumulative amount of the solvent is not different ($p>0.05$, one-way ANOVA) from the cumulative amount of PGML and PGMC that permeated after 6 hours. However, the percentage of PG permeated is significantly lower ($p<0.05$, one-way ANOVA)

than the values obtained for PGML and PGMC. This may indicate that the relatively low amount of NA permeated from PG may be because of the low permeation of this solvent.

The percentages of applied dose of NA and solvents permeated, washed and extracted from the membrane after 6h are shown in Figure 4.13.

The highest ($p<0.05$, one-way ANOVA) percentage of NA permeated from IPM and DPPG, even though the lowest ($p<0.05$, one-way ANOVA) percentage of these solvents (not different from PG) permeated over the course of the study. However, high percentages of IPM and DPPG (77.61 ± 5.33 and 54.70 ± 6.13, respectively) were extracted from the silicone membrane, indicating that the presence of the solvents in the membrane is enough to enable NA permeation. It is not surprising that most of the applied doses of IPM and DPPG were found in the membrane, since these two solvents have a higher affinity for the hydrophobic silicone membrane than for the hydrophilic receptor phase.

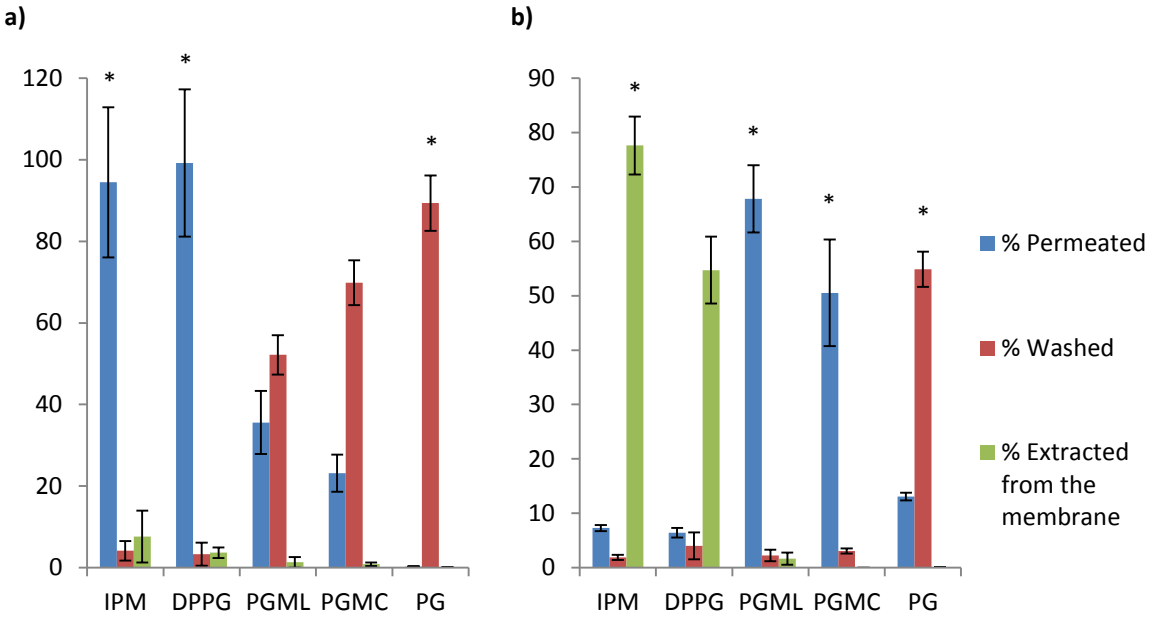


Figure 4.13 Percentages of applied dose permeated, washed and extracted from the membrane after 6h for a) nicotinamide and b) solvents, mean ± SD, n=5, *p<0.05

The highest ($p<0.05$, one-way ANOVA) percentage of solvent permeation was found for PGML and PGMC (67.78±6.18 and 50.51±9.79, respectively). Also, the permeation profiles indicate rapid penetration of these solvents, with more than 50% permeated after the first two hours of the experiment. These results indicate a depletion of PGML and PGMC from the donor phase, which in turn leads to a relatively low percentage of NA permeated and high percentage washed from the membrane at the end of the study.

The highest ($p<0.05$, one-way ANOVA) percentage of solvent and NA washed from the membrane was obtained for PG. Even though this solvent permeates though the silicone membrane to some extent (approximately 10%), only a small percentage of NA (0.30±0.03%) permeated from this solvent. This may be because of high solvent hydrophilicity and very low affinity for the membrane, resulting in too low solvent uptake into the membrane to enable NA permeation. This is in line with the low percentage of solvent extracted from the membrane at the end of the study, which was also observed for PGML and PGMC.

4.4 Results - pig ear skin studies

4.4.1 Solvent uptake studies - pig ear skin

The results of solvent uptake studies are shown in Table 4.10 and Figure 4.16.

Table 4.10 Solvent uptake into the model membranes, mean ± SD, n=3

Solvent	Solubility parameter [$cal^{1/2}/cm^{3/2}$]	Molecular weight [g/mol]	Solvent uptake into stratum corneum [%]
IPM	8.22	270.45	18 ± 6
DPPG	8.66	356.54	28 ± 8
PGML	9.44	258.40	39 ± 9
PGMC	9.85	230.31	47 ± 9
PG	14.07	76.09	81 ± 21

The highest uptake into the SC was obtained for PG and a linear relationship was found between the SC solvent uptake and δ values for the chosen solvents (r^2=0.96).

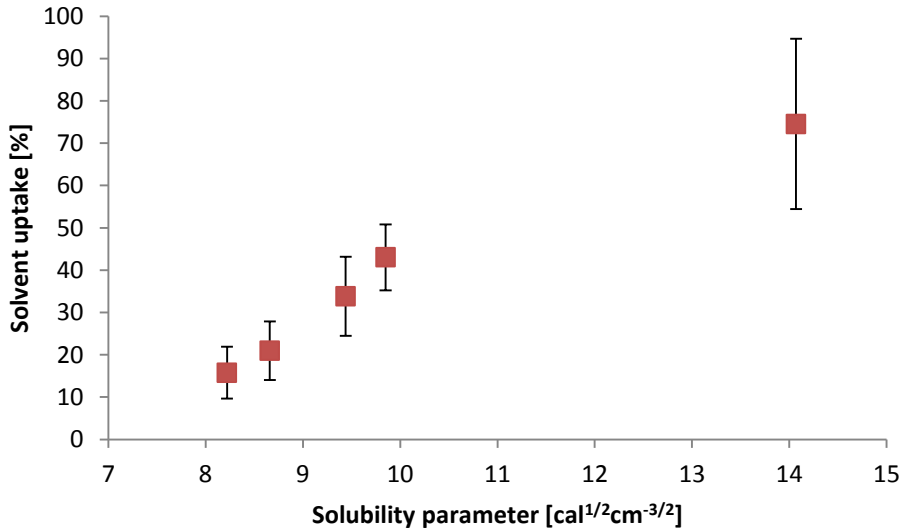


Figure 4.14 Solvent uptake and solubility parameter, mean ± SD, n=3

4.4.2 Nicotinamide partitioning

The investigation of the influence of solvents on NA partitioning into the SC was performed to provide insight into the drug affinity for this environment. The partition coefficient of NA was calculated according to Equation 4.2. The results are shown in Table 4.11.

Table 4.11 Nicotinamide partitioning into the stratum corneum, mean ± SD, n=3

Solvent	Concentration of NA in the solvent before uptake [mg/g]	Concentration of NA in the membrane [mg/g]	Concentration of NA in the solvent after uptake [mg/g]	Partition coefficient	Increase in the mass of the membrane [%]
IPM	3.1	7.5 ± 2.7	3.0 ± 0.0	2.5 ± 0.9	11.0 ± 0.4
DPPG	4.3	5.3 ± 0.6	4.0 ± 0.1	1.3 ± 0.2	14.2 ± 1.1
PGML	40.6	5.8 ± 1.1	38.2 ± 0.3	0.2 ± 0.0	26.6 ± 10.1
PGMC	56.3	4.7 ± 0.3	58.2 ± 2.0	0.1 ± 0.0	31.1 ± 21.9
PG	300.4	14.0 ± 0.7	302.8 ± 7.7	0.05 ± 0.0	58.2 ± 15.6

The highest partition coefficient ($p<0.05$, one-way ANOVA) was obtained for IPM and DPPG. The lowest partition coefficient ($p<0.05$, one-way ANOVA), was obtained for PG.

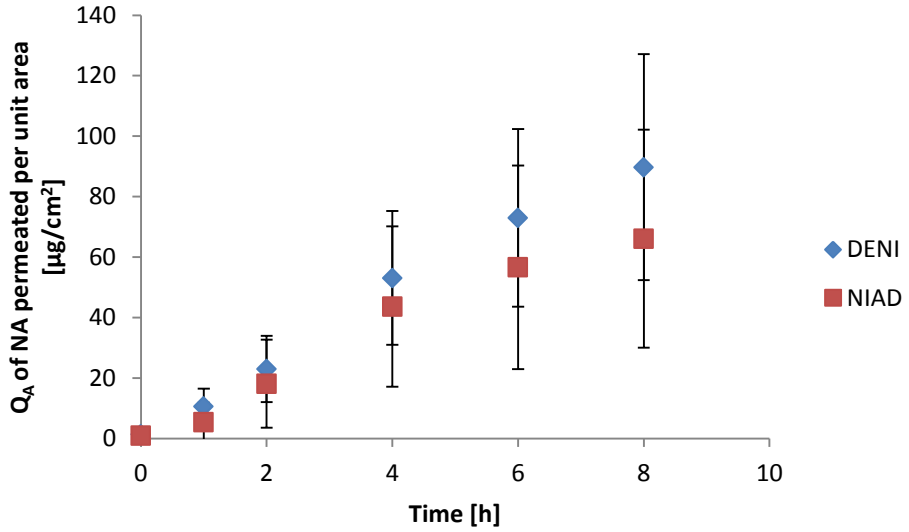
4.4.3 Nicotinamide finite dose permeation studies in pig ear skin - prototype formulations

The permeation profiles of DENI and NIAD formulations obtained in the finite dose pig ear skin study are shown in Figure 4.15.

The DENI and NIAD formulations showed similar permeation characteristics in the pig ear skin studies. The differences between Q_A and the amount of NA washed, extracted and permeated from each formulation were not statistically significant (t-test, $p>0.05$). The high standard deviation values are most probably because of differences in the skin thickness or integrity and are usually encountered when working with animal or human tissue [20, 48].

After the 8 h study, approximately 20% of the applied dose permeated through the skin. Most of the dose was left on the skin surface (the washing fraction of the mass balance study). Only a small percentage of NA was found in extracts from the epidermis and the dermis, indicating that DENI and NIAD formulations do not enhance NA retention in the skin.

a)



b)

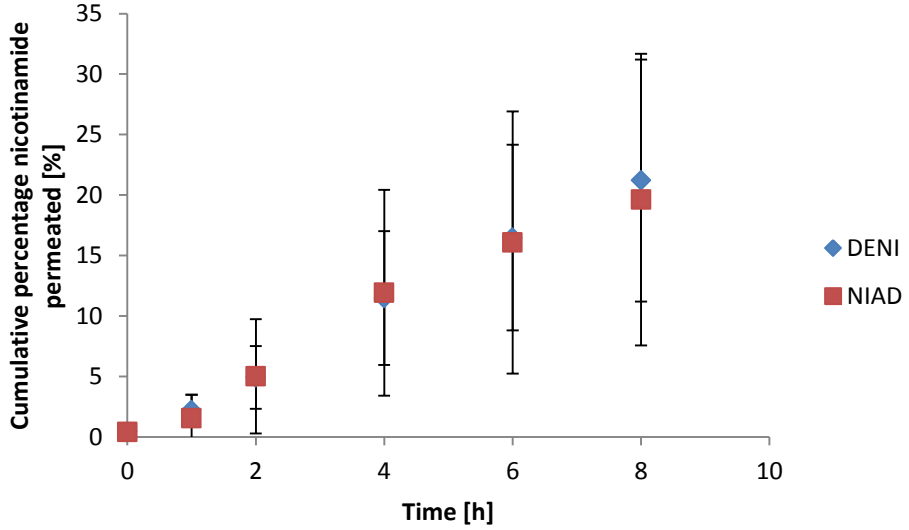


Figure 4.15 Finite dose permeation profiles of prototype DENI and NIAD formulations across pig ear skin: a) cumulative amount of nicotinamide, b) percentage of nicotinamide permeated (data shown as mean ± SD, n=5)

The permeation data were fitted into the Scientist® model for finite dose skin permeation studies. However, it was impossible to obtain fixed values of P_1 and P_2. Thus, the comparison between the formulations in the finite dose pig skin study was performed on the basis of the mass balance between the skin and the cell compartments. The summary of the results is shown in Table 4.12.

Table 4.12 Permeation parameters for finite dose studies in pig ear skin for DENI and NIAD formulations (data shown as mean ± SD, n=5)

Parameter [unit]	DENI	NIAD
Q_A [µg/cm^2] after 8h	89.7 ± 37.4	66.1 ± 36.1
% of NA permeated	18.4 ± 9.2	17.0 ± 10.2
% washed from the membrane	39.2 ± 9.9	56.6 ± 15.8
% extracted from the epidermis	1.9 ± 1.3	3.1 ± 2.0
% extracted from the dermis	1.2 ± 1.0	2.0 ± 1.2
Epidermis retention [µg/g]	278.4 ± 135.1	337.7 ± 214.1
Dermis retention [µg/g]	15.6 ± 12.8	20.6 ± 4.9

4.4.4 Nicotinamide finite dose permeation studies in pig ear skin - simple solvent systems

Finite dose permeation studies across full thickness porcine skin were performed. Simple solvent systems saturated with NA, i.e. with the same thermodynamic activity of NA at the beginning of the study, were tested. The concentration of NA in each solvent system was determined by HPLC. To facilitate analysis, results obtained for single solvents are shown separately from binary and multicomponent solvent systems (MSS), containing three or four solvents.

4.4.4.1 Single solvents

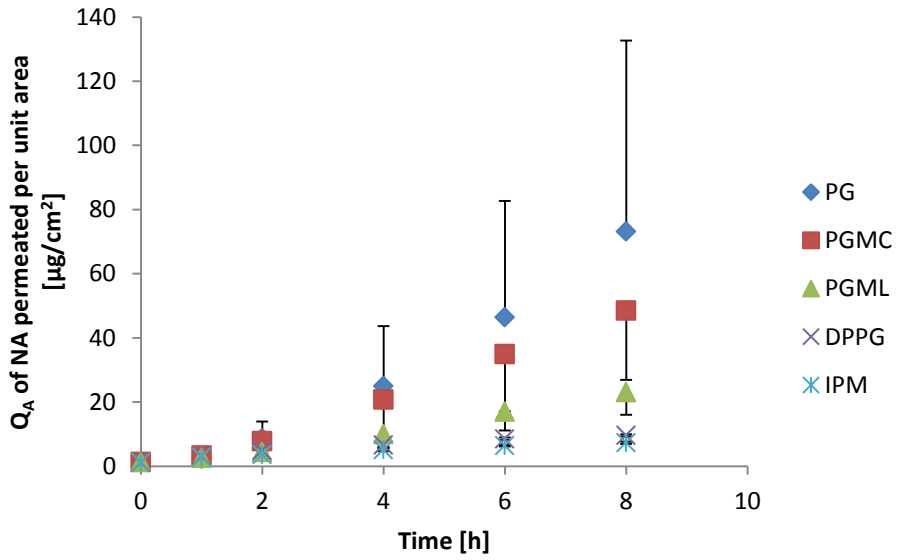
NA permeation from the following single solvents was examined in pig ear skin:

Table 4.13 Single solvents used in the finite dose pig ear skin studies

Solvent	Concentration of NA [mg/mL]	Solubility parameter [cal$^{1/2}$/cm$^{3/2}$]
IPM	0.68	8.22
DPPG	1.53	8.66
PGML	22.81	9.44
PGMC	38.17	9.85
PG	246.76	14.07

The NA permeation profiles obtained in the finite dose pig ear skin studies of single solvents are shown in Figure 4.16.

a)



b)

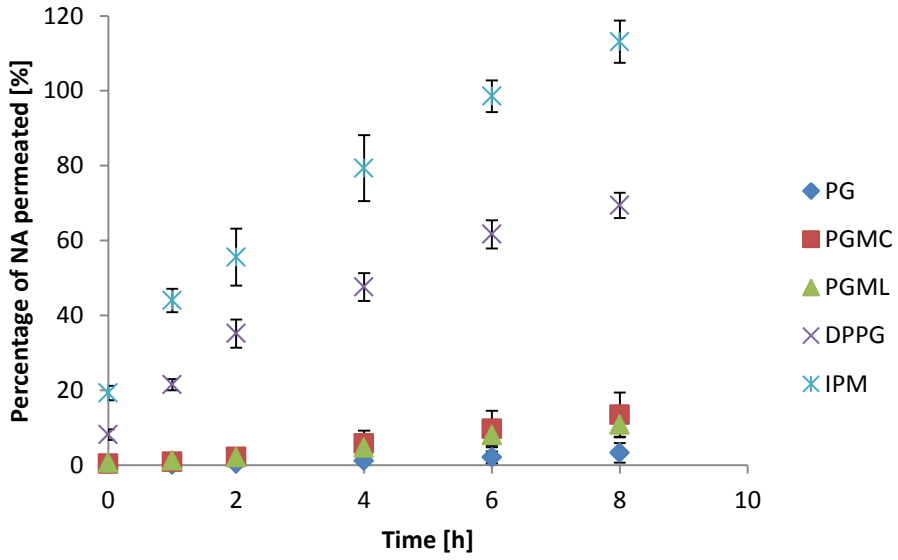


Figure 4.16 Finite dose pig ear skin NA permeation profiles for single solvents at 32 ± 0.5°C (mean ± SD, n=5); a) cumulative amount of nicotinamide, b) percentage of nicotinamide permeated

The cumulative amount (Q_A) of NA and the % of appied dose permeated after 8 h from each of the solvents tested are shown in Figure 4.17.

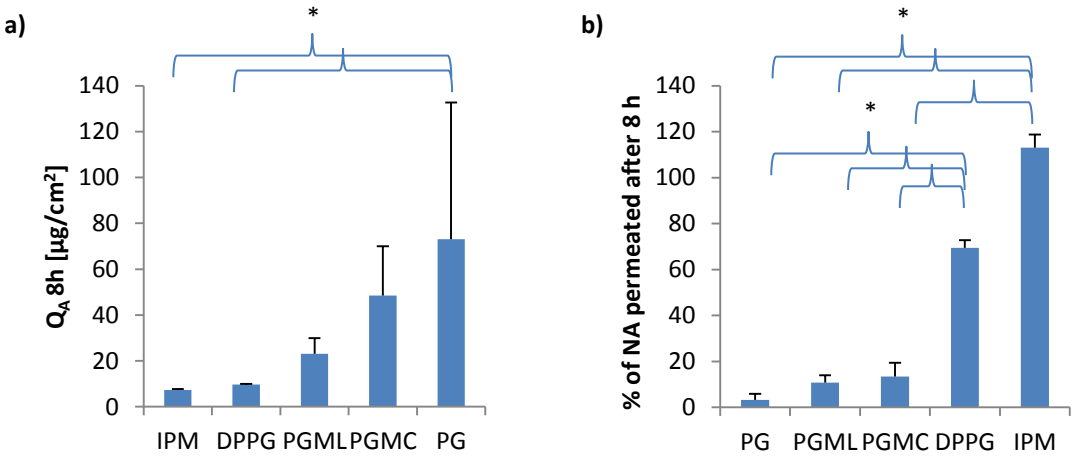


Figure 4.17 Cumulative amount of NA (a) and % of the applied dose (b) permeated after 8 h from single solvents in the finite dose permeation studies in pig ear skin at 32 ± 0.5°C (data shown as mean ± SD, n=5, *p<0.05)

For IPM the majority of the dose permeated over the course of the study when compared with the other solvents (one-way ANOVA, p<0.05). However, the smallest Q_A of NA, which was significantly different from PG (one-way ANOVA, p>0.05), was obtained for IPM. IPM has high penetration enhancing potential for NA, but low solubility of NA in this solvent resulted in a small total amount delivered across the skin. A similar effect can be observed for DPPG which was significantly different from PG in terms of Q_A (one-way ANOVA, p<0.05). On the other hand, the lowest % of NA permeated is observed for PG and is significantly different from IPM and DPPG (one-way ANOVA, p<0.05). This is because of the high NA solubility in this solvent, i.e. a high amount of NA is needed to reach saturation in PG. Thus, a high dose of the drug is applied to the skin in the saturated PG solution and only a small part of this dose permeates during the experiment. In the middle of this range of solvents are PGML and PGMC, for which it was possible to deliver a relatively small total amount of NA across the membrane, reflected in approximately 10% of applied dose. Thus, two groups of solvents significantly different from each other (one-way ANOVA, p<0.05) can be distinguished on the basis of the percentage of NA permeated after the 8 h experiment. DPPG and IPM belong to the group with comparatively higher percentages permeated whereas PG, PGML and PGMC result in low percentages of NA found in the receptor solution at the end of the study.

The influence of the selected solvents on NA permeation can be illustrated with the permeability coefficient (k_p), which compensates for the difference in solubility of NA in the solvents. IPM has the highest k_p for NA across the skin (one-way ANOVA, p<0.05). The second

highest k_p was obtained for DPPG and was significantly different from all the other solvents (one-way ANOVA, p<0.05). PG, PGML and PGMC resulted in the lowest k_p values not significantly different from each other (one-way ANOVA, p>0.05). The k_p obtained for single solvents is shown in Figure 4.18.

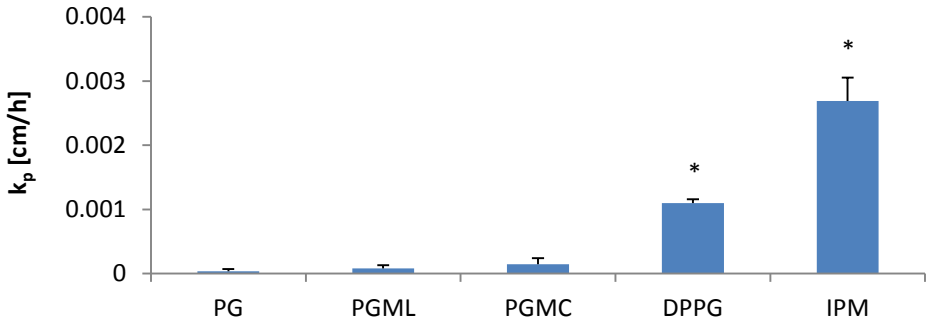


Figure 4.18 Permeability coefficients for the finite dose NA permeation studies in pig ear skin for single solvents at 32 ± 0.5°C (data shown as mean ± SD, n=5, *p<0.05, significantly different from other solvents)

In the development of a topical NA formulation for atopic dermatitis treatment NA retention in the deep layers of the skin rather than its permeation through the membrane is preferable. At the same time, it is desirable that as little as possible of the applied dose remains on the skin surface at the end of the application process. Thus, data on the percentage of NA permeated, washed from the skin and retained in skin layers were obtained and are shown in Figure 4.19.

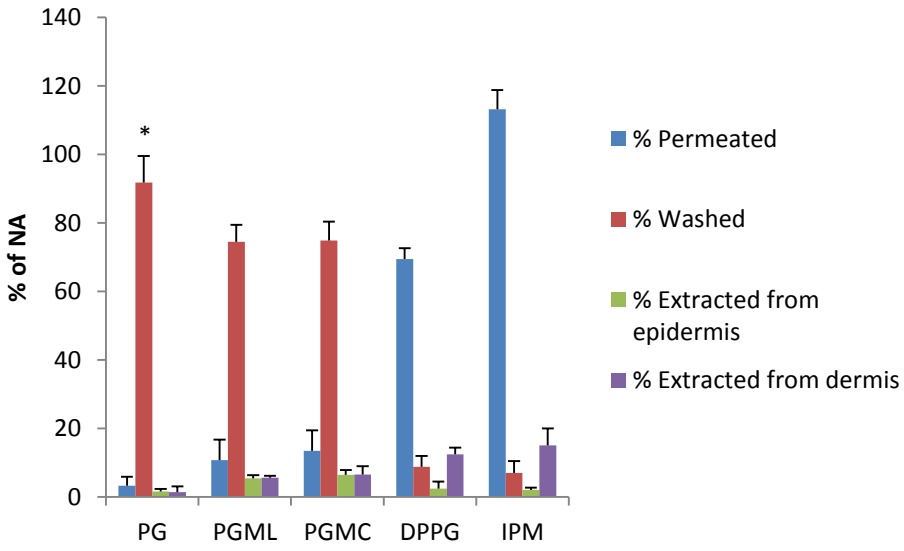


Figure 4.19 Percentages of NA permeated, washed from the skin and extracted from the epidermis and dermis in the finite dose permeation studies in pig ear skin for single solvents at 32 ± 0.5°C (data shown as mean ± SD, n=5, *p<0.05, significantly different from other solvents)

In the case of PG, approximately 90% of the applied dose remains on the skin surface and this value is significantly higher from all other solvents tested (one-way ANOVA, $p<0.05$). The smallest percentage of NA left on the skin surface at the end of the permeation studies was found for IPM and DPPG when compared with the other solvents (one-way ANOVA, $p<0.05$). These two solvents were not different from each other in terms of the percentage of NA remaining on the skin surface at the end of the study (one-way ANOVA, $p>0.05$). PGML and PGMC are in the middle of this range of solvents (approximately 80% of the dose was washed from the skin surface at the end of the study) and are not significantly different from each other (one-way ANOVA, $p>0.05$). These results are consistent with the permeation data, i.e. a small percentage of NA permeated resulted in a high percentage recovered from the skin surface.

When the overall amount of NA retained in the epidermis was calculated [µg/g], the lowest values were found for IPM and DPPG (one-way ANOVA, $p<0.05$). These two solvents were not statistically different from each other in terms of NA retention in the epidermis (one-way ANOVA, $p>0.05$). PG and PGMC were the solvents with the highest epidermal retention potential for NA (one-way ANOVA, $p<0.05$). A significantly higher (t-test, $p<0.05$) retention of NA was found for the epidermis than for the dermis for all the solvents, which is because of the higher mass of the dermis and its greater thickness. Delivery in IPM and DPPG resulted in smaller dermal retention values of NA, when compared with PG (one-way ANOVA, $p<0.05$). PGML and PGMC showed moderate NA retention potential and were not statistically different from any of the solvents tested (one-way ANOVA, $p>0.05$). These results are illustrated in Figure 4.20.

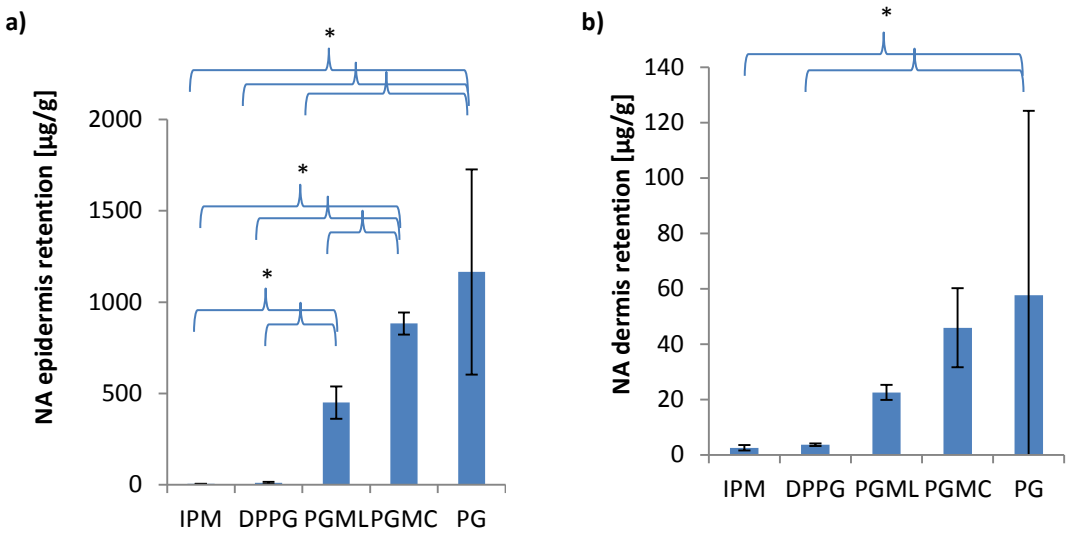


Figure 4.20 NA retention in epidermis (a) and dermis (b) in the finite dose permeation studies in pig ear skin for single solvents at 32 ± 0.5°C (mean ± SD, n=5, *p<0.05)

4.4.4.2 Binary solvent systems

Various authors have examined a number of drugs and have shown that the percutaneous absorption is affected synergistically when using solvent combinations [49-52]. Also, the importance of balanced drug solubility in the vehicle for optimal delivery has been emphasized [53, 54]. Very high solubility of NA in PG may be one of the reasons for the high percentage of the dose remaining on the skin surface at the end of the permeation study. A large amount of the drug remaining on the skin surface and not penetrating into the skin is not desirable from an economic and therapeutic point of view. The concentration of NA used in topical formulations usually ranges from 2 to 5% [55-64]. Thus, the solvent ratio in the binary systems was chosen so that the NA concentration in the vehicle varied around these values and did not exceed 10% (i.e. 100 mg/mL). To achieve this, diminishing the ratio of PG incorporated into the solvent systems was necessary because of the very good solubility of NA in this solvent. Thus, single solvents were combined into the binary solvent systems listed in Table 4.14. The concentration of NA in the saturated solutions applied to the skin was determined by the HPLC method described previously and is also shown in Table 4.14.

Table 4.14 Binary solvent systems used in the finite dose pig ear skin studies

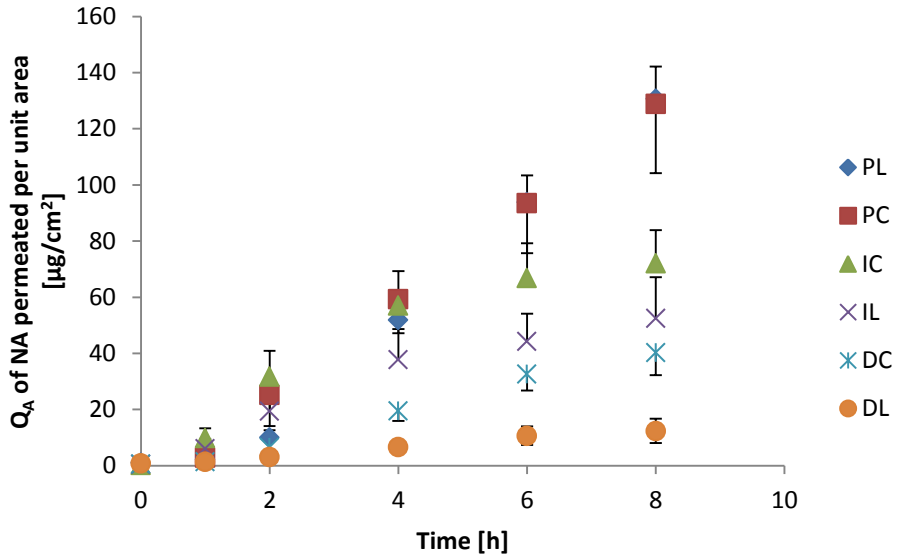
Solvent system	Ratio of solvents used	Concentration of NA [mg/mL]	Solubility parameter [$cal^{1/2}/cm^{3/2}$]
IPM:PGML (IL)	50:50	8.07	8.83
DPPG:PGML (DL)	50:50	9.17	9.05
IPM:PGMC (IC)	50:50	12.70	9.03
DPPG:PGMC (DC)	50:50	15.09	9.25
PG:PGML (PL)	10:90	36.14	9.90
PG:PGMC (PC)	10:90	64.73	10.27

The NA permeation profiles obtained in the finite dose pig ear skin studies of binary solvent systems are shown in Figure 4.21.

The cumulative amount of NA which permeated after 8 h from each of the solvent systems is shown in Figure 4.22. The k_p values obtained for solvent systems are also illustrated.

Binary systems incorporating 10% of PG in combination with PGML or PMGC (PL and PC) result in the highest Q_A of NA when compared with the other systems tested (one-way ANOVA, $p<0.05$). On the other hand, IC and IL are significantly different (one-way ANOVA, $p<0.05$) from the other solvent systems in terms of percentage of NA permeated, resulting in a higher percentage of NA delivered across the skin (see Figure 4.23). When k_p values were compared, the only significant (one-way ANOVA, $p<0.05$) differences were found between IL and binary solvent systems incorporating DPPG. A synergistic effect of the solvent mixtures on NA permeation was not observed. The k_p values obtained for the binary systems were lower or not significantly different from the values for IPM or DPPG (one-way ANOVA, $p<0.05$).

a)



b)

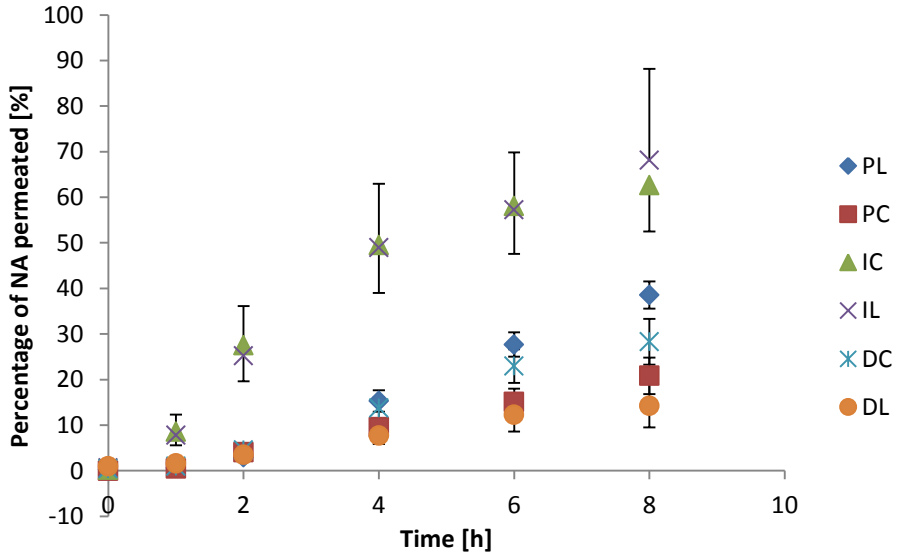


Figure 4.21 Finite dose pig ear skin NA permeation profiles for binary solvent systems at 32 ± 0.5°C (mean ± SD, n=5); a) cumulative amount of nicotinamide, b) percentage of nicotinamide permeated

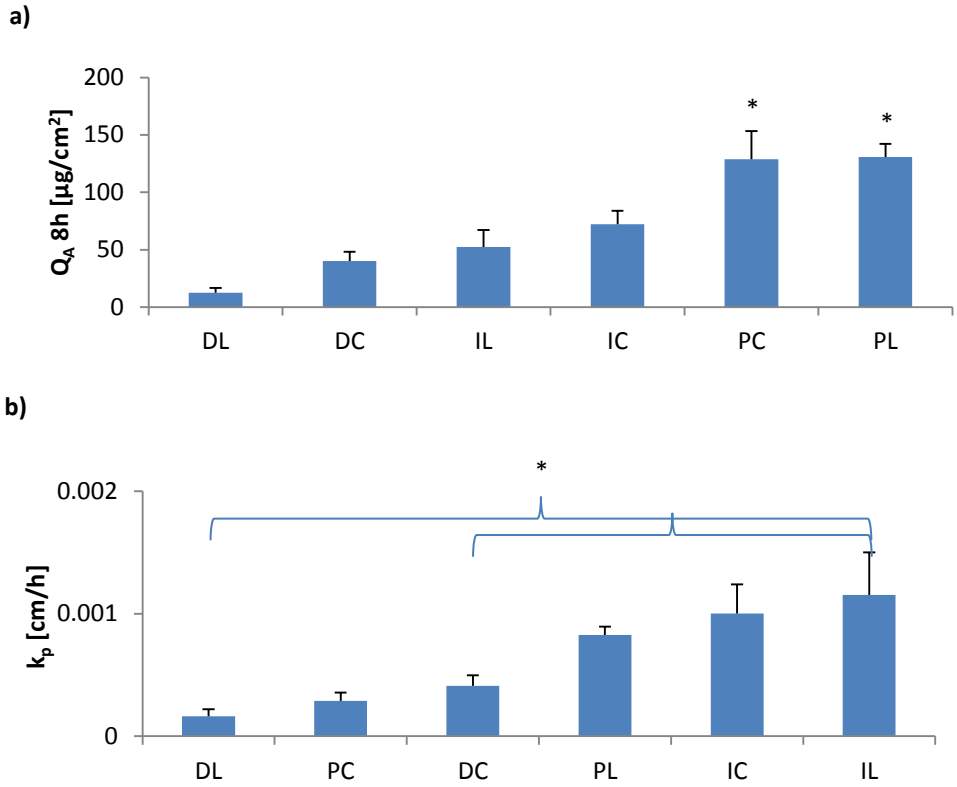


Figure 4.22 a) Cumulative amounts of NA, b) permeability coefficients obtained for binary solvent systems in the finite dose permeation studies in pig ear skin at 32 ± 0.5°C (data shown as mean ± SD, n=5, *p<0.05)

The results of the mass balance studies are shown in Figure 4.23. The highest % of NA was washed from the skin surface for the PC system (one-way ANOVA, $p<0.05$). The lowest % of NA left on the skin surface at the end of the permeation studies was found for IL and IC when compared with the other solvents (one-way ANOVA, $p<0.05$) and it was not significantly different from IPM and DPPG which resulted in the lowest % residue from the single solvents (one-way ANOVA, $p>0.05$). All the binary solvent systems resulted in significantly lower % residues of NA when compared with PG (one-way ANOVA, $p<0.05$).

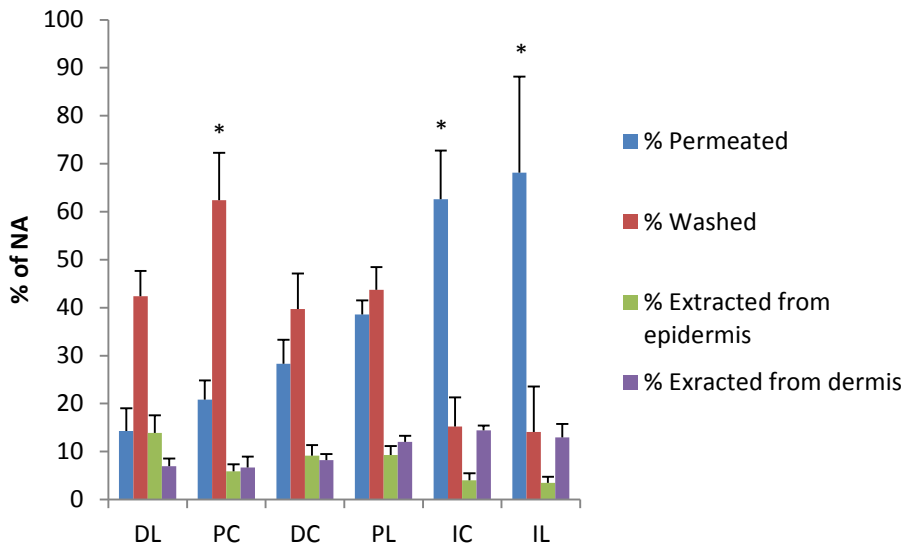


Figure 4.23 Percentages of NA permeated, washed from the skin and extracted from the epidermis and dermis in the finite dose permeation studies in pig ear skin for binary solvent systems at 32 ± 0.5°C (data shown as mean ± SD, n=5, *p<0.05)

The highest retention of NA in the epidermis and the dermis (one-way ANOVA, $p<0.05$) was observed for binary solvent systems which incorporated 10% of PG with PGML or PGMC (PL and PC). However, PL and IC were not significantly different from each other in terms of NA retention in the dermis (one-way ANOVA, $p>0.05$). The results are shown in Figure 4.24.

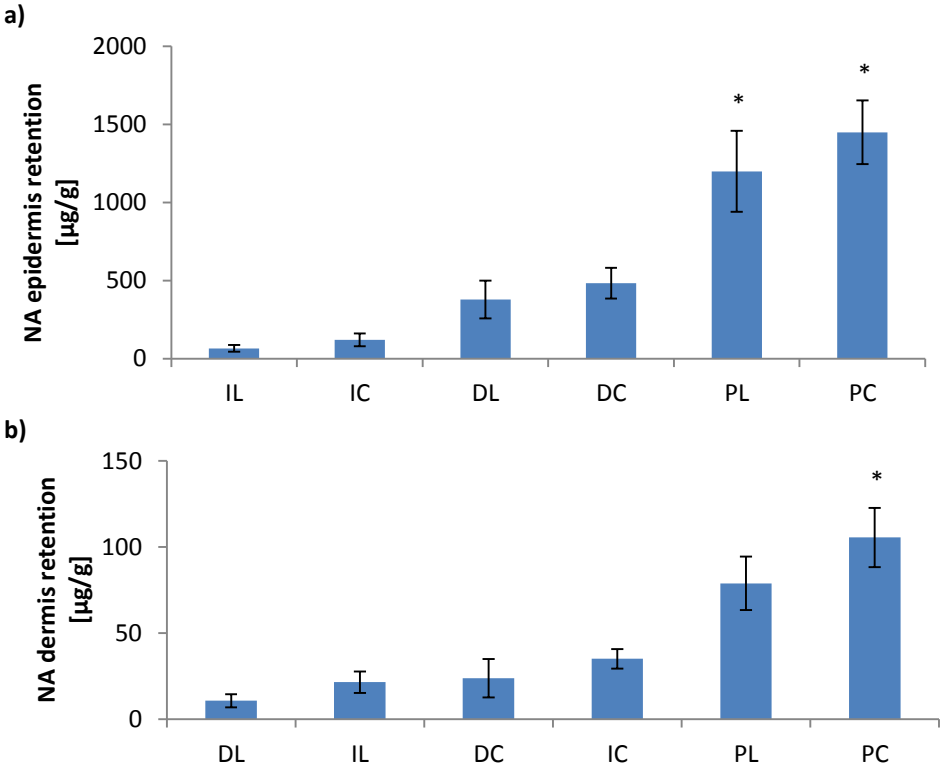


Figure 4.24 NA retention in epidermis (a) and dermis (b) in the finite dose permeation studies in pig ear skin for binary solvent systems at 32 ± 0.5°C (mean ± SD, n=5, p<0.05)

NA retention in the skin layers following the application of PL and PC was higher than for the single solvents, however statistical significance was reached only for PC when dermis retention was taken into account (one-way ANOVA, $p<0.05$). Similarly, for other binary solvent systems no statistically significant increase in epidermis and dermis retention of NA was observed in comparison with single solvent systems (one-way ANOVA, $p>0.05$). These results indicate that the synergistic effect of binary solvent systems on NA retention in the skin layers is in most cases not significant.

It is worth noting that high epidermis and dermis retention of NA obtained for solvent systems incorporating 10% PG coincides with the similarity of the δ value of these solvent systems (9.90 and 10.27 $cal^{1/2}/cm^{3/2}$ for PL and PC respectively) to the δ of the skin (approximately 10 $cal^{1/2}/cm^{3/2}$). This may indicate that optimal adjustment of the δ value of the vehicle may lead to improved retention of NA in the skin following topical application.

4.4.4.3 Ternary and quaternary solvent systems

Since binary solvent systems with 10% PG showed promising effects in terms of NA skin retention and there is a possibility of a greater synergy when more than two solvents are used; the following ternary and quaternary solvent systems were investigated:

Table 4.15 Ternary and quaternary solvent systems

Solvent system	Ratio of solvents used	Concentration of NA [mg/mL]	Solubility parameter [$cal^{1/2}/cm^{3/2}$]
IPM:PGML:PG (ILP)	45:45:10	26.35	9.35
IPM:PGMC:PG (ICP)	45:45:10	37.98	9.54
DPPG:PGML:PG (DLP)	45:45:10	28.41	9.55
IPM:PGML:PGMC:PG (ILCP)	30:30:30:10	39.58	9.66
DPPG:PGMC:PG (DCP)	45:45:10	36.09	9.74
DPPG:PGML:PGMC:PG (DLCP)	30:30:30:10	42.21	9.79

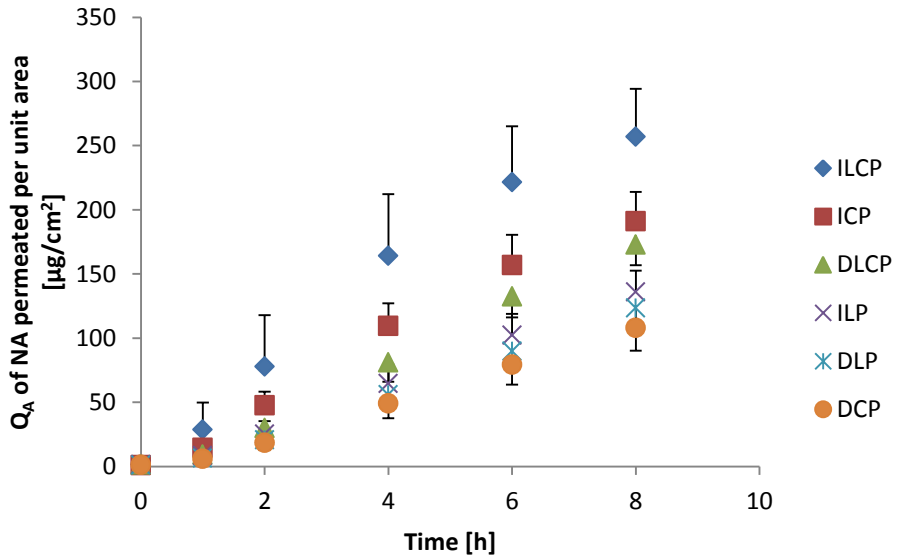
The solvents were selected so that each multicomponent solvent system comprised of:

- PG, which is a hydrophilic solvent with good solubilising properties of NA and a δ value close to the δ of the drug,
- one (for ternary solvent systems) or two (for quaternary solvent systems) solvents with δ values closest to skin, i.e. PGML and/or PGMC,
- one of the solvents in which NA is poorly soluble and which has a δ value lower than skin, i.e. IPM or DPPG.

The proportion of PG in all the multicomponent solvent systems (MSS) was fixed at 10%, because of the high solubility of NA in this solvent and thus a high amount of NA that would be needed to reach saturation of PG. The other solvents were incorporated into the systems in equal proportions.

The NA permeation profiles obtained in the finite dose pig ear skin studies of multicomponent solvent systems are shown in Figure 4.25.

a)



b)

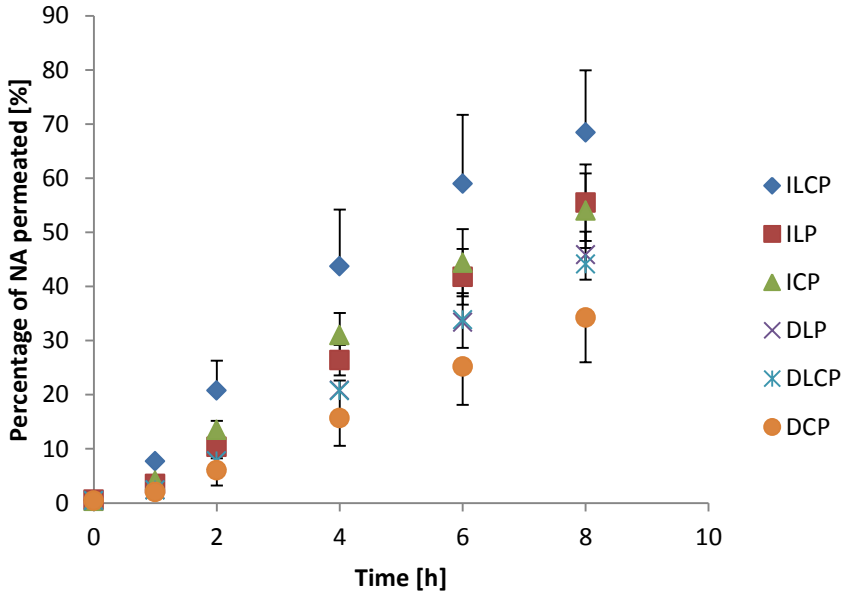


Figure 4.25 Finite dose pig ear skin NA permeation profiles for multicomponent solvent systems at 32 ± 0.5°C (mean ± SD, n=5); a) cumulative amounts of nicotinamide, b) percentages of nicotinamide permeated

The Q_A permeated after the 8h experiment from the MSS and the k_p values are shown in Figure 4.26. The highest Q_A was obtained for the ILCP solvent system and it was significantly higher than for all the single, binary and multicomponent solvent systems tested (one-way ANOVA, p<0.05). Also, the highest % of the applied dose permeated after 8h from the MSS was obtained for ILCP and it was significantly different from DCP, DLP and DLCP (one-way ANOVA, p<0.05) (see Figure 4.27). The k_p values obtained for the MSS were comparable. The only statistically significant (one-way ANOVA, p<0.05) differences were between ILCP vs. DCP and DLP vs. DCP. The k_p values obtained for the MSS were not significantly (one-way ANOVA, p>0.05) higher from the values obtained for IPM or DPPG alone, indicating that the synergistic effect of multicomponent formulations on NA permeation is not significant.

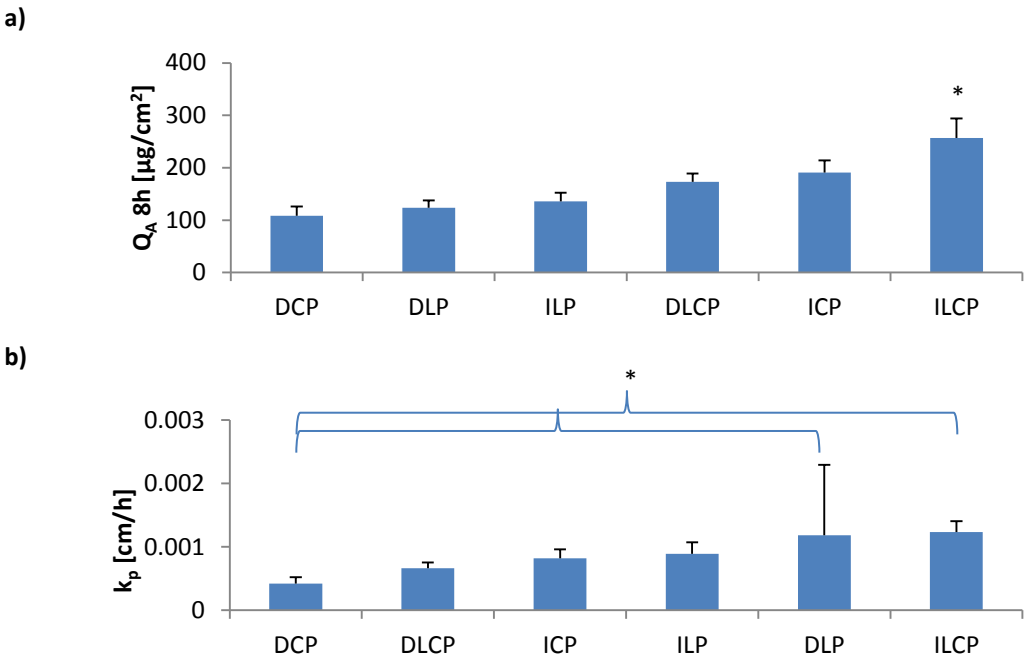


Figure 4.26 a) Cumulative amounts of NA, b) permeability coefficients obtained for multicomponent solvent systems in the finite dose permeation studies in pig ear skin at 32 ± 0.5°C (mean ± SD, n=5, *p<0.05)

The percentage of NA washed at the end of the study from the skin surface was similar for all the multicomponent solvent systems (MSS). The highest percentage of NA remaining on the skin surface at the end of the study was found for DCP and it was significantly different from ILP and ILCP (one-way ANOVA, p<0.05). The lowest % of NA was found for ILP, however it was significantly different only from DCP (one-way ANOVA, p<0.05). The percentage of NA washed from the skin surface obtained for ILP, ILCP, DLP and ICP was not significantly different from IPM and DPPG, which showed the lowest percentage of NA remaining at the end of the study

(one-way ANOVA, p>0.05). All the MSS resulted in a significantly lower % residue of NA when compared with PG (one-way ANOVA, p<0.05).

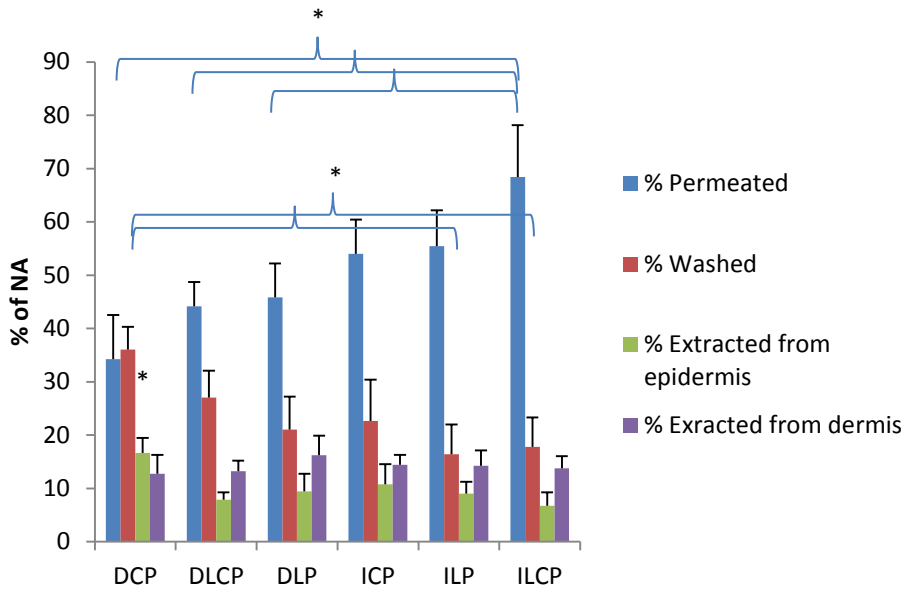


Figure 4.27 Percentages of NA washed from the skin in the finite dose permeation studies in pig ear skin for ternary and quaternary solvent systems at 32 ± 0.5°C (mean ± SD, n=5, *p<0.05)

The results of NA retention in the epidermis and the dermis are shown in Figure 4.28. From the MSS tested, the highest retention of NA in the epidermis was obtained for DCP when compared with the other MSS (one-way ANOVA, p<0.05) both in terms of percentage of NA extracted and in terms of the amount of NA present in this skin layer after DCP application. Combining DPPG, PGMC and PG (45:45:10) resulted in a significant increase in NA retention in the epidermis when compared both with single solvents and binary solvent systems (one-way ANOVA, p<0.05), indicating a possible synergistic effect of these three solvents used in combination. This is possibly because of the optimal δ value of DCP, which is close to the δ value of the skin (9.74 and 10 $cal^{1/2}/cm^{3/2}$, respectively).

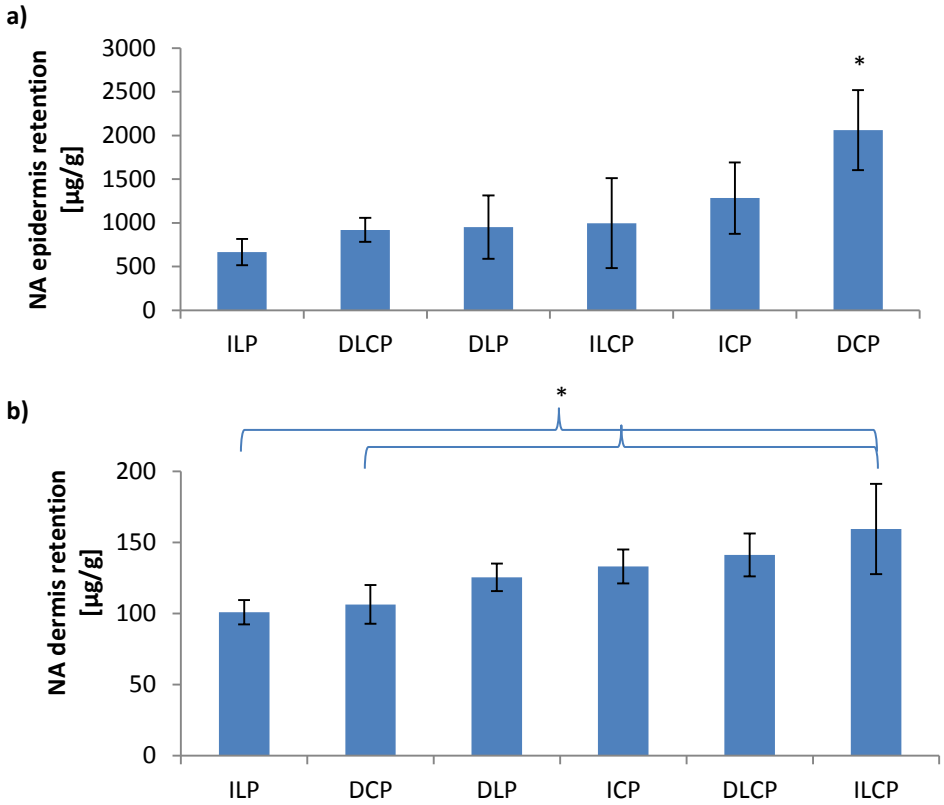


Figure 4.28 NA retention in: a) epidermis, b) dermis in the finite dose pig ear skin permeation studies for ternary and quaternary solvent systems at 32 ± 0.5°C (mean ± SD, n=5, *p<0.05)

Quaternary solvent systems showed the best dermal retention of NA. The results were significantly different (one-way ANOVA, $p<0.05$) in the case of ILCP vs. ILP and DCP. However, no statistically significant differences (one-way ANOVA, $p>0.05$) in the fraction of NA applied (see Table 4.15) which was retained in the dermis was found between the multicomponent solvent systems tested. Quaternary solvent systems resulted in a higher retention of NA in the dermis when compared with almost all binary systems and single solvents (one-way ANOVA, $p<0.05$). Only one binary solvent system, i.e. PC, showed similar (one-way ANOVA, $p>0.05$) potential for NA retention in the dermis when compared with DLCP. The δ values are 9.66, 9.79 and 10.27 $cal^{1/2}/cm^{3/2}$, for ILCP, DLCP and PC respectively. However, the concentration of NA in PC (approximately 60mg/mL) is higher than in the quaternary solvent systems (approximately 40mg/mL) (see Table 4.14 and Table 4.15, respectively). Thus, a comparatively higher overall amount of NA is applied to the skin in PC. This may also be the reason for high amount of NA found in the dermis at the end of the study after the application of this binary solvent system.

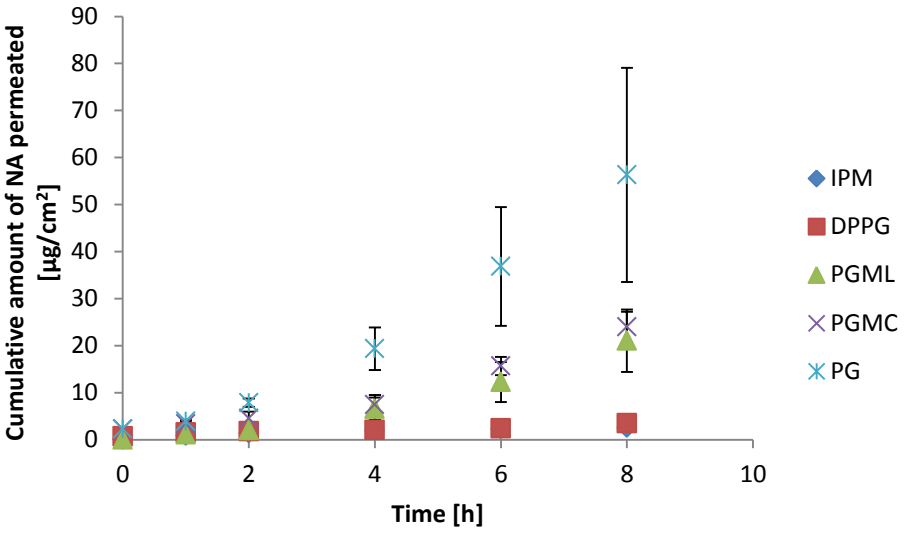
4.4.5 Solvent and nicotinamide permeation - finite dose pig ear skin studies

Finite dose permeation studies across full thickness pig ear skin were performed. NA and solvent permeation profiles are shown in Figure 4.29.

Similarly to the silicone membrane data, the lowest cumulative amount of NA ($p<0.05$, one-way ANOVA) permeated from IPM and DPPG, which is in line with the low solubility of NA in these solvents and thus, the low dose applied to the donor compartment. High lipophilicity of IPM and DPPG renders them poor solvents for the drug and is also responsible for the lack of permeation of these solvents across full thickness pig ear skin. This is because of the presence of a hydrophilic layer of dermis which hinders the permeation of lipophilic substances. In the *in vivo* situation, an abundant blood supply in the dermis acts as a sink and allows for the permeation of lipophilic chemicals into the systemic circulation. However, the dermis is not perfused *in vitro* and thus, it is difficult to mimic the *in vivo* permeation of lipophilic solvents.

The highest cumulative amount of NA which permeated ($p<0.05$, one-way ANOVA) was obtained for PG, which is in line with the data reported in section 4.4.4. Also, this was the only vehicle from the chosen solvents that permeated across full thickness pig ear skin. This is not surprising, since PG is the most hydrophilic of the solvents tested and thus, it is able to penetrate through the hydrophilic layer of dermis.

a)



b)

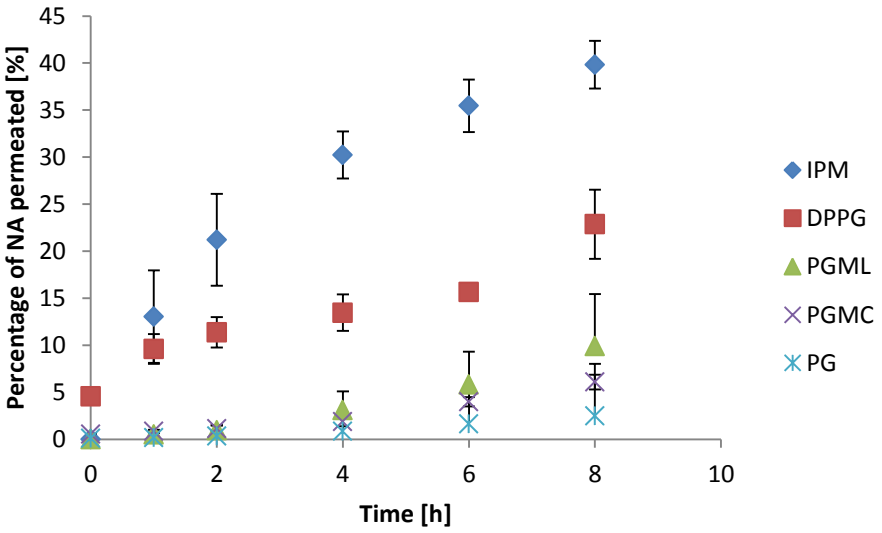
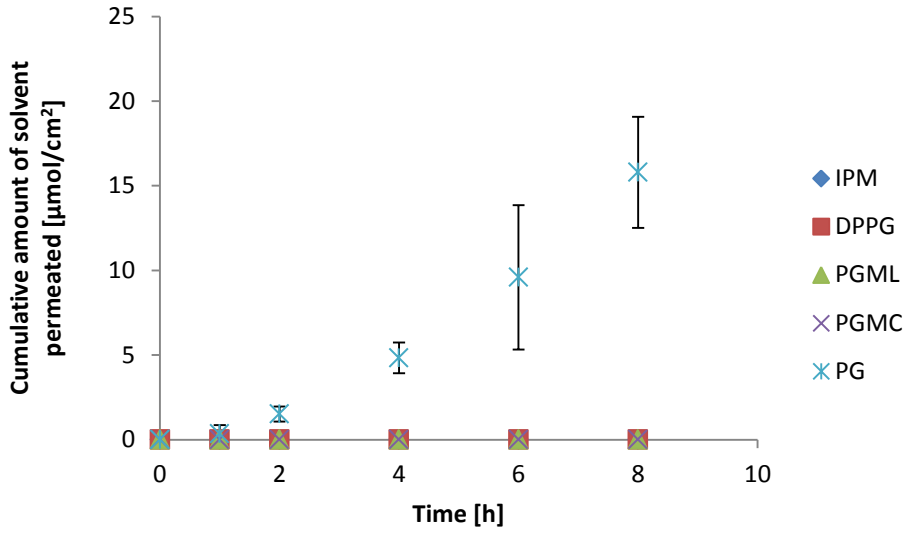


Figure 4.29 Finite dose pig ear skin permeation profiles for nicotinamide a) cumulative amount of nicotinamide, b) percentage of nicotinamide permeated at 32 ± 0.5°C (mean ± SD, n=5)

a)



b)

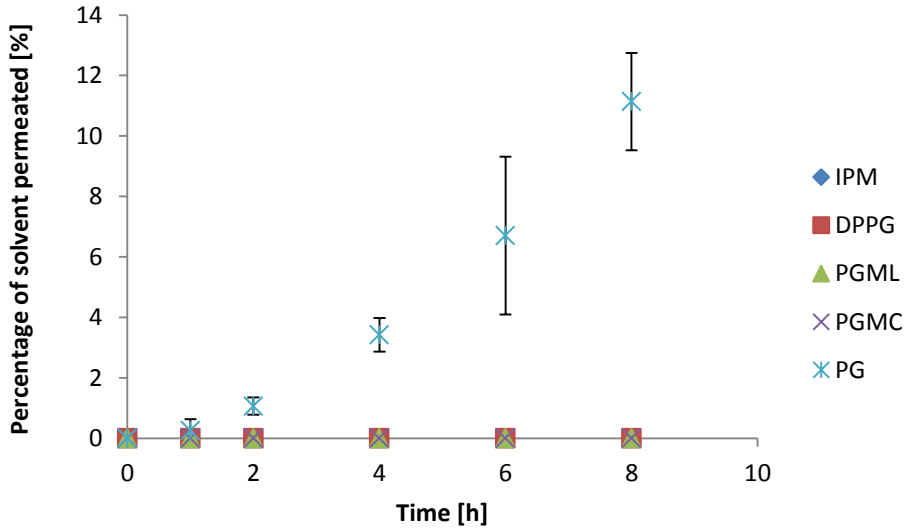


Figure 4.30 Finite dose pig ear skin permeation profiles for solvents a) cumulative amount of solvent, b) percentage of solvent permeated at 32 ± 0.5°C (mean ± SD, n=5)

The percentages of applied doses of NA and solvents permeated, washed and extracted from the pig ear skin after 8h are shown in Figure 4.31.

The highest ($p<0.05$, one-way ANOVA) percentage of NA permeated from IPM, followed by DPPG, PGML, PGMC and PG, which is in line with the results obtained in the studies, where only NA permeation was monitored. This order may be because of differences in NA solubility in these solvents with the lowest solubility for IPM and the highest for PG. Even though highest cumulative amount of NA permeated from PG, it is still a relatively a small percentage of the dose that was applied to the donor compartment of a Franz cell.

The results of solvent analysis suggests that even though no permeation was observed for IPM, DPPG, PGML and PGMC, the presence of these solvents in the skin (approximately 2-5% of the dose applied) was enough to enable drug transport across the membrane. The stratum corneum is considered the main barrier for permeation [65], thus the presence of solvents in this skin layer is important for NA penetration, because the process occurs only for the drug in solution. Once the drug reaches the hydrophilic environment of the dermis, it may be dissolved in water present in this layer and thus, transported into the receptor fluid. Nevertheless, the high percentage of NA extracted from the skin in the case of IPM and DPPG (40.11±11.58 and 37.29±3.59, respectively) indicates that some of the drug is entrapped in the skin and does not reach the receptor fluid, probably because of the retention of these solvents in the skin.

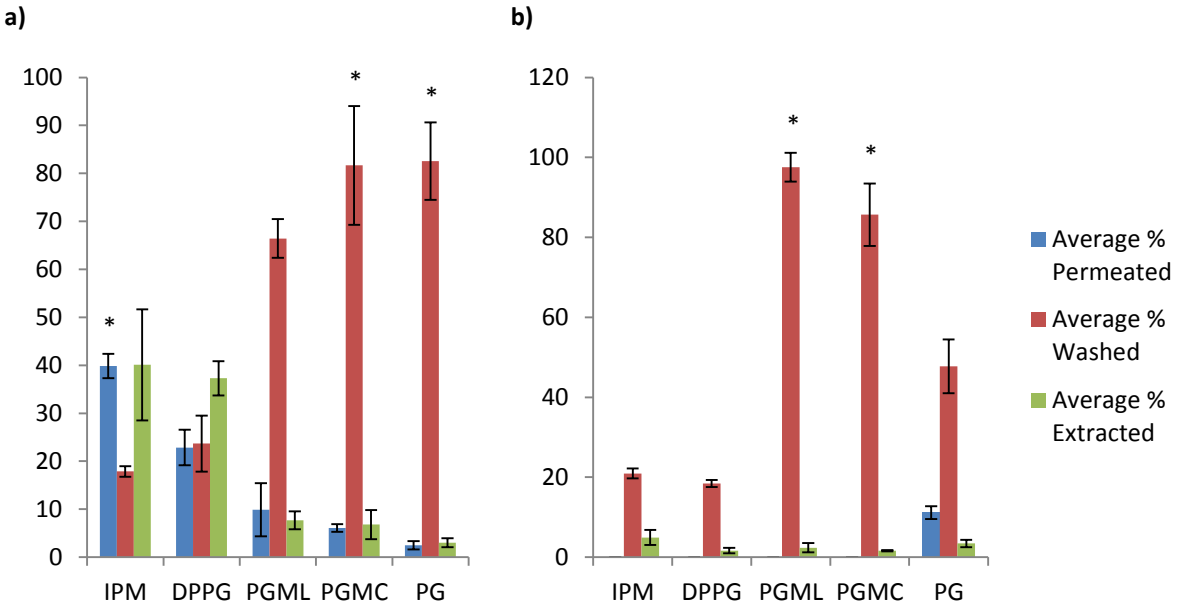


Figure 4.31 Percentage of applied dose permeated, washed and extracted from the pig ear skin after 8h for a) nicotinamide and b) solvents, mean ± SD, n=5, *p<0.05

The highest percentage of NA washed from the skin ($p<0.05$, one-way ANOVA) was obtained for PGMC and PG, followed by PGML. This is in line with the high percentages of these solvents that were washed from the skin at the end of the study. For IPM and DPPG, the washing fraction contained approximately 20% of the solvents applied. Nevertheless, this value was higher than the percentages of the solvents extracted from the skin. There was a low percentage of total recovery obtained for these two solvents (25.90±1.82 and 20.11±0.64, for IPM and DPPG respectively) which could be elucidated in the future. Evaporation studies, such as dynamic vapour sorption or thermogravimetric analysis, may be performed in the future to give more insight into this phenomenon.

4.5 Discussion

4.5.1 Solvent uptake studies

Uptake studies provide information on the affinity of the solvent for the membrane. Several authors have correlated solvent sorption into silicone membrane with the results of permeation studies, pointing out to the importance of the vehicle interaction with the membrane in the permeation process [3, 8, 10, 12, 66]. Thus, solvent uptake studies into pig ear stratum corneum and silicone membrane were performed to assess the extent of the interaction between the model membranes and the chosen solvents and their potential to increase NA delivery across this membrane.

Solvents with δ values close to the δ of silicone membrane (7.5 $cal^{1/2}/cm^{3/2}$) [39] showed higher uptake into this membrane (approximately 58% and approximately 16%, for IPM and DPPG respectively). The exception was MO, which was not incorporated into the silicone membrane despite its lipophilicity and δ value of 7.44 $cal^{1/2}/cm^{3/2}$. The reason for this may be the high molecular weight (MW) of MO components. In a study by Cross et al. [67], the highest sorption of solvents into the silicone membrane was observed for solvents with δ values ranging from 7 to 9.5 $cal^{1/2}/cm^{3/2}$, which is in line with the findings from this study (δ values of 8.22 and 8.66 $cal^{1/2}/cm^{3/2}$, for IPM and DPPG respectively). More hydrophilic solvents with higher δ values were not incorporated into the lipophilic membrane (uptake less than 1%). Previous studies also showed that for very large molecules, solvent uptake into the silicone membrane may be also dependent on their molecular size and shape [14, 67]. The same study by Cross et al [67] did not find a correlation between solvent sorption into the silicone membrane and the δ value. Thus, the authors postulated that MW is an important factor for membrane sorption predictions, which is in line with the data for MO - a mixture of long chain alkanes. Also, Dias et al. [14] observed a similar relationship to the one found here for a range of different solvents.

The results of solvent uptake studies indicate that there is a major difference between SC and silicone in terms of the ability of these two membranes to absorb solvents with different properties. Even though SC is perceived as a lipophilic membrane, the uptake of the chosen solvents into the SC increased with their hydrophilicity. On the other hand, a reverse situation was observed for silicone membrane. It may be postulated, that the molecular size plays an even greater role in the solvent uptake process into the SC when compared with silicone membrane. This may be because of less tightly packed structured of the polymer when compared with the compact structure of the stratum corneum. The molecular size of IPM and

DPPG (MW 270.44 and 356.54 g/mol, respectively) pose more difficulties for absorption into the SC, whereas molecules with smaller size (for example the MW of PG is 76.09g/mol) are more easily taken up by the membrane. Thus, in the case of the SC, solvent MW, its molecular size and solubility parameter may influence the solvent uptake.

In general, high solvent sorption into the membrane will cause changes in its physicochemical properties and influence the diffusion and partitioning of nicotinamide. This in turn, will influence the permeation of the drug. This phenomenon is discussed below.

4.5.2 Nicotinamide partitioning

It was observed that NA dissolved in lipophilic solvents (IPM and DPPG) has higher membrane/solvent partition coefficient values than NA dissolved in more hydrophilic solvents (One way ANOVA, $p<0.05$) both for silicone membrane and pig ear stratum corneum (see section 4.3.2 and 4.4.2, respectively).

For the silicone membrane, the results may be explained by the low solubility of NA in IPM and DPPG and the high uptake of these solvents into the membrane. A high membrane/solvent partition coefficient indicates higher affinity of the drug for the membrane than for these highly lipophilic solvents. The low partition coefficient obtained for PG indicates higher affinity of the drug for this hydrophilic solvent than for the silicone membrane or SC. It is worth noting that for all solvents tested the amount of NA found in the silicone membrane was comparable, despite large concentration differences between the NA solutions in these solvents. This may be because of the differences in the membrane uptake of these solvents, i.e. solvents with high NA solubility showed low affinity for the membrane and solvents with low NA solubility were readily taken up into the silicone.

For SC, the phenomenon can be solely explained by solubility differences between the solvents, as their uptake into the membrane did not correlate with the amount of NA found in the SC at the end of experiment. This means that, even though a relatively high membrane concentration of NA was achieved for PG, its high solubility in this solvent and thus high concentration in the solvent used in the experiment, did not allow for the achievement of a high partition coefficient value.

4.5.3 Nicotinamide permeation from prototype formulations

Two different nicotinamide formulations were tested: NIAD – a simple aqueous gel containing mainly water and modified acrylic polymer as a gelling agent; and DENI – a two phase system – water in oil emulsion in which IPM and MO are the lipid phase and water is the hydrophilic

phase, emulsified with sorbitan laurate surfactant (hydrophilic-lypophilic balance (HLB) of 8.6). In DENI, glycerol acts as the formulation humectant. Moreover, both of the prototype formulations contain pH regulators (trietanoloamine, citric acid) and phenoxyethanol as a preservative. Taking into account the differences in the prototype formulations composition, it was expected that they would impact nicotinamide permeation differently.

In vitro testing of topical formulations is recommended before *in vivo* studies are performed [68, 69]. Also, the controlled conditions of *in vitro* permeation studies give a better insight into the influence of the vehicle on the percutaneous absorption of the drug when compared with the more complex *in vivo* situation. Thus, the performance of the prototype formulations in terms of percutaneous absorption of NA was evaluated *in vitro* prior to the *in vivo* studies described in the next chapter. Because of incorporation of NA into two different vehicles, a difference in the thermodynamic activity of the drug in these two formulations and an influence of vehicle components on the *in vitro* NA permeation were expected.

In the infinite dose study across silicone membrane, the DENI formulation achieved significantly (t-test, $p<0.05$) higher values of flux and permeability coefficient. This may result from the presence of IPM in this formulation, which is known for its penetration enhancing properties [14, 67]. The presence of surfactant could be another cause of higher nicotinamide skin penetration from DENI. Also, IPM and MO may play a role in diminishing NA solubility in the vehicle and increasing thermodynamic activity of the drug [1]. The solubility of NA both in IPM and MO is low, when compared with water, which is the main ingredient in the NIAD formulation. Thus, incorporation of these solvents into DENI results in higher thermodynamic activity of NA when compared with NIAD.

In several studies, transport across silicone membrane was correlated with the solvent uptake into the membrane [3, 8, 10, 12, 66, 67]. IPM has high affinity for the lipophilic membrane. On the contrary, water, which is the main solvent in the NIAD formulation, is not sorbed into the silicone membrane. Thus, a higher affinity of IPM for the silicone membrane increases its potential for NA delivery. In a study by Twist and Zatz [3], enhanced flux of parabens was attributed to higher solubility of the model permeants in the membrane when applied in solvents readily taken up by the silicone. Because water is not sorbed into the silicone membrane and NA has a very high affinity for this hydrophilic solvent, NA does not partition readily into the membrane from NIAD. On the other hand, membrane uptake of IPM present in DENI should increase the partitioning of the drug into this membrane. Additional studies on NA partitioning into the silicone membrane from the chosen solvents show that the NA

membrane/solvent partition coefficient is significantly (One way ANOVA, $p<0.05$) higher for IPM than for hydrophilic solvents. This approach may explain the significantly (t-test, $p<0.05$) higher partition coefficient obtained for the DENI formulation in the infinite dose silicone membrane permeation study.

Viscosity is one of the factors influencing diffusion. The more viscous the formulation the slower is the diffusion process [70]. It was established in previous studies that formulation modifiers can not only increase drug flux through the membrane, but also influence the release rates of the drug from the formulation [71]. *In vitro* studies showed that the increase in the viscosity of a formulation decreased the rate of retinoic acid release into the receptor phase [71]. During the application of prototype NA formulations it was observed that NIAD was less viscous than DENI, which may be because of the lack of lipophilic components in the vehicle. Thus, a lower diffusion coefficient obtained for NA from DENI may be caused by the presence of the lipophilic compounds in the formulation. Tests of formulation viscosity, along with release testing, may confirm this.

A significantly higher Q_A (t-test, $p<0.00001$) was found after 8h in the finite dose silicone study when compared with the infinite dose experiment. The values of 120±21 and 118±5 μg/cm^2 in the finite dose study and 29±4 and 23±3 μg/cm^2 in the infinite dose study were obtained, for DENI and NIAD respectively. The discrepancies between occluded and non-occluded conditions were also observed for rooperol tetra-acetate permeation across silicone membrane [72]. The delivery of the drug differed significantly (at the 95% confidence level) from a hydrophilic gel and a water-in-oil formulation under occluded conditions. However, no significant (at 95% confidence level) difference was observed for the formulations under non-occluded conditions. The gel contained primarily water (approximately 75%) and propylene glycol (PG) (25%), while the water-in-oil formulation comprised of 25% water, 25% of PG and 50% of lipophilic components (including 12% of MO). Rooperol tetra-acetate is highly lipophilic, thus the authors suggested that higher flux values obtained from the gel under occluded conditions are because of the favoured release of the drug from hydrophilic bases. In contrast, in the NA infinite dose study across silicone membrane the highly hydrophilic NA permeates better from DENI, which contains lipophilic components. Apart from the uptake of IPM into the membrane and the increased thermodynamic activity caused by MO and IPM, the expected favoured release from the lipophilic formulation may be another reason for better NA delivery from DENI.

In a study by Pefile et al. [72], the difference between the results obtained under occluded and non-occluded conditions was attributed to water evaporation from the vehicle, and thus an increase in its lipophilicity and changes in the thermodynamic activity of the drug in the vehicle. Similarly, Tanaka et al. [7] obtained different results for hydrocortisone butyrate propionate flux across silicone membrane under occluded and non-occluded conditions. The authors also related these differences to the thermodynamic activity changes of the drug in the vehicle occurring under 'open' conditions caused by water and ethanol evaporation from the vehicles. Water is the main solvent used in both prototype NA formulations tested in this study and NA is highly soluble in this solvent. Thus, water evaporation could increase the thermodynamic activity of NA [1], explaining the higher Q_A found for the finite dose studies when compared with the infinite dose studies across the silicone membrane.

In the finite dose silicone and pig ear studies, no significant differences (t-test, $p>0.05$) in the permeation and drug retention in the model membranes between NIAD and DENI were observed. As mentioned above, these results are most probably because of changes occurring during the finite dose studies. These experiments are performed under dynamic conditions, where the formulation can be modified by various factors such as solvent evaporation and depletion [73, 74]. However, because of the differences in the vehicle components, compensation effects are possible. At the end of the finite dose studies a thin dry residue was observed where NIAD was applied, in contrast to the greasy and moist formulation residue for DENI. Since NIAD contains mainly water, the effect of increased thermodynamic activity of the drug because of the evaporation of the solvent from the donor compartment may be more pronounced for this formulation. Thus, water evaporation from NIAD may compensate for the penetration enhancement caused by the presence of IPM and MO in the DENI formulation. Solvent evaporation studies, such as dynamic vapour sorption or thermogravimetric analysis, may give more insight into the evaporation of water from the prototype formulations and may provide more insight into the thermodynamic activity changes occurring during the finite dose studies. However, it must be kept in mind that the complexity of the prototype formulations may hinder such analysis. Thus, studies on simpler solvent systems may be more helpful in elucidating the mechanisms underlying the permeation process of NA from the prototype formulations.

It is worth noting that in the finite dose silicone study more than half of the dose penetrated over the 24h time period. This indicates that NA diffuses through the membrane readily despite its hydrophilicity. Also a relatively high percentage (approximately 20%) of the applied dose was found in the receptor solution after 8h of NA permeation through pig ear skin. The

molecular diffusion coefficient is inversely related to the MW of the diffusant [75] and smaller molecules were shown to penetrate the skin more readily [76]. Thus, the small dimensions of NA, reflected in its MW (122,13g/mol) and molecular volume (108.13$Å^3$) [77], may be the reason for relatively high drug permeation across the silicone membrane and the pig ear skin. Nevertheless, NA hydrophilicity causes a lack of NA retention in the lipophilic silicone membrane. Similarly, NA was not retained in the epidermis and the dermis. Thus, the results obtained for DENI and NIAD formulations may indicate that solvents used in the prototype formulations do not cause NA retention in the model membranes.

4.5.4 Nicotinamide permeation from simple solvent systems

The interactions between the vehicle, the drug and the skin need to be understood for the rational development of the topical formulation. Theoretical calculations and *in vitro* studies are helpful in elucidating these relations. An appropriate choice of solvents for the optimal NA delivery was attempted based on the theoretical solubility parameters and *in vitro* studies. The chosen solvents encompass a wide range of solubility parameters: from the most lipophilic MO to the most hydrophilic water (δ of 7.74 and 22.97 $cal^{1/2}/cm^{3/2}$, respectively). Values obtained for the skin and the model drug lie in the middle of this range. However, the literature δ value for the skin (approximately 10 $cal^{1/2}/cm^{3/2}$) indicates its lipophilicity, while NA is a hydrophilic compound (δ=13.85). When a solvent with an appropriate δ value is chosen, it may increase NA solubility in the membrane, by shifting the δ value of the skin towards the solubility parameter of the drug [78]. Increased solubility in the skin means increased affinity of the drug for the membrane and thus, increased percutaneous transport of the drug [79-81]. This hypothesis was further investigated in the *in vitro* permeation studies.

Infinite dose studies across silicone membrane were performed for simple solvent systems. The highest k_p was obtained for IPM and the lowest for water, PG and their 50:50 mixture (one-way ANOVA, $p<0.05$). The J_{ss} ranged from 11±1 to 188±31 μg/cm^2/h, for PG and IPM respectively. High J_{ss} values were obtained for IPM, DPPG and mixtures of IPM with DPPG, PGML and PGMC. These systems have close δ values to that of silicone membrane (7.5 $cal^{1/2}/cm^{3/2}$) [39]. This was also reflected in the high IPM and DPPG sorption into the silicone membrane obtained in the solvent uptake studies. At the same time, a high NA flux was related to the low solubility of the drug in the solvent system. This effect was observed by previous authors for the permeation of another derivative of nicotinic acid (nicorandil) across an artificial membrane [42]. These results are also in line with other literature where it is noted that good solubility of a drug in a vehicle indicates its high affinity for this vehicle and thus its lower permeation [82]. NA is hydrophilic, thus its solubility in lipophilic solvents, such as IPM

and DPPG, is low. At the same time, these are the solvents that have a high potential to penetrate the lipophilic silicone membrane. The solvent uptake into the silicone membrane was correlated with drug permeation by previous authors [3, 8, 10, 12, 66] and data presented above confirm this approach. A high membrane interaction of solvents with low δ values was observed by Dias et al. [14] and resulted in a high flux of a hydrophilic drug (caffeine) from these solvents. A linear relationship was found between the solvent uptake and caffeine flux. A high uptake of aliphatic alcohols into silicone membrane resulted in an increased solubility and diffusivity of parabens in the membrane when compared with water and PG [3]. Thus, the increased permeation of NA across the silicone membrane observed for solvents with a high membrane uptake may be because of the increased diffusion and solubility of NA in this membrane. Dias et al. [13] found that for caffeine, IPM was one of the solvents which were postulated to penetrate the skin lipids and to alter the drug solubility in the membrane (influencing its partitioning). This increase in partitioning may be the reason for the highest k_p of NA observed for IPM when compared with the other solvent systems (one-way ANOVA, $p<0.05$). Studies on the partitioning behaviour of NA between the silicone membrane and the chosen solvents described above support this concept, as indeed the highest partition coefficient was obtained for IPM.

Finite dose studies more closely mimic the real *in vivo* application conditions and allow for the quantification of the drug in the skin layers. Thus, further studies were performed using finite doses of formulations with full thickness pig ear skin as a model membrane. The focus was on establishing solvent systems with the highest NA retention in the skin layers and the lowest percentage of NA washed from the skin surface at the end of the 8h permeation study. Also, the permeability coefficients, the applied concentration of NA and the solubility parameters of the solvent systems tested are shown in Table 4.16.

Table 4.16 The results of finite dose pig ear permeation studies from simple solvent systems (mean ± SD, n=5)

Solvent	kp ± SD [cm/h] ($x10^{-3}$)	Epidermis retention ± SD [ug/g]	Dermis retention ± SD [ug/g]	% Washed ± SD	C_{NA} [mg/mL]	δ $cal^{1/2}/cm^{3/2}$
IPM	2.7 ± 0.3	4 ± 1	3 ± 1	7 ± 3	0.68	8.22
DPPG	1.1 ± 0.1	10 ± 6	4 ± 0	9 ± 3	1.53	8.66
IL	1.1 ± 0.3	65 ± 21	21 ± 6	14 ± 9	8.07	8.83
IC	1.0 ± 0.2	120 ± 42	35 ± 6	15 ± 6	12.70	9.03
DL	0.2 ± 0.1	379 ± 121	11 ± 4	42 ± 5	9.17	9.05
DC	0.4 ± 0.1	483 ± 99	24 ± 11	40 ± 7	15.09	9.25
ILP	0.9 ± 0.2	667 ± 150	101 ± 9	16 ± 6	26.35	9.35
PGML	0.1 ± 0.0	450 ± 88	23 ± 3	74 ± 5	22.81	9.44
DLP	1.2 ± 1.1	954 ± 362	126 ± 10	21 ± 6	28.41	9.55
ILCP	1.2 ± 0.1	998 ± 514	159 ± 32	18 ± 6	39.58	9.66
DCP	4.2 ± 0.1	2061 ± 457	106 ± 14	36 ± 4	36.09	9.74
DLCP	0.7 ± 0.1	921 ± 138	141 ± 15	27 ± 5	42.21	9.79
PGMC	0.1 ± 0.1	883 ± 60	46 ± 14	75 ± 6	38.17	9.85
PL	0.8 ± 0.1	1199 ± 260	79 ± 15	44 ± 5	36.14	9.90
ICP	0.8 ± 0.1	1284 ± 408	133 ± 12	23 ± 8	37.98	10.27
PC	0.3 ± 0.1	1449 ± 204	105 ± 17	62 ± 10	64.73	11.96
PG	0.04 ± 0.03	1165 ± 561	58 ± 67	92 ± 8	246.76	14.07

The relationship between the solubility parameter (δ) and the permeability coefficient (k_p) obtained for the tested solvent systems is shown in Figure 4.32.

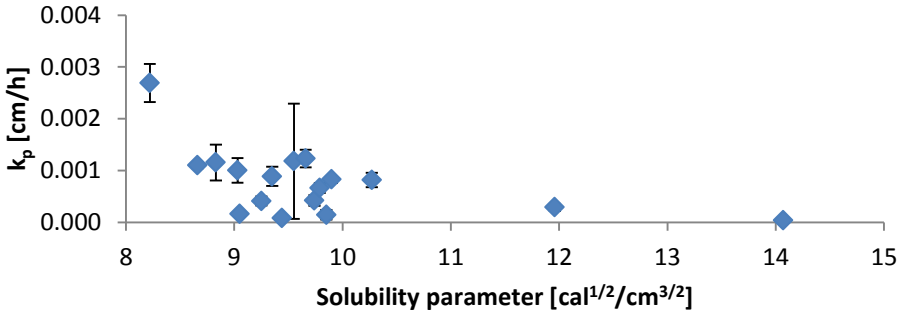


Figure 4.32 Relationship between the solubility parameter and the permeability coefficient in the finite dose pig ear studies for simple solvent systems (mean ± SD)

The importance of the δ value in skin permeation was emphasised by previous authors. Liron and Cohen [78] observed maximum skin k_p values for alkanoic acids with the δ value close to 10 $cal^{1/2}/cm^{3/2}$. Also, higher k_p values in human skin *in vitro* were obtained for substances with the δ value close to 10 $cal^{1/2}/cm^{3/2}$ for a series of narcotic analgesics [83]. In the NA pig ear skin permeation studies, the highest (one-way ANOVA, $p<0.05$) k_p was observed for IPM when compared with all solvent systems tested. Although high k_p values were also observed for binary solvent systems incorporating IPM and PGML or PGMC, synergistic effects between the solvents were not observed. Also, no synergistic effect was found for ternary and quaternary solvent systems, i.e. k_p values obtained for these solvent mixtures were lower than for IPM (one-way ANOVA, $p<0.05$). These results indicate that, from the chosen solvents, IPM is the best solvent in terms of increasing the skin permeability of NA, despite the fact that its δ value is far from the δ value of the drug (8.22 and 13.85 $cal^{1/2}/cm^{3/2}$, for IPM and NA respectively). A strong interaction of IPM with the skin observed by previous authors in DSC and X-ray diffraction studies [84] together with the close δ value of this solvent to that of skin may be the reason for this. Similar DSC and diffraction studies performed for the other solvents tested may elucidate their influence on the skin.

The retention of NA in the skin is crucial for its efficacy in the treatment of dry skin conditions such as atopic dermatitis (AD). An optimal adjustment of the δ value of the vehicle to that of the drug and the skin should lead to the optimised retention of the drug in the tissue. The skin retention data for the single solvents show that PG, as a solvent with the solubility parameter (14.07 $cal^{1/2}/cm^{3/2}$) closest to the δ value of NA (13.85 $cal^{1/2}/cm^{3/2}$), is capable of increasing drug retention both in the epidermis and the dermis. Nevertheless, the high NA solubility in PG renders this solvent not cost-effective, because a high amount of NA in the formulation is required to reach saturation (i.e. maximal thermodynamic activity) of the drug in PG [1]. Hence, a high percentage of NA remains on the skin surface at the end of 8h permeation. Also, rapid permeation of PG and thus solvent depletion may be the reason for the high percentage of NA washed from the skin surface [11, 52, 85]. Also, it has to be noted that, the drug retained in the skin would be ultimately removed by the blood supply. On the other hand, the use of solvents with relatively low δ values, such as IPM and DPPG (8.21 and 8.66 $cal^{1/2}/cm^{3/2}$ respectively), results in a low dose of NA applied (because of low solubility) and the lowest concentration of the drug in the skin layers (one-way ANOVA, $p<0.05$). PGML and PGMC are the solvents with δ values closest to the δ value of skin (9.44, 9.85 and 10 $cal^{1/2}/cm^{3/2}$ respectively). At the same time, NA solubility in these solvents is high enough to obtain comparatively good drug concentration in the skin layers, but the amounts are not significantly

different when compared with PG, IPM and DPPG (One-way ANOVA, $p>0.05$). These results are in line with previous findings [4, 86] and theoretical predictions [1], where a balance between the drug concentration in the vehicle and the partition coefficient between the vehicle and the skin is needed for optimal delivery. Thus, PGML and PGMC use in NA delivery is a rational compromise between high doses that can be achieved with PG and the high permeability obtained with IPM and DPPG.

Topical products for AD treatment are usually used up to three times a day [87]. Thus, the formulation is applied approximately every 8h. A build-up of NA which did not permeate during that time may occur in the case of a high percentage of the dose left on the skin surface. For example, approximately 75% of NA was present on the skin surface at the end of the study for PGML and PGMC. Increased skin retention and decreased flux of NA was reported when the molecule was formulated in combination with salicylic acid [31]. The authors suggested that this may be because of changes in the partition behaviour of the two drugs present in a transdermal film. Similar changes in partitioning may be postulated when combinations of solvents are used. Thus, the next step was to investigate if better epidermal concentrations with a lower percentage of NA left on the skin surface at the end of application period may be achieved by using binary and multicomponent solvent systems. The amount of NA retained in the dermis and the epidermis together with the percentages of NA washed from the skin surface obtained for all the simple solvent systems are summarised in Table 4.16.

Because of the high solubility of NA in PG, the concentration of this solvent in the solvent mixtures was set at 10%. This allowed for a lower concentration of NA in the saturated solution. Thus, the drug was used more effectively, because a lower amount of NA was needed to achieve maximal thermodynamic activity in the vehicle. A 10% addition of PG into single solvents (PGML and PGMC) resulted in an increased retention of the drug in both analysed skin layers when compared with the single solvents, however in most cases statistical significance was not reached (one-way ANOVA, $p>0.05$). The addition of DPPG to the binary solvent systems resulted in a significant increase in epidermal NA retention only for the DCP system when compared with binary systems (one-way ANOVA, $p<0.05$). In the case of binary and ternary solvent systems tested in the current study, PG may play an important role in increasing NA affinity for the skin and addition of the other solvents (PGML, PGMC and DPPG) optimises this effect. This may be because of the optimal adjustment of the δ value of the vehicle to the δ value of the epidermis and the drug. As can be seen in Table 4.16, the δ value of solvents with the highest epidermal retention of NA ranged from 9.74 to 14.07 $cal^{1/2}/cm^{3/2}$ However, there was no significant (one-way ANOVA, $p>0.05$) effect on epidermal retention for

the multicomponent solvent systems other than DCP when compared with binary solvent systems and single solvents. Thus, synergistic effects cannot be postulated for the other multicomponent solvent systems.

The use of quaternary solvent systems resulted in the best dermal retention of NA when compared with binary systems and single solvents (one-way ANOVA, $p<0.05$). These results indicate that there may be a synergistic effect of combining the solvents with different δ values (and thus different affinities for the skin) on NA retention in the dermis. High dermal retention of NA was obtained for the solvent systems with the δ value ranging from 9.35 to 11.96 $cal^{1/2}/cm^{3/2}$ What is more, a significantly lower % of NA was left on the skin surface following the application of all binary and multicomponent solvent systems when compared with PG (one-way ANOVA, $p<0.05$), indicating that combining the solvents may result in more cost-effective formulations. The relationship between the δ value and the retention of NA in the skin is shown in the figures below.

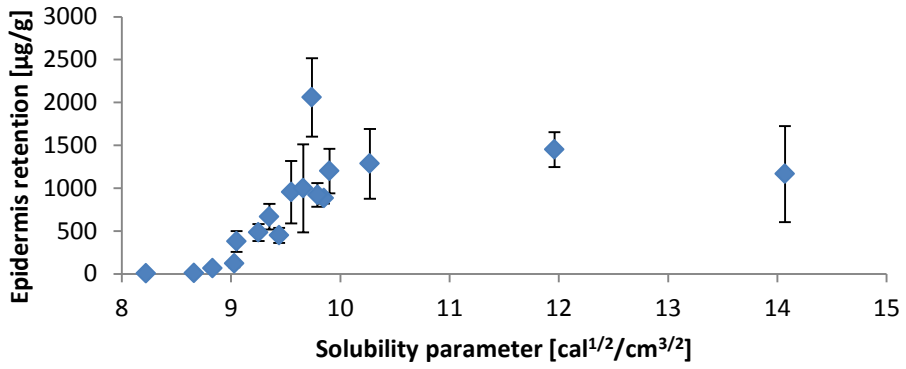


Figure 4.33 Relationship between the solubility parameter and NA retention in the epidermis in the finite dose pig ear studies for simple solvent systems (mean ± SD)

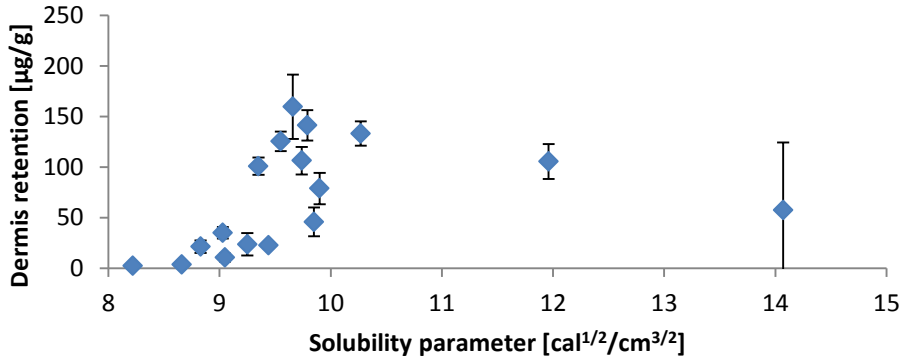


Figure 4.34 Relationship between the solubility parameter and NA retention in the dermis in the finite dose pig ear studies for simple solvent systems (mean ± SD)

4.5.5 Solvent permeation studies - simple solvent systems

The affinity of the solvent for the skin layers may be reflected in the uptake, permeation and retention of the solvent in the skin or model membrane. This in turn may be related to the drug permeation and its residence in the membrane. Thus, there is a need for monitoring not only the drug but also the solvent fate for better understanding of the processes that govern drug permeation and penetration enhancement [2].

In the finite dose silicone permeation studies, a relationship between rapid solvent permeation and decreased drug penetration caused by solvent depletion from the donor compartment could be seen for the PGML and PGMC vehicles. While approximately half of PGMC and two thirds of PGML permeated, reaching a plateau, the fraction of NA found in the receptor solution after the 6 hours study was approximately 30% of the original dose. A similar situation was observed by other authors when solvent depletion (via rapid evaporation) from the vehicle caused a decrease in the flux of a model drug [88, 89]. High solvent retention in the membrane, observed for IPM and DPPG, was related to the high percentage of NA which permeated from these vehicles. This indicates that the presence of these solvents in the membrane enabled the drug penetration from the donor compartment into the receptor phase. For PG, a high percentage of the solvent was washed from the membrane surface and also a high percentage of NA remained in the donor compartment at the end of the study, suggesting that low permeation of this hydrophilic solvent and low membrane retention hinders drug permeation across silicone membrane. Lower silicone permeation of PG when compared with another lipophilic solvent (octyl salicylate) was observed previously [36], indicating that lipophilic solvents penetrate silicone membrane more easily.

In the finite dose full thickness pig ear skin permeation studies, the highest percentage of NA permeated from IPM, followed by DPPG, PGML, PGMC and PG, which is in line with the results obtained in the studies, where only NA permeation was monitored. This trend may be because of differences in NA solubility in these solvents with the lowest solubility for IPM and the highest for PG. Even though a high cumulative amount of NA permeated from PG, it is still only a small percentage of the dose that was applied to the donor compartment of a Franz cell. Similarly to the silicone data, the high percentage of NA washed from the skin surface was related to the high percentages of PGML and PGMC that remained in the donor compartment indicating that solvent permeation is necessary for the drug to be transported into and through the skin. On the other hand, the results of solvent analysis may suggest that even

though no permeation was observed for IPM, DPPG, PGML and PGMC, the presence of these solvents in the skin (approximately 2-5% of the dose applied) was enough to assist the drug transport across the membrane. The stratum corneum is considered to be the main barrier for permeation [65], thus the presence of solvents in this skin layer is important for NA penetration because the process occurs only for the drug in solution. Once the drug reaches the hydrophilic environment of the dermis, it may be dissolved in the aqueous environment of this layer and thus, transported into the receptor fluid. Nevertheless, the high percentage of NA extracted from the skin in the case of IPM and DPPG (40±12 and 37±4, respectively) indicates that some of the drug is entrapped in the skin and does not reach the receptor fluid, probably because of the retention of these solvents in the skin.

The lack of permeation observed for lipophilic solvents in the full thickness pig ear studies is most probably because of the hydrophilic nature of the dermis, which effectively represents a barrier for the transport of these solvents. Studies performed on heat-separated skin may be helpful in addressing this issue. Nevertheless, the small amount of solvents present in the skin enabled NA permeation to some extent.

To conclude, the above mentioned results indicate, that a balance between solubility of the drug in the solvent and solvent affinity to the skin is desirable for an optimal topical delivery.

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5 Evaluation of prototype nicotinamide formulations *in vivo*

The composition of the vehicle is crucial for the development of an effective and safe nicotinamide (NA) formulation which may be used in atopic dermatitis (AD) treatment. The most common ingredients of the vehicles used in the treatment of dry skin conditions contain water, humectants, occlusive materials and emollients formulated into an ointment or cream with the aid of adjuvants [1]. Thus, four principal excipients from these groups were included in the prototype NA formulations: water, glycerol (GLY) as a humectant, liquid paraffin (mineral oil, MO) as an occlusive agent and isopropyl myristate (IPM) as an emollient and penetration enhancer. A formulation containing these excipients (Doublebase™ gel, Dermal Laboratories Ltd, UK) is available on the UK market [2].

Doublebase™ gel is indicated in dry skin conditions such as AD [2, 3]. Doublebase™ gel was shown to be superior to two other marketed formulations (Ultrabase® cream and Diprobase® cream) in terms of decreasing transepidermal water loss (TEWL) and increasing skin hydration in healthy volunteers after a single application [4]. Also corneometry readings were significantly ($p<0.0001$) higher for the Doublebase™ gel when compared with non-treated skin and with the two formulations after repeated application in a two-day test [4]. Moreover, after a seven-day application of the Doublebase™ gel, skin hydration improved significantly ($p<0.05$) in women with self-perceived dry skin on their lower legs when compared with non-treated skin and skin treated with Aqueous Cream BP [5]. Taking into account the efficacy of the Doublebase™ gel in alleviating dry skin conditions and improving skin hydration, the excipients present in this formulation were used in the development of the NA formulation for AD treatment.

As mentioned before, the influence of NA on the skin state was to be tested separately from the other excipients present in the formulations, which may also influence the skin. Thus, two prototype formulations containing 4% NA were prepared by Dermal Laboratories Ltd., UK: DENI contained all of the above mentioned components, while NIAD was formulated into a gel containing primarily water.

The prototype NA formulations were tested *in vitro* in the previous chapter. The results of finite dose experiments indicate that there is no statistically significant difference (t-test, $p<0.05$) between the two formulations in terms of NA permeation across silicone membrane and porcine skin. However, not only the drug, but also the formulation components may influence the state of the skin and the final formulation performance *in vivo* [6-12].

The skin state may be assessed *in vivo* with a wide array of non-invasive and minimally invasive techniques. Tape stripping is one of the techniques which allows for the investigation of morphological and physiological properties of the tissue [13, 14]. It also enables the assessment of percutaneous penetration and skin retention of the drug quantified on the tapes [15-17]. The amount of the stratum corneum (SC) removed with consecutive tapes can be an indicator of cell cohesion [18, 19]. Since the SC consists mainly of protein (approximately 75%) [20], the protein content measurement is used to quantify the amount of the SC stripped from the skin surface [21-23].

Another technique is transepidermal water loss (TEWL) measurement, which gives information on the skin barrier function to prevent water loss [24]. Combining tape stripping with TEWL measurements can give information on the skin resistance to damage [25, 26] and the depth of the SC that is reached with the stripping procedure [27]. Also improvement or deterioration of the skin barrier function caused by topical products can be reflected in TEWL values [11, 28-30].

The investigation of corneocyte size and maturity gives information on the skin cell proliferation and maturation process [31]. Topically applied products may influence these processes. For example, changes in corneocyte surface area and cornified envelope maturation caused by Aqueous Cream BP topical application in healthy subjects were studied using this technique [28].

Changes in the SC protease activity (PA) are associated with skin barrier dysfunction [32-37]. The measurements of protease activity can give important information on the influence of the formulation on skin state, especially on the desquamation and inflammatory processes [28, 35].

The purpose of this study was to assess changes in the skin condition associated with the application of prototype topical formulations, studied in the previous chapter, and their placebos. The changes in measured parameters should indicate improvement or deterioration in the skin barrier function and health brought about by application of topical formulations. The amount of protein stripped from the skin surface, the corneocyte surface area, the corneocyte maturity, activities of selected SC proteases and the TEWL were measured. Changes in these parameters caused by the application of the formulations to the mid ventral forearm of healthy volunteers were characterised. Also the influence of the formulation composition on NA retention in the skin *in vivo* was investigated. The observation of these

effects, together with the assessment of skin delivery of the model compound, may enable the design of improved topical products.

5.1 Materials

Prototype formulations (DENI and NIAD) and their placebos (DE and AD respectively) were supplied by Dermal Laboratories Ltd., UK. The composition of DENI and NIAD formulations is shown below. In the placebo formulations NA was replaced with purified water.

Table 5.1 Composition of DENI formulation

Material	% w/w
Nicotinamide EP	4.0
Isopropyl Myristate EP	
Paraffin Liquid EP	
Glycerol EP	
Modified acrylic polymer (Pemulen^{TM} HSE TR1)	
Sorbitan Laurate EP	
Triethanolamine BP 2000	
Phenoxyethanol EP	
Water Purified EP	

Table 5.2 Composition of NIAD formulation

Material	% w/w
Nicotinamide EP	4.0
Modified acrylic polymer (Pemulen^{TM} HSE TR1)	
Sorbitan Laurate EP	
Citric Acid Anhydrous EP	
Phenoxyethanol EP	
Triethanolamine BP 2000	
Water Purified EP	

Table 5.3 Materials used in the *in vivo* studies

Material	Supplier
Laboratory water purifier (OPTION 3, 75 litre volume)	USF Elga, UK
PBS tablets (DulbeccoA, pH 7.3±0.2 at 25°C)	Oxoid, UK
Nicotinamide (NA)	Dermal Laboratories Ltd., UK
HPLC grade solvents: Acetonitrile (AcCN), Water	Fisher Scientific, UK
HPLC vials with flat bottom inserts and caps with rubber seal	Fisher Scientific, UK
Plastic syringes (1, 2 and 5mL) and needles	Fisher Scientific, UK
Microscope cover glasses	Fisher Scientific, UK
Surgical scalpels, scissors and tweezers	Fisher Scientific, UK
Acetone	Fisher Scientific, UK
Dessicator	Fisher Scientific, UK
Magnesium perchlorate desiccant	Acros Organics, UK
Sodium dodecyl sulphate (SLS)	Sigma Aldrich, UK
Ethylenediaminetetraacetic acid	Sigma Aldrich, UK
Acetic acid	Sigma Aldrich, UK
Nile red	Sigma Aldrich, UK
Trizma® hydrochloride buffer solution (Tris pH 8.0)	Sigma Aldrich, UK
Triton-100	Sigma Aldrich, UK
PAP Pen for immunostaining 5 mm tip width	Sigma Aldrich, UK
DL-dithiothreitol (DDT)	Fluka Analytical, UK
Dimethyl sulfoxide (DMSO) (>69 % D: 1.67)	VWR International, UK
AnalaR Normapur Perchloric acid 70%	VWR International, UK
Polysine™ Microscope Slides	VWR International, Belgium
D-Squame tape (2.2cm diameter, 3.8cm^2 area)	CuDerm Corporation, USA
Standard D-Squame disc racks and pressure device	CuDerm Corporation, USA
Aminomethyl coumarin (AMC)	DSM Nutritional Products Ltd, Switzerland
Fluorogenic substrate for tryptase: Tos-Gly-Pro-Lys-AMC I-1370	DSM Nutritional Products Ltd, Switzerland
Fluorogenic substrate for kallikrein 5: Boc-Phe-Ser-Arg-AMC I-1400	DSM Nutritional Products Ltd, Switzerland
Fluorogenic substrate for kallikrein 7: MeOSuc-Arg-Pro-Tyr-AMC Pefafluor 180-23	DSM Nutritional Products Ltd, Switzerland
Fluorogenic substrate for plasmin: MeOSuc-Ala-Phe-Lys-AMC I-1275	DSM Nutritional Products Ltd, Switzerland

Primary monoclonal antibody, anti-human involucrin (clone SY5)	Cambridge Scientific, UK
Rabbit polyclonal antibody to mouse fluorescent IgG-H&L (whole molecule) fluorescein isothiocyanate (FITC) antibody	Abcam, UK
Eppendorf Research® pipettes	Eppendorf AG, Germany
Closed chamber Aquaflux® AF103	Biox Systems, UK
Squame Scan A850 infrared densitometer	Heiland Electronic,Germany
Fluorescence microscope, microphot-FZA with rhodamine dichroic filter	Nikon, UK
Analytical balances (accuracy 0.0001g and 0.0001mg)	Sartorius GMBH, Germany
Nikon D5100 Digital Single Lens Reflex camera	Nikon, UK
ImageJ® software	NIH-Image, USA
Orbital incubator S150	Stuart Scientific, UK
Centrifuge 5415R	Eppendorf, UK
HPLC Column Primesep A 250x4.6mm, Particle 5µm, 100Å	SIELC Technologies, USA
HPLC Column Symmetry C_{18}, 5um, 4.6x150mm	Waters, USA
SecurityGuard Cartridge with C_{18} (ODS, Octadecyl) 4mmLx3mm filter	Phenomenex, USA

5.2 Methods

5.2.1 Ethical approval

The study entitled '*The influence of topical formulation composition on test compound transport into the skin and on skin condition'* (Ref. No A01/2011) was approved by the National Research Ethics Committee London - Camberwell St Giles (REC Ref. No. 11/LO/1275).

5.2.2 Volunteer recruitment

Because of inter- and intra-individual variability, a high number of volunteers are needed to reach statistical significance in *in vivo* studies [38]. Most commonly 12 to 20 subjects have been included to study formulation effects on skin [39-50]. Thus, 20 healthy adult volunteers were recruited for this study. Subjects with any previous or on-going skin disease, any skin abnormalities (tattoos, birth marks, scars etc.) at the test sites, known sensitivity to any of the formulation ingredients or taking or using medication purchased over the counter in pharmacies or prescribed by a healthcare practitioner which may have an effect on the skin were excluded from the study. Written informed consent was obtained from all participants prior to taking part, after they were given at least 24 hours to read the Participant Information Leaflet and had ample opportunity to discuss any questions they had. Before the study, participants were asked to complete a questionnaire stating their age, gender, ethnic background and skin state. 8 male and 12 female volunteers aged 23-55 years (average 29±9 years), all Caucasian, participated in the study.

5.2.3 Application of test formulations

Bi-lateral formulation application sites located on the left and right volar forearms measured approximately 25cm^2. They were demarcated using a laminated template (Figure 5.1). There were 2 application sites for each arm (total of 4 per volunteer) and 1 untreated (control) site allocated randomly on one of the forearms (Figure 5.2).

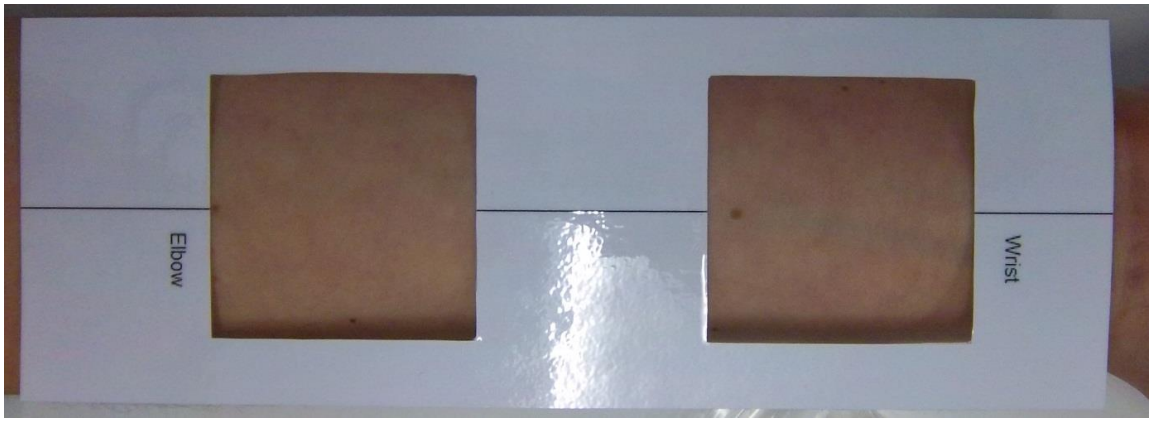

Figure 5.1 Laminated template aligned on the forearm

Individual applications comprised 50µL (i.e. 2µL/cm^2) of the test formulations dispensed by volunteers from 1mL syringes and then gently massaged into the designated treatment site. Topical products in atopic dermatitis treatment are usually indicated for up to three times a day application [51]. Thus, the formulations were applied by the subjects three times a day to the specified skin sites for a treatment period of 21 days (a period which takes into account the stratum corneum turnover time). Information on participants' compliance with the application regime was recorded in Participant's individual Diaries and collected by the investigator at the end of the study (average compliance was 90±12).

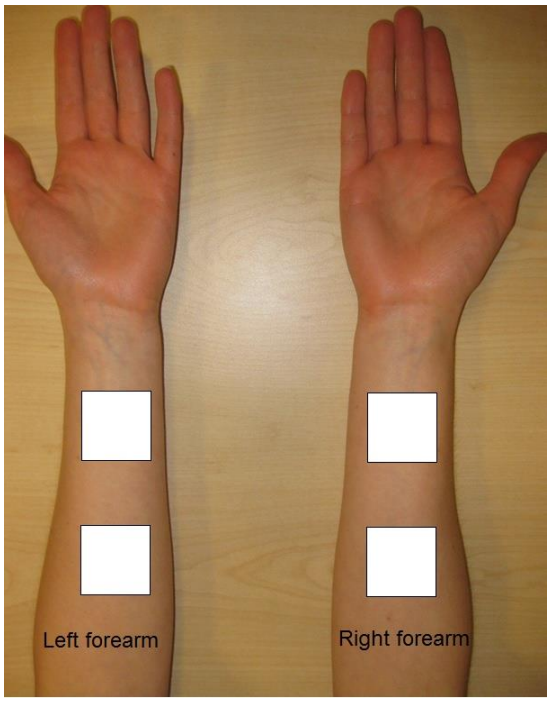

Figure 5.2 Application sites

Volunteers were asked to refrain from applying any topical products to the skin areas under investigation during the 24 hours prior to the study and during the treatment regimen until the day of measurements. There were no other restrictions regarding normal day-to-day life of participants, including clothing, washing, bathing, showering, sports participation etc. After 21 days of application, a one-day wash-out period was allowed, when no formulation was applied, and the measurements (tape stripping and TEWL) were performed on the 23rd day of the study. The measurements were performed in the laboratory, in ambient conditions at a temperature of 25.8±1.7°C and a relative humidity of 44.6±3.6%. Before the measurements, volunteers acclimatised in these conditions for at least 15 minutes.

5.2.4 Tape stripping

After completing the formulation application regimen, the skin was stripped consecutively 20 times at each of the 4 treated sites and 1 control site with D-Squame® discs (CuDerm Corporation, USA). In total 5 skin areas on the volar forearms were sampled. The D-Squame® tape was placed on the skin at a constant pressure of 225g/cm^2 applied with a D-Squame pressure device (CuDerm Corporation, USA) for 5 seconds and then the tape was removed.

5.2.5 Protein content determination

Protein content [µg/cm^2 of tape] was quantified as an indirect way of measuring the amount of the SC removed with each tape. After the tape stripping procedure the tapes were placed

on the standard D-Squame® racks (CuDerm Corporation, USA) and the absorption readings were taken with an infrared densitometer (SquameScan 850A, Heiland Electronic,Germany).

The amount of protein was calculated according to the equation validated by Voegeli et al. [52]:

$$C_{\text{protein}} = 1.366A - 1.557$$

Equation 5.1

where:

C_{protein}- concentration of the protein on the tape [µg/cm^2]

A - absorption [%].

5.2.6 Transepidermal water loss measurements

TEWL was measured at each of the five designated skin areas before tape stripping (baseline) and after each 5 tapes with a closed chamber Aquaflux® AF103 (Biox Systems, London, UK). ΔTEWL, i.e. the difference between the TEWL measurement of the stripped area and the baseline measurement, was calculated.

The tape stripping and TEWL measurement procedure is shown in Figure 5.3.

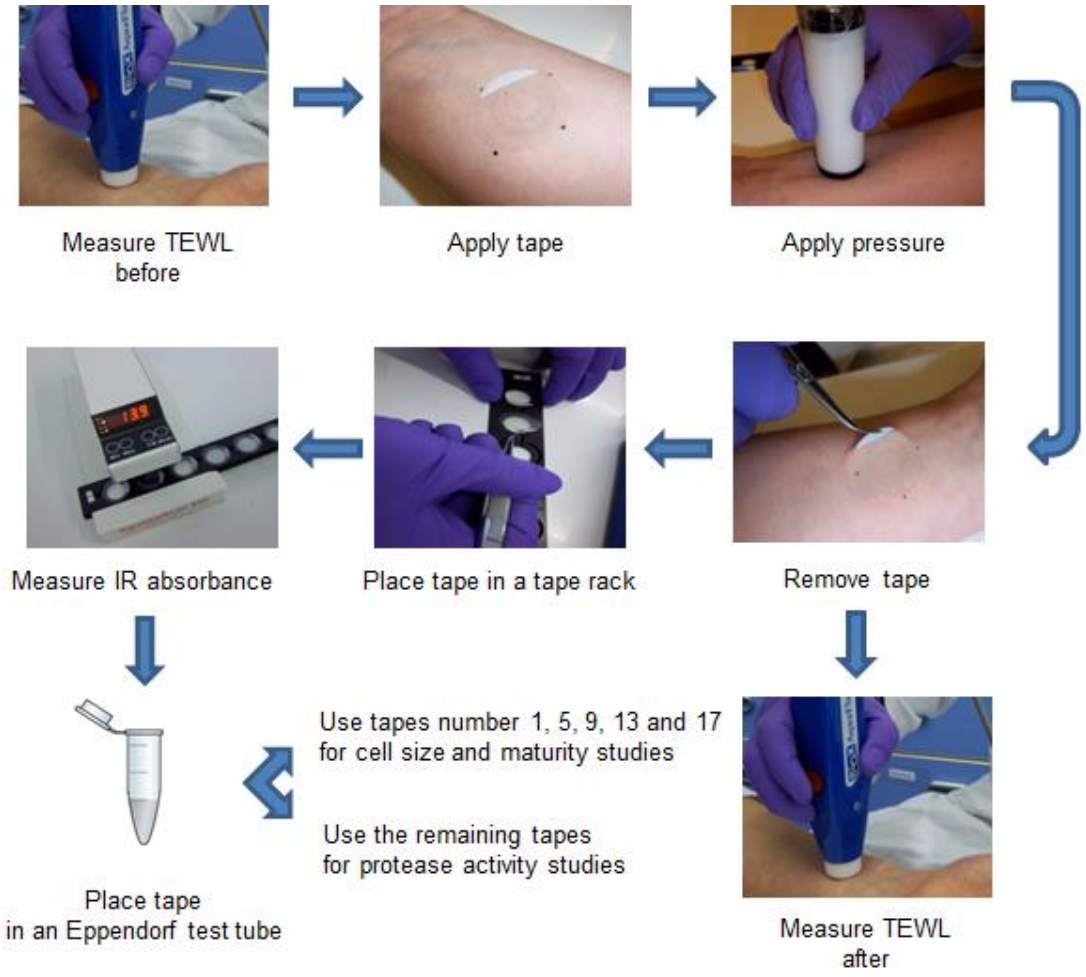

Figure 5.3 Tape stripping and TEWL measurement procedure

5.2.7 Corneocyte size and maturity measurements

Tapes number 1, 5, 9, 13 and 17 were used for corneocyte size and maturity measurements. Corneocyte maturity and corneocyte surface area were measured by immunofluorescence and Nile red staining method described below. The method was previously developed and described by Hirao et al. [53].

Each tape was transferred into a separate glass vial and approximately 1mL of the dissociation buffer comprising of 2% sodium dodecyl sulphate (sodium lauryl sulphate, SLS), 20mM DL-dithiothreitol, 5mM ethylenediaminetetraacetic acid and 0.1M Tris-hydrochloride buffer pH 8.0 was added to each vial. The tapes were extracted at approximately 100°C for 5-10min. Extracts were then transferred into Eppendorf tubes and centrifuged at 6000rpm for 10min. The supernatant was removed and the extraction procedure was repeated three times. The cellular precipitate was suspended in 100µL of the dissociation buffer.

2-5µL of the cell suspension was applied to a microscope slide (WVR International, Belgium) and left to dry at room temperature. The applied suspension was encircled with a liquid blocker PAP Pen (Sigma Aldrich, UK) to avoid loss of cells and solutions from the slide. 15µL of the primary monoclonal antibody anti human involucrin (clone SY5, Cambridge Scientific, UK) 1:100 in sterile purified water was then applied and the slides were left overnight in the desiccator at 4°C. Next, 15µL of the Anti-Mouse IgG (whole molecule)−FITC secondary antibody H&L produced in rabbit (Abcam, UK) 1:50 in PBS was applied and the slides were left for 1h in a dark room at room temperature. After rinsing the slides with PBS, 20µg/mL solution of Nile red in 75% glycerol (prepared from 500µg/mL stock solution in acetone) was applied to the slides for 10min at room temperature in the dark room. The slides were rinsed with PBS, covered with cover slips and observed under a fluorescence microscope (Nikon, UK). Images of Nile red-stained (red pixels) and immuno-stained (green pixels) cells were taken with a Nikon D5100 camera (Nikon, UK), magnified 10x, 2.3 pixels per µm, resolution 4928x3264 pixels. ImageJ® software (Version 1.44p, NIH-Image, USA) was used to measure the RedGreenBlue pixel ratio (to analyse cell maturity) and number of pixels (to investigate corneocyte surface area from Nile red stained pictures) as described before [54]. Cell maturity was also analysed by calculating the ratio between red and green cells counted on the green fluorescence image. A similar method of cell maturity assessment was described by Hirao et al. [53].

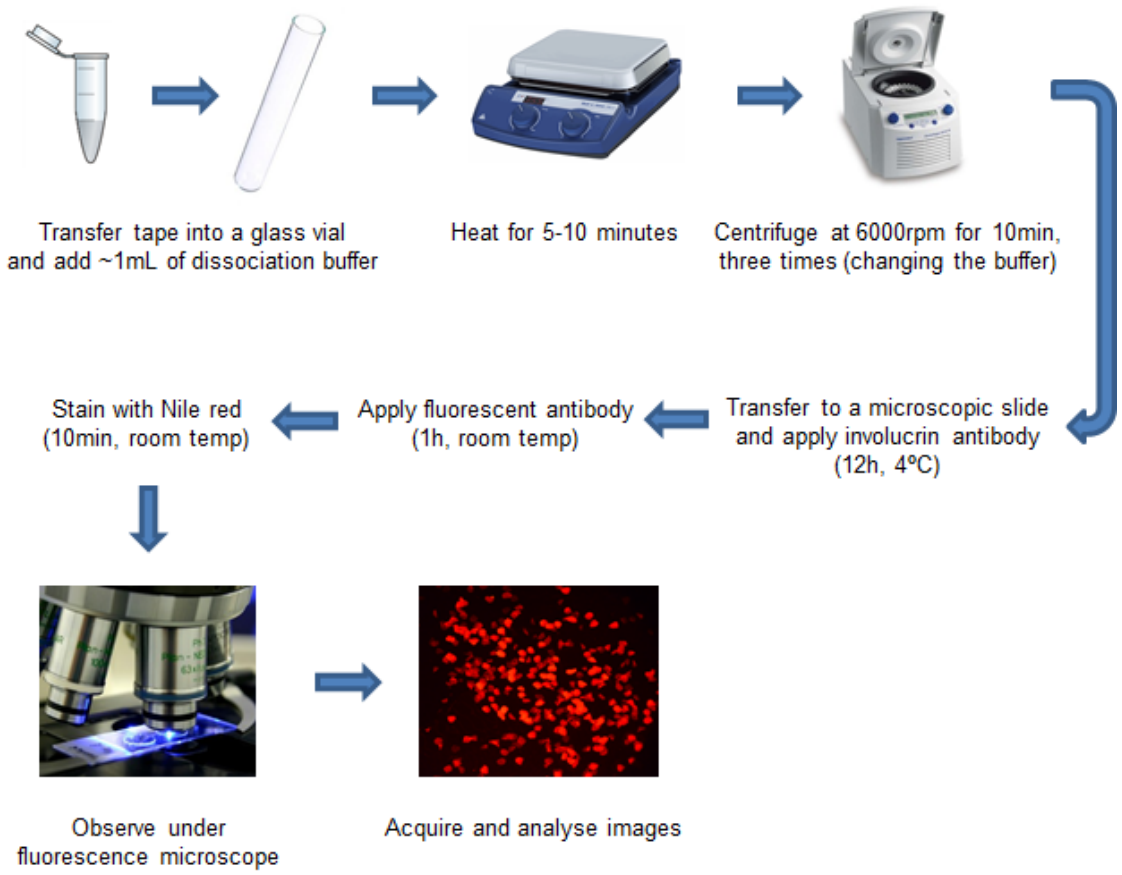

Figure 5.4 Cell size and maturity measurement procedure

5.2.8 Protease activity measurements

Protease activity (PA) was measured by the HPLC assay of the fluorescent product (aminomethyl coumarin, AMC) of enzymatic reactions involving peptide substrates. The method was previously described by Voegeli et al. [55]. Tapes not utilised for corneocyte size and maturity measurements were used for PA determination.

Each tape was placed in a separate Eppendorf tube and extracted with 750µL of the buffer (pH 8.0) containing 0.1M Tris-HCl and 0.5% TritonX-100 (15 min., 25°C, 1000rpm) in the orbital incubator S150 (Stuart Scientific, UK). 400µL of extracts from three tapes were pooled (2-4, 6-8, 10-12, 14-16, 18-20). 250µL of pooled extracts was transferred into 4 Eppendorfs. 1.25µL of 5mM fluorogenic substrates dissolved in DMSO (final substrate concentration 25µM) was added, each to a different Eppendorf. The solutions were mixed (2h, 37°C, 1000rpm, orbital incubator). After 2h incubation, 250µL of 1% acetic acid was added to the reaction mixture. The reaction mixture was transferred into a HPLC vial and analysed by HPLC for the AMC

content. The HPLC method for AMC quantification was validated prior to the studies (coefficient of variance<10-15%, r^2>0.999) and is described in Table 5.4.

Table 5.4 Parameters of the HPLC method for AMC quantification

Column	**Symmetry Shield RP18, 5µm, 4.6x150mm** (Waters, Milford, MA, USA)
Guard column	**SecurityGuard Cartridge System with C_{18} (ODS, Octadecyl) 4mmLx3mm filter** (Phenomenex, USA)
HPLC System	**HP Series** 1050 quaternary pump, HP Series 1050 autosampler, HP series 1100 degasser, Waters 470 Fluorescence Detector
Software	**PRIME software** Ver.4.2.0., HPLC Technology Co. Ltd, UK
Mobile phase 1	Acetonitrile:Water:Trifluoroacetic acid (30:70:0.1, v/v/v) - KLK5
Mobile phase 2	Acetonitrile:Water:TFA (25:75:0.1, v/v/v) - TRY, PLA
Mobile phase 3	Acetonitrile:Water:TFA (20:80:0.1, v/v/v) - KLK7
Injection volume	20µL
Flow rate	1 mL/min
Temperature control	40^{o}C
Fluorescence detection wavelength	442nm for emission, 354nm for excitation
Retention time of AMC	~3min
Analysis time	~10min
Calibration curve range	1-10ng/mL (standard concentrations: 1; 2,5; 5; 7,5; 10ng/mL of AMC prepared from a 1000ng/mL stock solution by dilution with the reaction buffer)

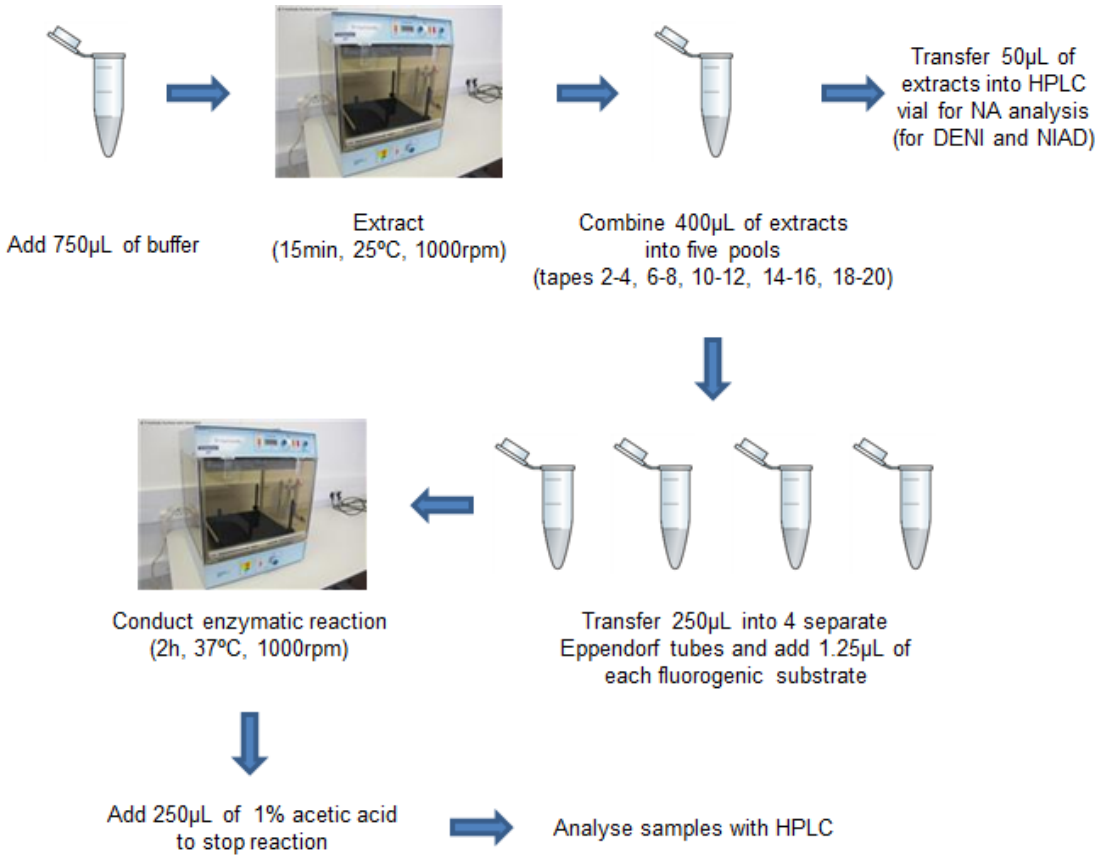

Figure 5.5 Protease activity measurement

5.2.9 Nicotinamide content

NA skin retention was determined for the sites treated with DENI and NIAD formulations. NA content in the tapes was determined with the HPLC method described in section 3.2.5. Extracts from PA measurements (before pooling) were taken. The extraction method was validated prior to the study (as described in the appendices).

5.2.10 Statistical analysis

Statistical significance was determined using IBM SPSS Statistics version 19 software (SPSS Inc., an IBM Company). Normality was assessed using the Kolmogorov-Smirnov statistical test with Sig.>0.05 indicating normality. A one-way ANOVA with Tukey post-hoc test ($p<0.05$) was performed to compare differences in total protein content, mean TEWL, ΔTEWL, cell size and cell maturity in the tapes taken from 4 test sites and 1 control site. A probability of $p<0.05$ was considered statistically significant. A Kruskal-Wallis test with a Mann-Whitney U post-hoc test and Bonferroni correction was performed for KLK5, TRY, PLA and KLK7 activity comparisons

because of the lack of normal distribution of these data. A probability of $p<0.005$ was considered statistically significant. A correlation between cell maturity determined with ImageJ® software and the cell counting method was assessed with Spearman's rho correlation test. A probability of $p<0.05$ was considered statistically significant. All results are presented as the mean ± standard error of mean (SEM) (n=20) unless otherwise stated.

5.3 Results

5.3.1 Protein content

The amount of protein taken with each of the tapes was quantified according to the method described in section 5.2.5. In general, less protein was stripped with increasing tape number which is in line with previous findings [21, 22, 54, 55]. The average amount of protein stripped from the investigated sites with each tape is shown in Figure 5.6.

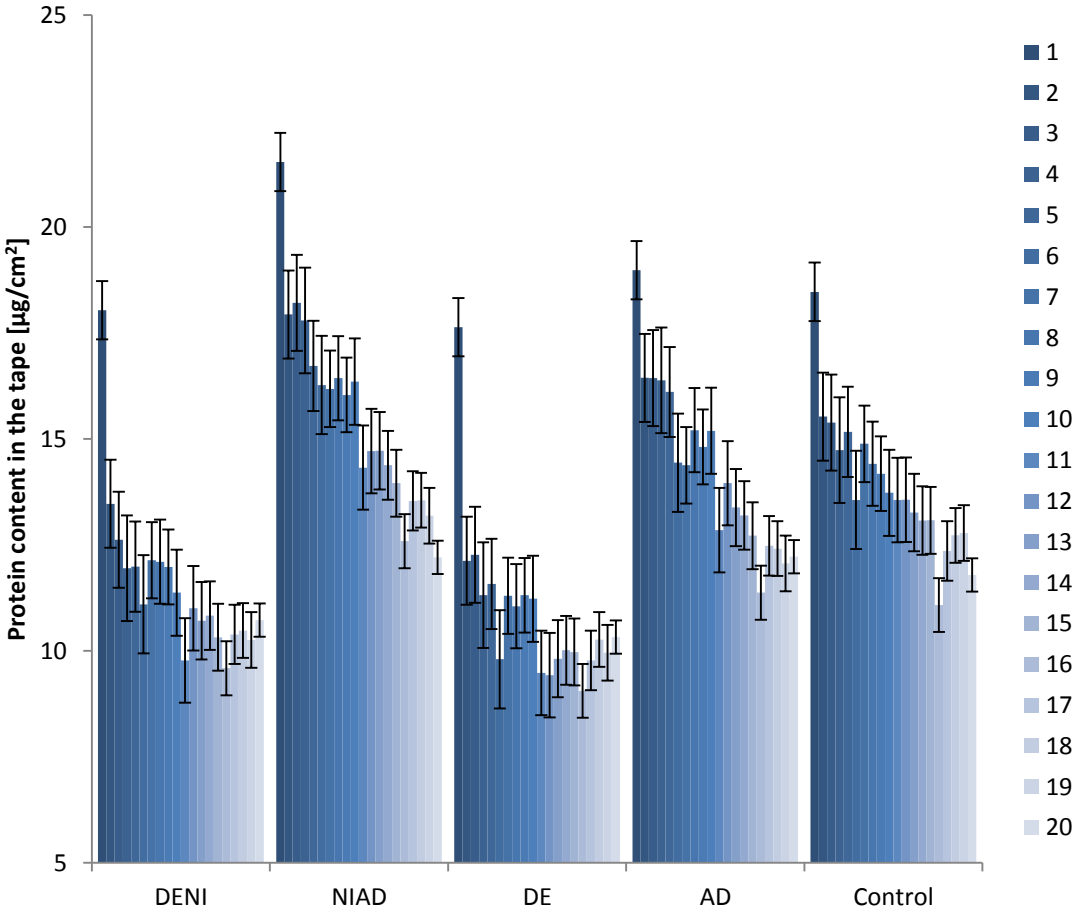

Figure 5.6 Amount of protein stripped with each tape (data shown as mean ± SEM, n=20)

The total amount of protein [µg/cm^2] stripped from each measured site was calculated and is shown in Figure 5.7.

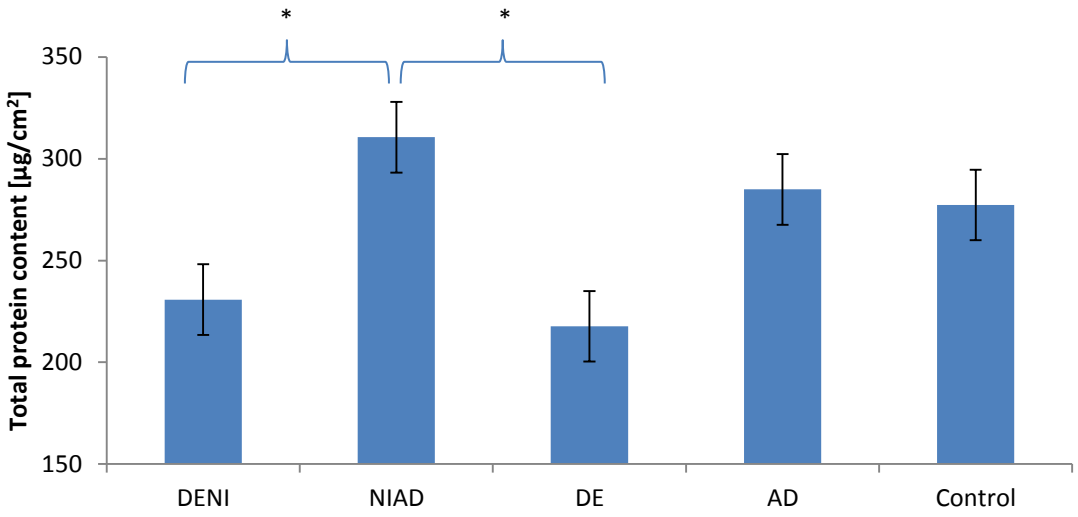

Figure 5.7 Total protein taken from the measurement sites (data shown as mean ± SEM, n=20), the measurement sites significantly different from each other are marked with an asterisk (*p<0.05).

A statistically significant (one-way ANOVA, $p<0.05$) difference in total protein content was found between DENI vs. NIAD and DE vs. NIAD formulations. A lower amount of protein was found in tapes stripped from the application sites of formulations containing lipophilic excipients (DENI, DE).

5.3.2 Transepidermal water loss

TEWL [g/m^2/h] was measured five times at each of the areas of interest (before the tape stripping and after each five tapes). As previously reported [21, 54], TEWL increased with the removal of the SC layers. This is illustrated in Figure 5.8. The effect was more pronounced for the sites treated with placebo formulations and the control site, however statistical significance was not reached (one-way ANOVA, $p>0.05$).

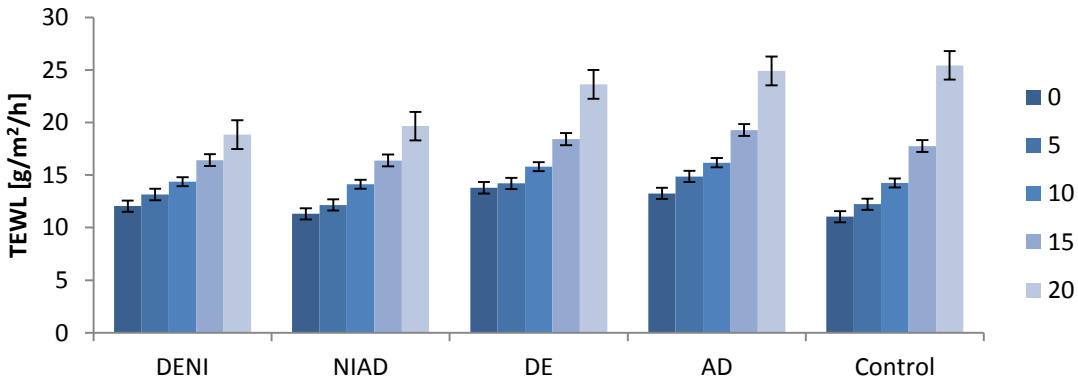

Figure 5.8 TEWL obtained for each formulation at baseline (0) and after taking every five tapes (5, 10, 15, 20) (data shown as mean ± SEM, n=20)

ΔTEWL between the TEWL measurement of the stripped area and the baseline measurement was calculated and is illustrated in Figure 5.9. Higher differences from the baseline TEWL were found for the control and AD application sites, especially after 15 and 20 tapes were taken. However, statistical significance was not reached (one-way ANOVA, $p>0.05$).

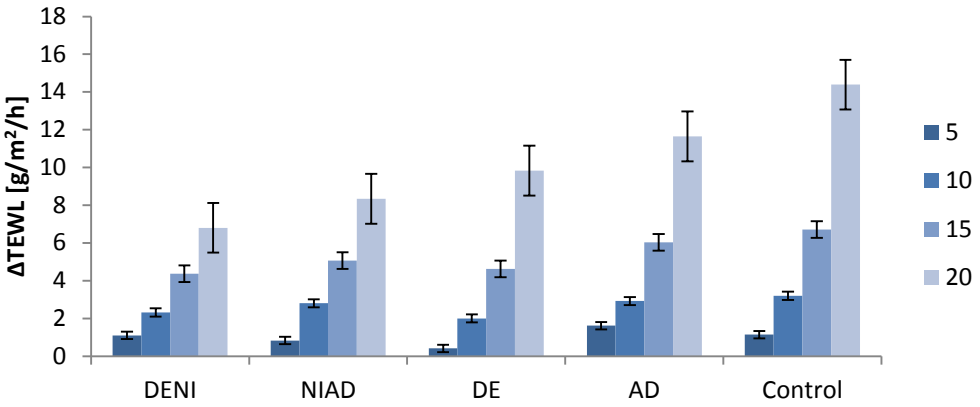

Figure 5.9 ΔTEWL between the TEWL after the tape stripping procedure and the baseline TEWL (data shown as mean ± SEM, n=20)

The average TEWL values obtained for each measurement site were calculated. A statistically significant difference (one-way ANOVA, $p<0.05$) for mean TEWL was found between the NIAD formulation and its placebo (AD), with higher values obtained for the latter (see Figure 5.10).

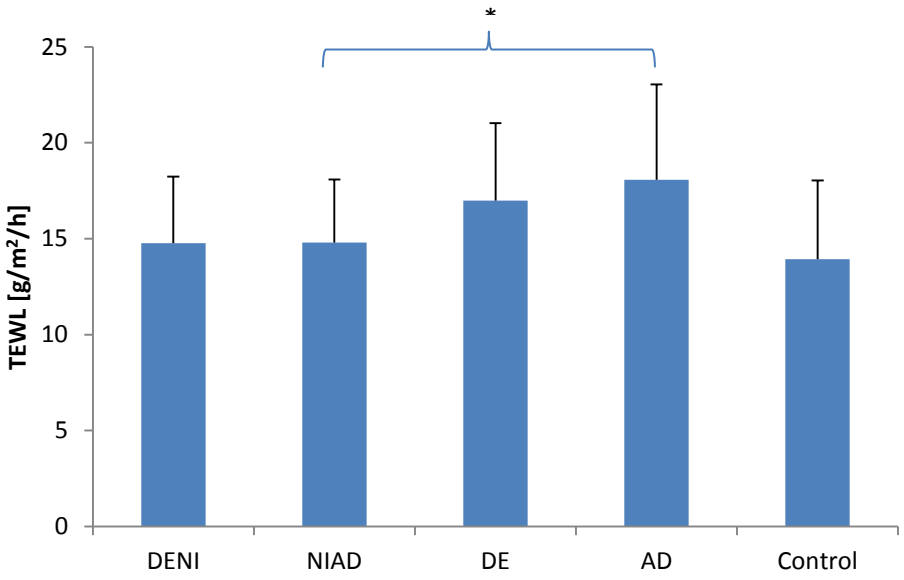

Figure 5.10 Average TEWL obtained for each formulation (data shown as mean ± SEM, n=20, *p<0.05)

5.3.3 Corneocyte size

Corneocyte size was measured for tape number 1, 5, 9, 13 and 17. In most cases, there was a decrease of the cell surface area with the tape number, observed also by Mohammed et al. [54]. The results for each site of interest are shown in Figure 5.11.

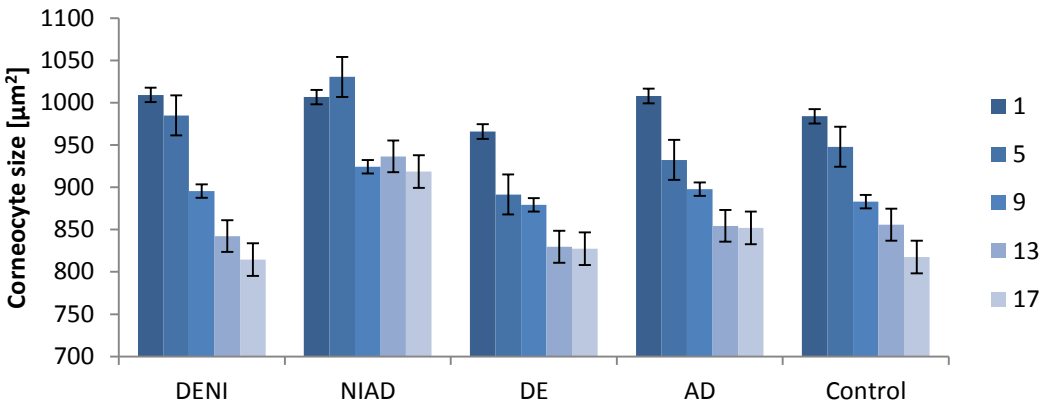

Figure 5.11 Corneocyte size (data shown as mean ± SEM, n=20)

The average cell surface area was calculated for each measurement site. Statistically significant (one-way ANOVA, $p<0.05$) differences were found between average cell surface area obtained from NIAD vs. DE and NIAD vs. control sites with larger cells for NIAD in both cases (see Figure 5.12).

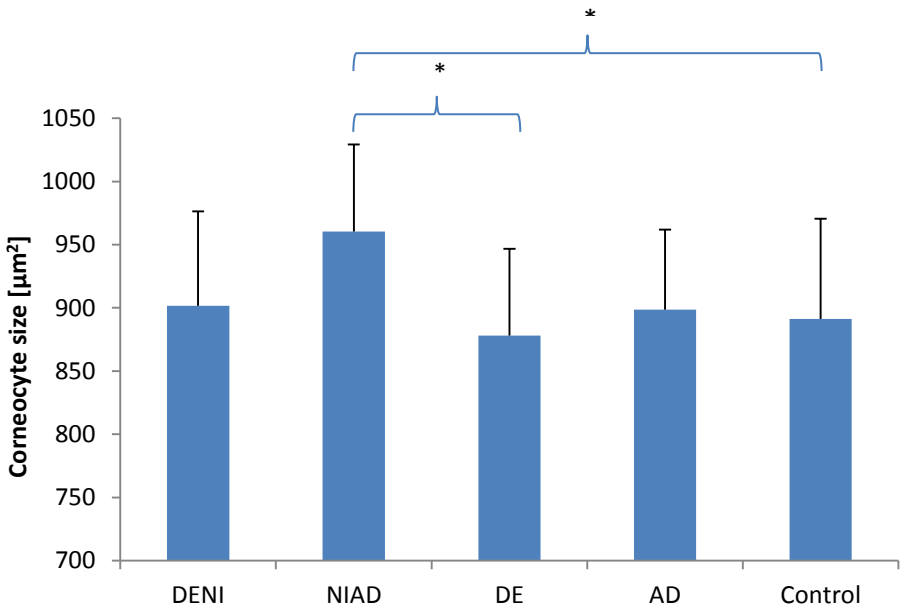

Figure 5.12 Average corneocyte size (data shown as mean ± SEM, n=20, *p<0.05)

5.3.4 Corneocyte maturity

Corneocyte maturity was measured for tape number 1, 5, 9, 13 and 17 in two ways:

- by ImageJ® software calculation of the red/green pixels ratio,
- by visual determination of mature (red) and immature (green) cells and calculation of the red/green cell number ratio.

The results for each site of interest are shown in Figure 5.13. In general, the maturity of corneocytes decreased with the depth of stratum corneum (reflected in the increasing tape number), which is in line with previous findings [53, 54]. A statistically significant difference (one-way ANOVA, $p<0.05$) in cell maturity was found when the NIAD formulation was compared vs. placebo (AD) according to the cell counting method for tape 9. More mature cells were observed for the NIAD formulation. There was a moderate correlation between both methods used to calculate the corneocyte maturity assessed with Spearman's rho 2-tailed test ($r=0.38$, $p=0.000$).

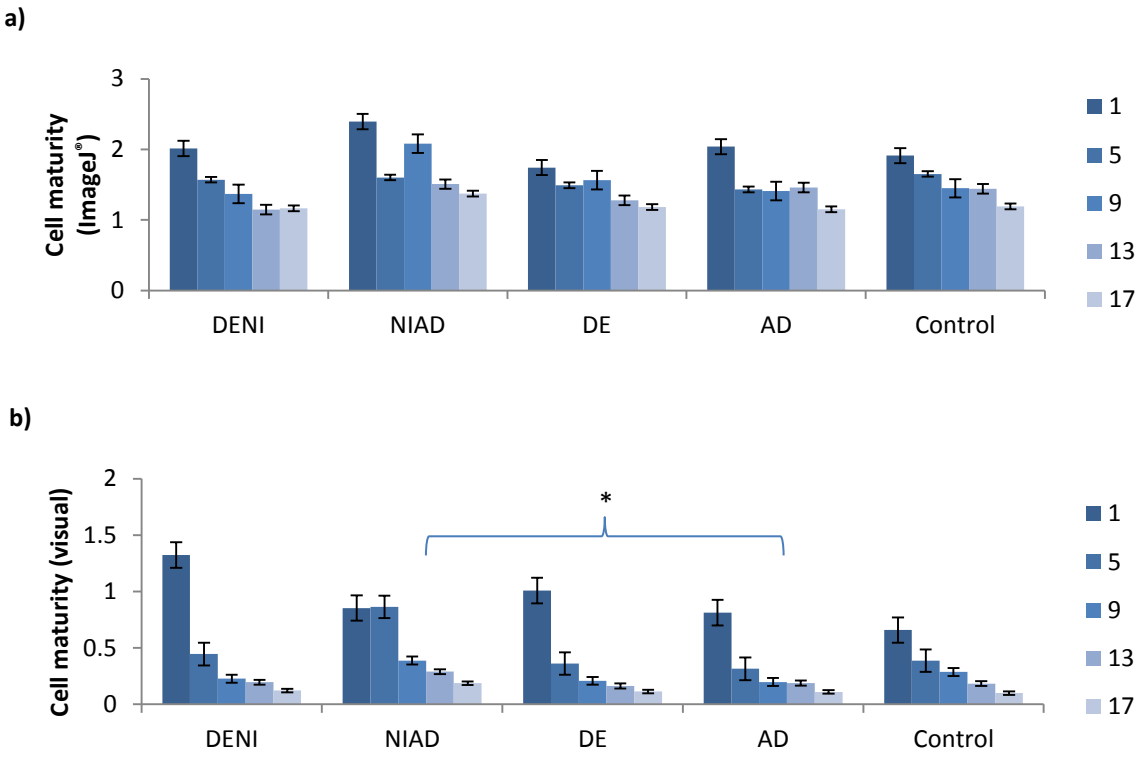

Figure 5.13 Corneocyte maturity a) ImageJ® method, b) visual determination and cell counting method (data shown as mean ± SEM, n=20, *p<0.05)

5.3.5 Protease activity

The activity of four proteases: kalikrein 5 (KLK5), tryptase (TRY), plasmin (PLA) and kalikrein 7 (KLK7) was measured at each of the sites for five pools of three tapes.

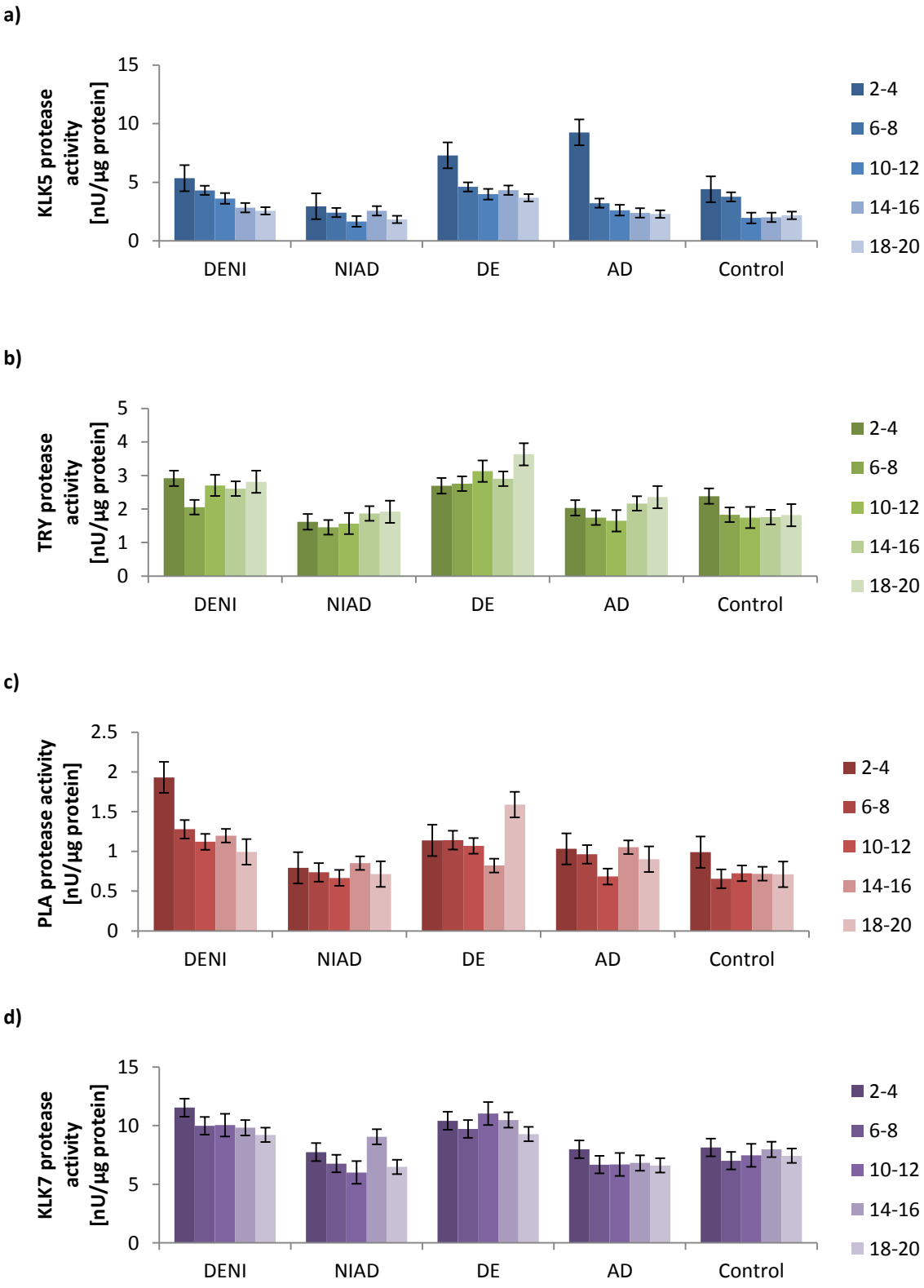

Figure 5.14 Protease activity a) KLK5, b) TRY, c) PLA, d) KLK7 (mean ± SEM, n=20)

The summary of results for each protease is shown in Figure 5.14.

Total PA was calculated (i.e. the sum of the activity in the five pools of tapes taken from one measurement site) and is shown in Figure 5.15. Statistically significant (Kruskal-Wallis, $p<0.005$) differences were found between DENI vs. NIAD and DE vs. NIAD formulations for the total TRY and KLK7 enzyme activities. Lower activities of both enzymes were found for NIAD formulation.

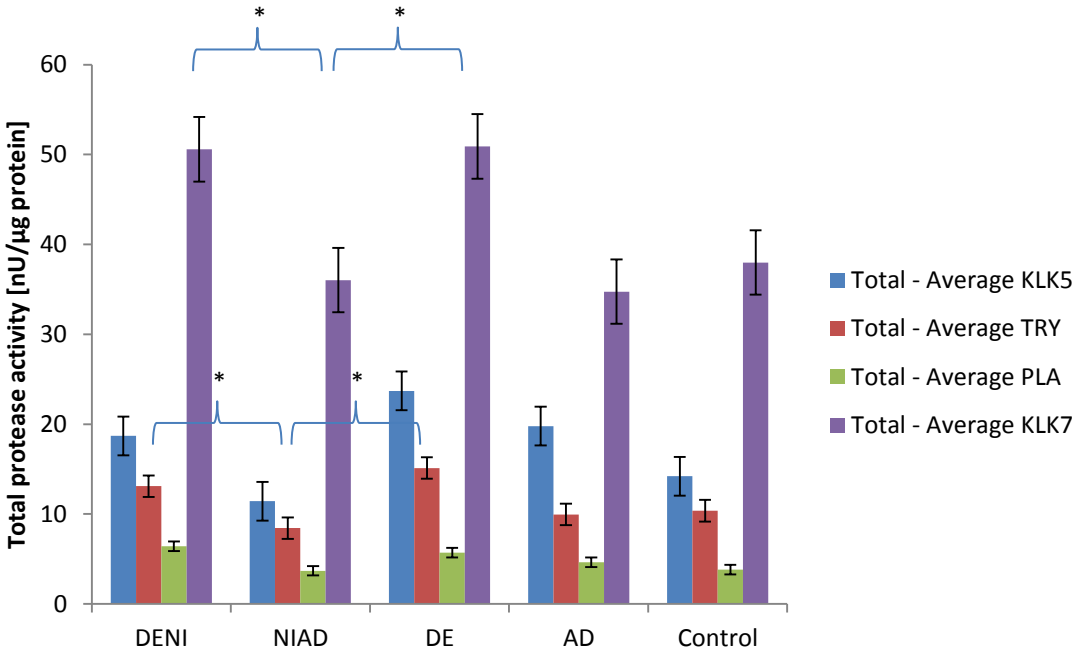

Figure 5.15 Total protease activity (data shown as mean ± SEM, n=20, *p<0.005)

5.3.6 Nicotinamide content

NA was analysed in the extracts from the tapes taken from the skin areas where DENI and NIAD formulations were applied. The extracts of three tapes were pooled together giving five pools for each treatment site per volunteer.

High inter-individual variability was observed for NA content in the tapes with results ranging from 0.010 to 0.741 and from 0.006 to 0.675 µg of NA/µg protein, obtained for DENI and NIAD respectively. Thus, the DENI/NIAD ratio of NA found in the tapes was calculated for each volunteer. The average DENI/NIAD ratio for NA content in the pools of tapes is shown in Figure 5.16. The DENI/NIAD ratio for total NA content obtained in this study was 1.27±0.76 (mean ± SEM, n=20) indicating similarity of DENI and NIAD in terms of NA retention in the outermost layers of the SC.

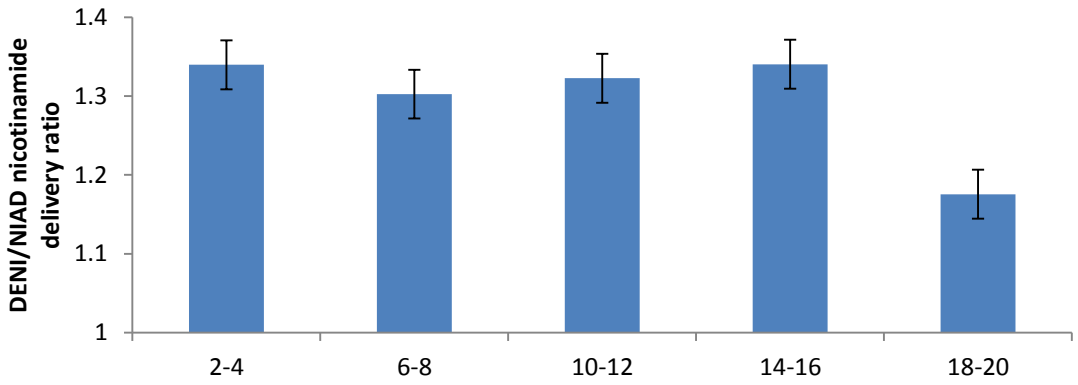

Figure 5.16 DENI/NIAD ratio for total nicotinamide content from pooling of tapes (data shown as mean ± SEM, n=20)

5.4 Discussion

The prototype NA formulations were developed by Dermal, Ltd., UK and were tested *in vivo*. To separate the vehicle effect from the influence of NA on the skin condition, 4% NA was incorporated into two different vehicles. One of the formulations contained mainly water (NIAD), while the other was composed of other excipients, which are commonly used in topical formulations indicated in the treatment of dry skin conditions (DENI). Also placebo formulations, AD and DE respectively, where NA was substituted with purified water, were prepared to give a greater insight into the vehicle influence on the state of the skin. The prototype formulations and their placebos were tested *in vivo* in a healthy volunteer study.

The amount of protein quantified in the tapes is an indicator of skin cohesion [18, 19]. For example, protein content was used to assess the decrease in skin cohesion caused by treatment with a washing emulsion and water [56]. The more cohesive the skin the more difficult it is to strip it with an adhesive tape. Thus, the lower (one-way ANOVA, $p<0.05$) amount of protein found in tapes stripped from the application sites of lipid containing formulations (compared with NIAD) suggests that DENI and its placebo improve skin cohesion. This may be because of the lipophilic components present in these test formulations. Caussin et al. [57] found that lipophilic and hydrophilic moisturisers have different effects on the SC. Lipophilic moisturisers increased skin hydration as opposed to the hydrophilic preparations. In another study, it was shown that lipophilic excipients form separate domains in the SC, which may be the reason for their positive effects on skin [58]. Nevertheless, the lower amount of protein found for tapes taken from DENI and DE treated sites may be also attributed to

changes in the adhesion to the tapes caused by the presence of the lipophilic vehicle. The influence of vehicle on the amount of the SC removed with adhesive tapes was observed by Tsai et al [59] with significantly (one-Way ANOVA, $p<0.05$) higher values obtained for an alcoholic solution when compared with semisolid formulations. Similar results were obtained by Lademann et al. [60] for ethanol-based and oily formulations, with deeper layers of the skin reached after the tape stripping of the skin treated with the first formulation. There was no statistically significant (one-way ANOVA, $p>0.05$) difference in terms of protein content between DENI and its placebo (DE), suggesting that NA present in the first formulation does not influence the amount of protein stripped with the tapes.

Significantly lower average TEWL values (one-way ANOVA, $p<0.05$) were obtained for NIAD when compared with its placebo (AD). This may indicate that NA present in the NIAD formulation has a positive effect on skin integrity, rendering it more robust against water loss and disruption by tape stripping. These results are in line with data obtained by Tanno et al. [61]. In a study on volunteers with dry skin, significantly lower TEWL values were observed after 4 weeks treatment with a NA solution compared with its placebo. NA was found to increase ceramide and free fatty acid levels and TEWL correlated with the level of ceramides. Also, Crowther et al. [40] attributed lower TEWL values to the increase in skin thickness caused by a NA containing moisturiser. On the other hand, Buraczewska et al. [7] found that a polymer hydrogel increased TEWL after a 7 week treatment (when compared with non-treated skin). What is more, the skin treated with this preparation was more prone to damage caused by SLS. In this study, no significant (one-way ANOVA, $p>0.05$) difference between mean TEWL was found between non-treated and AD-treated skin. However, possible skin barrier deterioration caused by AD, which is a simple hydrogel formulation, cannot be excluded. TEWL values at baseline and after stripping of 5 and 10 tapes were higher (although not significantly, one-way ANOVA, $p>0.05$) for AD when compared with the control site. This effect may be reversed by NA present in the NIAD formulation. This is reflected in significantly (one-way ANOVA, $p<0.05$) lower mean TEWL values obtained for NIAD when compared with AD.

Glycerol (GLY) is a humectant commonly used in topical moisturisers. It was shown to accelerate skin barrier recovery [42]. Administered topically, orally and intra-perineurally, GLY improved skin properties of aquaporin-3-deficient mice [62]. However, a significant decrease ($p=0.0252$) in TEWL was found only after 4-weeks treatment with a GLY-containing formulation when compared with placebo in the atopic patient group. Further treatment showed that the two formulations were not different in terms of improving skin integrity and severity of the disease (SCORAD) [63]. Also, no improvement in barrier function after GLY-based moisturiser

treatment was observed by other authors [9, 11, 64]. The combination of GLY and NA was more effective in improving the skin barrier function [40]. Atrux-Tallau et al. [65] found that low (up to 2%) and high (3-10%) doses of glycerol applied after acute chemical skin irritation with sodium lauryl sulphate caused an increase or persistence of high TEWL in damaged skin. Because of its polarity, GLY integrates into the hydrophilic regions of orthorombically packed lipids and increases the distance between the lipid lamellae [66]. This interaction with the SC may be the reason for the penetration enhancing effects of GLY observed for nicotinic acid [67]. However, it may also be responsible for an increased water loss through the skin because of the opening of its structure. Thus, presence of GLY may be the reason for the lack of significant (one-way ANOVA, $p>0.05$) positive effect of NA on TEWL in DENI formulation when compared with other formulations.

Larger corneocytes were found for NIAD treated skin when compared with DE and control sites (one-way ANOVA, $p<0.05$). Holzle and Plewig [31] established that smaller and irregular corneocytes are related to damaged or diseased skin. Also the corneocyte surface area was correlated with the cell maturity [54]. Thus, the results obtained in this study may indicate that NA present in the NIAD formulation slows down the corneocyte turnover time allowing for larger cell formation. However, this potential effect on turnover was not reflected in corneocyte maturity measurements. There was almost no statistically significant difference (one-way ANOVA, $p>0.05$) in this parameter between the sites treated with NA containing formulations and their placebos or control sites.

GLY penetrates the SC and interacts with the hydrophilic regions of the SC lipids [66]. The short- and long-term effects of GLY treatment on the corneocyte size were assessed by previous authors with a modified tape stripping/fluorescence imaging method [14]. There was an increase in the corneocyte size observed 3 hours after GLY treatment, followed by a decrease after 7 hours, persisting up to 12 hours after the treatment. The authors suggested that this is because of the moisture initially brought to the skin surface from the deeper skin layers by GLY and moisture depletion after its evaporation from the skin surface. The study found that in general the surface areas of corneocytes increased after a 9-day glycerol treatment. However, the results obtained in the current study, indicate that after a 21-day treatment GLY, which is present at a relatively high concentration (5%) in DE and DENI formulations, may have a cell-shrinking effect because of its dehydrating influence on the deeper layers of the skin. This effect is only significant (one-way ANOVA, $p<0.05$) when no protective NA is present in the formulation, i.e. for the DE formulation. However, because of the complexity of formulations tested it is difficult to attribute the effect on corneocyte size to

GLY alone. An *in vivo* study with a simple GLY-based formulation performed according to the protocol described for prototype NA formulations may provide evidence of GLY's possible negative effects on corneocyte size.

NIAD treated skin showed lower total activity of TRY and KLK7 proteases (Kruskal-Wallis, $p<0.005$) when compared with the DENI and DE formulations. Desquamation is dependent on the relative humidity of the environment and the desquamatory enzymes activity is related to the water present in the stratum corneum. *In vitro* studies demonstrated that application of 10% GLY increases the activity of stratum corneum chymotrypsin-like enzyme (SCCE) [68]. Also, GLY increased the enzymatic digestion of corneodesmosomes in dry skin conditions [69]. Thus, It may be postulated that NA (found in NIAD formulation) diminishes TRY and KLK7 activity and for the DENI formulation, the decrease in TRY and KLK7 activity caused by NA may be masked by the effect of GLY.

KLK7 was reported to be more active in normal skin when compared with dry skin [70, 71]. At the same time, increased activity of key desquamatory enzymes (KLK5 and KLK7) was found for chemically (SLS) damaged dry skin [72, 73]. Voegeli at al. [37] correlated increased TEWL and decreased epidermal thickness with higher tryptase-like (TRY) protease activity (PA). This is in line with the findings from this study, where lower TRY activity for the skin site treated with NIAD formulation was associated with lower TEWL values for this site and larger corneocyte surface area. This suggests that the decrease in this PA, caused by NA present in the formulation, may improve the skin barrier to water loss.

Similar NA retention in the epidermis obtained for the *in vivo* study (DENI/NIAD ratio of 1.27 ± 0.76, mean ± SEM, $n=20$) is in line with the finite dose *in vitro* permeation studies described in the previous chapter. In the permeation studies, no significant difference (t-test, $p>0.05$) in terms of cumulative amount of NA permeated or retained in the skin after 24 or 8h (for silicone and pig skin, respectively) was found. These results may explain the lack of differences (DENI/NIAD ratio close to 1) between the formulations tested *in vivo*.

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6 Conclusions

6.1 Pre-formulation studies

The first stage of this work was to perform preliminary pre-formulation studies. The potential candidate solvents for the delivery of NA to the skin were chosen from the compounds commonly used in topical and transdermal formulations. Several solvents with a wide range of physicochemical properties were tested. Theoretical solubility parameters of the solvents were established. Based on solubility parameter (δ) calculation, solvents with δ value close to that of the skin (i.e. PGML, PGMC, DPPG and IPM) or nicotinamide (NA) (i.e. PG) were chosen to be investigated further in the *in vitro* studies. NA was stable in these solvents for 48h at 32˚C. Also, to simplify the experimental conditions, solvent systems, which were found to be miscible were tested *in vitro* in the uptake and permeation studies. Incorporation of a maximal amount of NA into the system, thus achieving a maximal thermodynamic activity, was possible after solubility studies were performed.

However, in the real life situation, neat solvents or their liquid mixtures would be rarely applied to the skin as a topical formulation. Additional excipients, such as humectants, gelling agents, stabilizers, antioxidants or preservatives, would be added to formulate the final product. Thus, prototype formulations (DENI and NIAD) containing several additional excipients from these groups were also tested *in vitro*. Since these more complicated formulations are more likely to be applied to diseased skin, than the simple solvent systems, their influence on the parameters indicating the skin condition was tested *in vivo*. Non-invasive and semi-invasive techniques such as TEWL measurements and tape stripping were used.

Knowledge of the basic physiochemical parameters of the formulation components, allowed for a critical analysis of the data. The conclusions from both *in vitro* and *in vivo* studies are described below.

6.2 *In vitro* solvent uptake and NA partitioning studies

Solvent uptake studies into silicone membrane and pig ear stratum corneum were performed. The interaction between the silicone membrane and the chosen solvents was highest for the solvents with δ values close to the δ value of the membrane (IPM, DPPG). The exception was MO, a large molecule which was hardly incorporated into the silicone membrane, despite its δ value matching that of the membrane. In the case of stratum corneum (SC), the uptake of the chosen solvents into this membrane increased with their hydrophilicity (largest for a relatively

small molecule - PG), even though the membrane itself is perceived as a lipophilic one. It can be concluded that for the SC the molecular size is even more important for the solvent-membrane interaction than the δ value.

High sorption of IPM and DPPG into the silicone membrane and low solubility of NA in these solvents was reflected in the high partitioning of NA into this membrane. For SC, where the uptake of the solvent did not correlate with NA partitioning, it seems that high solubility of NA in PG could not be compensated by the uptake of this solvent into the SC and thus a high calculated membrane/solvent partition coefficient of NA was not achieved.

6.3 *In vitro* nicotinamide permeation studies from prototype formulations

The above-mentioned uptake and partitioning results are reflected in the permeation of NA from the prototype formulations. Two prototype NA formulations were developed by Dermal Laboratories Ltd. for the treatment of AD. 4% NA was incorporated into two vehicles: one containing primarily water (NIAD) and the other comprising also of IPM, MO and GLY (DENI). The composition of the vehicles was dictated by the need to separate the influence of NA from the other formulation components on the skin condition *in vivo*. Prior to the *in vivo* testing, *in vitro* experiments were conducted to assess the performance of the prototype formulations in terms of percutaneous absorption of NA.

In vitro studies performed with silicone membrane as a barrier for NA diffusion showed that NA permeated through the lipophilic membrane despite its hydrophilicity. Two prototype formulations tested showed different behaviour when applied as infinite and finite doses. In the infinite dose studies, significant differences (t-test, $p<0.05$) in permeation parameters were found between DENI and NIAD formulations. This may be because of the presence of lipophilic components in the DENI vehicle as opposed to the water-based NIAD formulation. These components may influence the viscosity and thus the diffusion of NA from the formulation.

Evaporation of water, the main solvent used in both formulations, occurred during the finite dose experiments. Thus, less solvent was available for the drug to be in solution and the thermodynamic activity, i.e. the drive for diffusion, increased. This situation was more pronounced for the NIAD formulation, because of its higher water content. Hence, the positive effect of lipophilic components present in DENI was compensated by a greater change in thermodynamic activity observed for NIAD. This may be the reason for the lack of significant (t-test, $p>0.05$) differences between the extent and the rate of NA permeation for both

formulations in the silicone and in the pig ear skin finite dose studies. This change in thermodynamic activity (for both formulations) may explain the higher cumulative amount of NA permeated in the finite dose experiment when compared with the infinite dose study.

To conclude, in the infinite dose studies, NA permeated from the DENI formulation to a larger extent when compared with NIAD. This was possibly because of the presence of lipophilic components in the DENI formulation, which were readily taken up by the silicone membrane (IPM), and which influenced NA thermodynamic activity and formulation viscosity (IPM, MO) and thus favoured NA release from this formulation. However, the prototype formulations were not different in terms of NA silicone and pig ear skin permeation in the finite dose studies. It may be postulated that some compensatory effects, such as water evaporation from the prototype formulations, play an important role in the NA permeation process from these formulations.

Drug retention in the outermost layers of the skin is desirable for topical products. In the finite dose studies conducted both with silicone and pig ear skin as model membranes, little or no NA was found in the membrane at the end of the study. The results obtained for silicone are not surprising, since NA is a hydrophilic compound with a low affinity for lipophilic membranes. Considering the vehicle components used to formulate the prototype products, IPM has a high affinity for the membrane, but its NA retention potential is hindered by low solubility of the drug in this solvent. NA is highly soluble in water but this hydrophilic solvent exhibits a low affinity for the silicone membrane. Similarly, low epidermal and dermal retention of NA was observed in the pig ear studies. This may be attributed to the lack of a solvent in DENI and NIAD vehicles which is able to increase NA affinity for the skin layers.

6.4 *In vitro* nicotinamide permeation studies from simple solvent systems

Since the target layer of the skin for AD is the epidermis, the retention of NA in this skin layer could be improved by implementing a rational approach to the design of NA topical formulations. Also, the complexity of the prototype formulations makes the elucidation of the exact mechanism underlying the NA permeation process difficult. Thus, NA permeation from simple solvent systems was studied.

In the infinite dose silicone membrane permeation studies, systems with δ values close to that of silicone membrane, high uptake into this membrane and low solubilizing potential for NA (i.e. IPM, DPPG and mixtures of IPM with DPPG, PGML and PGMC), achieved the maximal

steady state flux. It may be concluded that, because of the above-mentioned properties, these solvents increased NA diffusion and solubility in the model membrane.

NA is hydrophilic, thus its solubility in lipophilic solvents, such as IPM and DPPG, is low. At the same time, these are the solvents that have a higher potential to penetrate the lipophilic silicone membrane when compared with hydrophilic solvents (such as PG). The highest k_p across the silicone membrane in the infinite dose studies was obtained for IPM (t-test, $p<0.05$). This solvent has a δ value close to the δ value of the silicone membrane (7.5 $cal^{1/2}/cm^{3/2}$) [1]. Also, in the finite dose pig ear skin permeation studies the highest k_p was obtained for IPM (one-way ANOVA, $p<0.05$). No synergistic effects were observed when the selected solvents were used in combinations. This suggests that IPM alone interacts strongly with the skin and addition of other components to the system does not increase this effect. Thus, adjustment of the δ value so that it is closer to the δ value of the drug did not result in an enhanced permeation.

In the finite dose pig ear skin permeation studies, the focus was on establishing the formulation with the best potential to retain NA in the skin layers with the lowest loss of formulation at the end of application as shown by the percentage of NA present at the skin surface at the end of the study.

From the single solvents tested, PG has a high potential to increase the skin retention of NA, however the good solubility of NA in this solvent suggests that a high percentage of the drug is left on the skin surface at the end of 8h permeation study. A better epidermal retention with a lower percentage of the applied dose left on the skin surface at the end of the application period was achieved by using combinations of the chosen solvents. From the solvent systems tested, especially binary mixtures with 10% addition of PG were capable of retaining high NA doses in the skin. What is more, ternary and quaternary solvent systems resulted also in a high epidermal and dermal NA retention. However, the synergistic effect could not be achieved for all systems tested. Optimal adjustment of the δ value of the solvent system could be the reason for the improved retention of NA when the drug was applied in the multicomponent solvent systems. For the epidermis and the dermis, the peak retention was observed at the δ value around 9.7 $cal^{1/2}/cm^{3/2}$, which is in line with the proposed δ value of the skin [2].

In conclusion, IPM was the solvent with the highest NA penetration enhancing potential across silicone membrane and porcine skin. However, solvent systems with a δ value close to 10 $cal^{1/2}/cm^{3/2}$ resulted in the best NA retention in the skin. This effect may be attributed to the increased solubility of NA in the skin, and thus increased partitioning, when the drug is applied

in an appropriate solvent. Thus, adjusting the δ value by using solvent mixtures may be helpful in designing an effective NA formulation which would target the drug to the skin.

6.5 *In vitro* solvents permeation studies

As shown above, the similarity of solvent δ value to that of the skin or model membrane, may be reflected in the drug permeation and its residence in the membrane. To understand this phenomenon, solvent permeation studies were performed together with the permeation of NA.

There were several observations made in the silicone membrane permeation studies. First of all, solvent depletion from the donor compartment caused a decrease in the drug permeation. Secondly, high solvent retention in the silicone membrane was related to a high percentage of NA permeated during the study, indicating that solvent presence is necessary for drug transport across the membrane. Low permeation and low retention of the solvent resulted in low permeation of the drug.

Lipophilic solvents (IPM, DPPG, PGML and PGMC) did not permeate across full thickness pig ear skin, probably because of the presence of the hydrophilic barrier i.e. the layer of dermis. PG was the only solvent that permeated across pig skin and also the highest amount of NA ($p<0.05$, one-way ANOVA) permeated from this solvent when compared with the other solvents tested. Nevertheless, some NA permeated from these solvents because of their presence in the skin layers, again indicating that this might be an important factor for drug permeation. These solvents partition into the skin from the formulation, but not out into the receptor fluid. This increases the residence time of this solvent in the skin, enhancing the permeation.

6.6 Influence of prototype formulations on the skin *in vivo*

The data obtained in the *in vitro* studies suggest that determination of the δ value may be useful for the prediction of the interactions between the solvent and the model membrane, which can be further used to choose the solvents with best penetration enhancing or membrane retention properties. Nevertheless, it is not only the drug, but also the vehicle that influences the state of the skin. The vehicle composition is especially important in the treatment of inflammatory or dry skin disorders. Thus, the performance of the prototype formulations in terms of skin health improvement was investigated *in vivo.*

A healthy volunteer study was performed with prototype NA formulations (DENI, NIAD) and their placebos (DE, AD respectively) applied three times a day for three weeks. The following

differences were found between the formulations tested: a lower total protein content (one-way ANOVA, $p<0.05$) for DENI and DE (compared with NIAD), a lower mean TEWL values (one-way ANOVA, $p<0.05$) for NIAD (compared with AD), a larger mean corneocyte surface area (one-way ANOVA, $p<0.05$) for NIAD (compared with DE and control site), a lower total activity of TRY and KLK7 (Kruskal-Wallis, $p<0.005$) for NIAD (compared with DENI and DE).

It may be concluded that lipophilic components present in two of these formulations (DENI and DE) have a positive impact on skin cohesion. This effect is reflected in the lower (one-way ANOVA, $p<0.05$) amount of protein that was quantified in the tapes stripped from the skin treated with DENI and DE formulations (compared with NIAD formulation devoid of these lipophilic components).

On the other hand, NA containing formulations may positively influence skin integrity as assessed by TEWL measurements. Lower (One-way ANOVA, $p<0.05$) TEWL values were found for the NIAD formulation when compared with its placebo. Also, larger (one-way ANOVA, $p<0.05$) corneocytes were found in tapes stripped from the NIAD treated site (compared with control and DE treated site), indicating an influence of NA present in the formulation on corneocyte size. A correlation between the corneocyte surface area and TEWL was established by previous authors [3, 4]. However, for mean TEWL values and corneocyte area calculated for each volunteer a correlation could not be established in this study, possibly because of the relatively small sample size or because of different effects on epidermal turnover for the formulations studied here.

It may be also postulated that NA diminishes TRY and KLK7 activity. These enzymes were significantly less active (Kruskal-Wallis, $p<0.005$) for the NIAD formulation than for the DENI formulation and its placebo (DE). It may be speculated, that the absence of any effect of NA on cell size, TEWL and PA for the skin site treated with DENI formulation may be because of the dehydrating effect of GLY present in this vehicle in a relatively high concentration [5-8]. Investigating the effect of GLY alone on skin hydration, corneocyte size and PA *in vivo* may confirm this. Nevertheless, GLY is not the only component in the prototype NA formulations and incorporation of other components into the vehicle may also influence the skin.

The effect of NA on human skin has been previously reported by many authors [9-22]. Nevertheless, the results obtained in this study only partially confirm the ability of prototype NA formulations to influence positively the state of the skin. Simplifying the experimental conditions by the use of simple solvent systems may enable separation of the vehicle influence on skin state from the impact made by NA.

The only statistically significant difference (one-way ANOVA, $p<0.05$) between treated and control sites, was found for the size of corneocytes when control and NIAD application sites were compared. This shows that healthy skin, subject to no pre-treatment with damaging agents, is a self-regulating environment, difficult to influence by topically applied formulations. An *in vivo* study on atopic patients may provide stronger evidence of the influence of the formulations tested on the skin.

The data from this *in vivo* study are presented as a function of the tape number. However, in light of a recent publication where the spectroscopic and gravimetric determinations of the SC protein in the tapes were correlated [23], the results may also be shown as a function of the SC depth reached in the tape stripping procedure. Nevertheless, the complication in data analysis caused by different depths obtained for each volunteer cannot be ignored.

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7 Future work

Pre-formulation studies were conducted to obtain basic information about the optimal formulation components. What followed were the *in vitro* studies evaluating simple solvent systems in terms of uptake and permeation of both nicotinamide (NA) and excipients. Also, *in vitro* and *in vivo* studies were performed with prototype topical NA formulations intended for atopic dermatitis (AD) treatment.

Next, the simple solvent systems could be formulated into semisolid forms and their permeation and influence on skin state could be tested *in vivo* and *in vitro*. This would allow for their comparison with the prototype formulations containing a minimal amount of penetration enhancing solvents.

The prototype formulations influence on atopic patients' skin could be evaluated in a clinical trial using both AD scoring systems such as SCORAD and objective instrumental skin evaluation methods such as TEWL to determine the endpoints.

Finally, in order to understand the effect of NA on skin health, other biomarkers in skin should be considered, for example enzymes involved in processing of filaggrin and production of NMF such as bleomycin hydrolase and calpain 1.

A Appendices

A.1 Optimisation and validation of HPLC method for nicotinamide assay

HPLC was chosen as an appropriate analytical method for nicotinamide (NA) assay in the samples obtained during this project. The HPLC system parameters, optimal analysis conditions and equipment used in analysis are listed below.

Table A.1 Parameters of the HPLC method for nicotinamide quantification

Column	**Primesep A 250x4.6mm, Particle 5µm, 100Å** Part N° A-46.250.0510, Serial N° PALLCDN9 Lot N° 015-070B (SIELC Technologies, USA)
Guard column	**SecurityGuard Cartridge System with C18 (ODS, Octadecyl) 4mmLx3mm filter (Phenomenex, USA)**
HPLC System I	**Agilent Technologies 1100 Series HPLC system:** Degasser G1322A Serial N° JP63203343, Quat Pump G1311A Serial N° DE52700795, ALS G1313A Serial N° DE23920375, Col. Comp. G1316A Serial N° DE53401012, DAD 61315B Serial N° DE11113362;
HPLC system II	**Agilent Technologies 1200 Series HPLC system:** Degasser G1322A Serial N° JP73062725, Quat Pump G1311A Serial N° DE62965070, ALS G1329A Serial N° DE64766771, TCC G1316A Serial N° DE63068585, VWD 61314B Serial N° DE71361620;
Software for chromatograms analysis	**ChemStation for LC Systems software:** Copyright© Agilent Technologies 1990-2002 or 2001-2008, Rev. A.09.03[1417] or B03.02[341]
Mobile phase	**Mobile phase A (80):** 0.07% Perchloric Acid in Deionised Water (1mL of 70% Perchloric Acid in 1L), pH 2 **Mobile phase B (20)**: Acetonitrile (HPLC grade)
Injection volume	10µL
Flow rate	2 mL/min
Temperature control	40°C
UV detection wavelength	263nm
Retention time of nicotinamide	~9.5min
Analysis time	~15min
Calibration curve range	2-200µg/mL (6 standards of the concentrations of 2; 10; 20; 50; 100; 200µg/mL of nicotinamide prepared from a stock solution of a concentration of 1000µg/mL by dilution with PBS)

A.1.1 Optimisation of the analysis conditions - system suitability testing

According to the EMEA ICH guidelines, system suitability testing should be included in the process of analytical method development and the parameters to be established during this procedure depend on the type of analytical method that is validated [1]. The CDER *Reviewer Guidance; Validation of Chromatographic Methods* [2] gives detailed recommendations on this subject and this document was followed in the process of method development and optimisation.

The terms used for system suitability parameters calculations are listed below.

W_x - width of the peak determined at either 5% ($W_{0.05}$), 10% ($W_{0.1}$) or 50% ($W_{0.5}$) of the peak height (from the baseline),

f - distance between peak maximum and peak front at W_x,

t_0 - elution time of the void volume or a component that is not retained by the column,

t_R - retention time of the analyte,

t_W - peak width of straight sides of the peak extrapolated to the baseline measured at baseline.

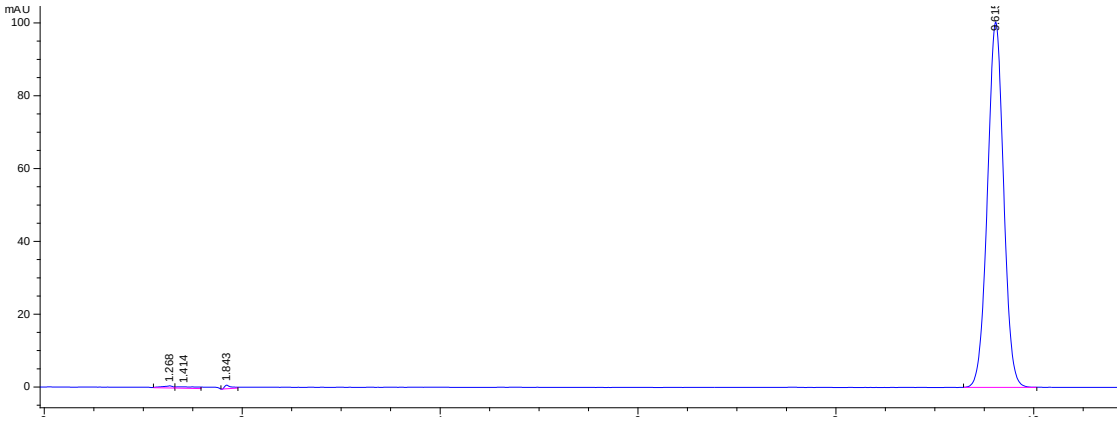


Figure A.1 Representative chromatogram of a standard sample used for the validation of HPLC method for nicotinamide assay (standard prepared in PBS at a concentration of 100µg/mL with the use of reference standard chemical compound [Nicotinamide EP])

The representative chromatogram of a standard sample is shown in Figure A.1 and the terms determined from this chromatogram are listed in Table A.2.

Table A.2 The terms for system suitability parameter calculations defined from the chromatogram of a standard sample

Term	Value [min]
$W_{0.05}$	0.36
$W_{0.1}$	0.32
$W_{0.5}$	0.16
$f_{0.1}$	0.16
t_0	1.843
t_R	9.615
t_W	0.72

A.1.1.1 Capacity factor (k')

Capacity factor measures where the peak, which represents the analysed compound is located with respect to the elution time of the non-retained components. A method of capacity factor calculation is presented below.

$$k' = \frac{t_R - t_0}{t_0}$$

Equation A.1

Capacity factor was calculated for the representative chromatogram of a standard sample which is shown in Figure A.1. The peak is well-resolved from the signal that comes from the non-retained components of the sample and the value of k' is above 2 ($k' > 2$), which is in agreement with CDER recommendations. The results are shown in Table A.4.

A.1.1.2 Precision/injection repeatability (RSD)

Injection repeatability indicates the performance of the HPLC system used for analysis at the time the samples are analysed. It is recommended that injection precision expressed as relative standard deviation (RSD) for at least five injections (n ≥ 5) of the same sample should be ≤ 1%. For the calculation of this parameter five consecutive injections of the same standard sample (standard prepared in PBS at a concentration of 100µg/mL with the use of reference standard chemical compound [Nicotinamide EP]) were performed and the RSD of the peaks areas and peak retention time was calculated according to the following equations:

$$RSD = \frac{SD}{Average\ peak\ area} 100$$

Equation A.2

$$RSD = \frac{SD}{Average\ retention\ time} 100$$

Equation A.3

Where:

RSD - Relative Standard Deviation

SD - Standard Deviation

The value obtained for analysed samples is in agreement with CEDR recommendations. The results are shown in Table A.3 and summarised in Table A.4.

Table A.3 Injection repeatability for a standard sample of a concentration of 100 µg/mL of nicotinamide

Injection	1	2	3	4	5	Average	SD	RSD [%]
Peak area	1129.2	1139.3	1137	1136.4	1138.2	1136.02	3.97	0.35
Retention time	9.54	9.62	9.62	9.61	9.61	9.60	0.03	0.36

A.1.1.3 Tailing factor (T)

The peaks obtained on the HPLC chromatogram should be as sharp as possible because the accuracy of quantification decreases with increase in peak tailing. If the peak is tailing it is difficult for the integrator to determine the boundaries of the peak and hence the calculation of the area under the peak is hindered. Integrator variables were pre-set for optimum calculation of the area for the peak of interest. The peak tailing factor was calculated for the representative chromatogram of standard sample according to the equation below and compared with CDER the recommendations ($T \leq 2$). The tailing factor obtained for representative chromatogram falls within the recommended values.

$$T = \frac{W_x}{2f}$$

Equation A.4

A.1.1.4 Theoretical number of plates (N)

The theoretical number of plates is a measure of column efficiency, i.e. how many peaks can be located per unit run-time of the chromatogram. Parameters which can affect the plate number include peak position, particle size of the column packing, mobile phase flow-rate and viscosity, the temperature of the column and the molecular weight of the compound analysed. Theoretical number of plates calculation utilised in the evaluation of the HPLC system for NA analysis is shown in the equation below.

$$N = 16\left(\frac{t_R}{t_W}\right)^2$$

Equation A.5

N depends on elution time but it is recommended that the value should be > 2000. The value calculated for the representative chromatogram meets this requirement.

A.1.1.5 Summary of the system suitability testing procedure

According to CDER recommendations for system suitability testing as a way of assuring the quality of chromatographic system performance, the minimum parameters to be tested are k', RSD and T for dissolution or release profile test methods. In the case of the HPLC method of NA assay additionally, the N parameter was determined. All the parameters tested meet the requirements set by the CDER *Reviewer Guidance, Validation of Chromatographic Methods [2]*. Because no compounds other than NA were analysed in one sample and one HPLC run, the resolution parameter was not calculated. The summary of system suitability test parameters is shown in Table A.4.

Table A.4 Summary of system suitability test parameters for HPLC method for nicotinamide assay

Parameter	Value calculated for the standard chromatogram	CDER recommended value
Capacity factor (k')	4.22	k' > 2
Injection repeatability (RSD)	Peak area: 0.35%, Retention time: 0.36%, n = 5	RSD ≤ 1%
Tailing factor (T) from $W_{0.5}$	1	T ≤ 2
Theoretical number of plates (N)	2853	N > 2000

A.1.2 Validation of the HPLC method for nicotinamide assay

The validation of the HPLC method for NA quantification was performed on the basis of the ICH *Note for Guidance on Validation of Analytical Procedures: Text and Methodology* (CPMP/ICH/381/95) issued by the European Medicines Agency [1]. According to this guideline the following validation characteristics should be considered when evaluating the assay of a compound of interest:

- Accuracy,
- Precision (Repeatability),
- Specificity,
- Linearity,
- Range.

Additional parameters, not perceived by the EMEA as mandatory for the validation of assay of a compound of interest include:

- Detection Limit,
- Quantitation Limit,
- Intermediate Precision,
- Reproducibility,
- Robustness.

A.1.2.1 Calibration curves

During the validation process calibration curves were obtained on each day of validation by HPLC analysis of each standard (freshly prepared on the day) performed at least in triplicate. The average areas of the peaks obtained for each standard were plotted against the concentrations of the standards. A calibration curve equation was determined using the method of least squares. Table A.5 contains the representative data used to plot the calibration curve, which is shown in Figure A.2.

Table A.5 Data obtained from HPLC analysis of the standards, used to calculate the calibration curve equation (see Figure A.2)

Injection	1	2	3	Average	SD	RSD [%]
Standard concentration [µg/mL]	Peak area					
200	2260.1	2264	2275.2	2266.4	7.84	0.35
100	1139.3	1137	1136.4	1137.6	1.53	0.13
50	569.7	566.2	567.2	567.7	1.80	0.32
20	227.1	228.5	225.9	227.2	1.30	0.57
10	114.7	115.1	114.7	114.8	0.23	0.20
2	23	22.5	22.7	22.7	0.25	1.11
0.2	2.1	2.3	2.3	2.2	0.12	5.17

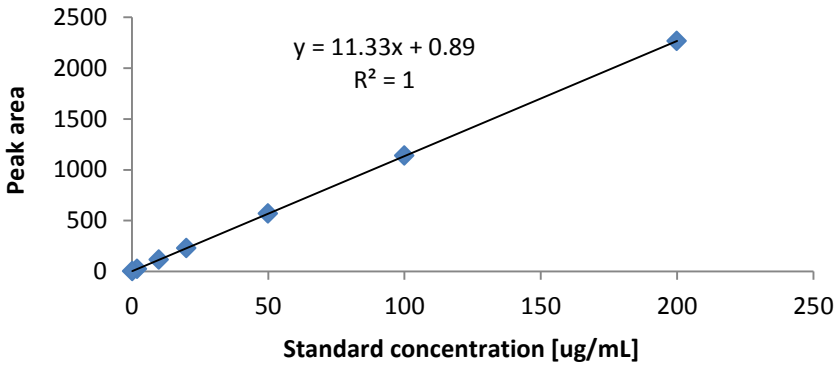


Figure A.2 Calibration curve plotted from the data listed in Table A.5

The following characteristics of the HPLC method for assay of NA were evaluated and compared with the ICH *Guidance* recommendations.

A.1.2.2 Specificity

The specificity of the analytical method is the ability to assess without any doubt the compound of interest in the sample that is analysed. It means that the presence of other chemicals from the sample matrix or introduced during the sample preparation do not affect the performance of the analysis.

To assure specificity of the HPLC method which is being validated two representative chromatograms provided in Figure A.3 and Figure A.13 were compared. No interference is expected in the analysis from the matrix present in the sample, since the blank sample gives a steady baseline reading throughout the analysis time, with no peaks visible at the retention time of NA. The discrimination between the blank and standard sample is easy to perform as can be seen in the examples shown below. The analyte shows no interference from other sample components.

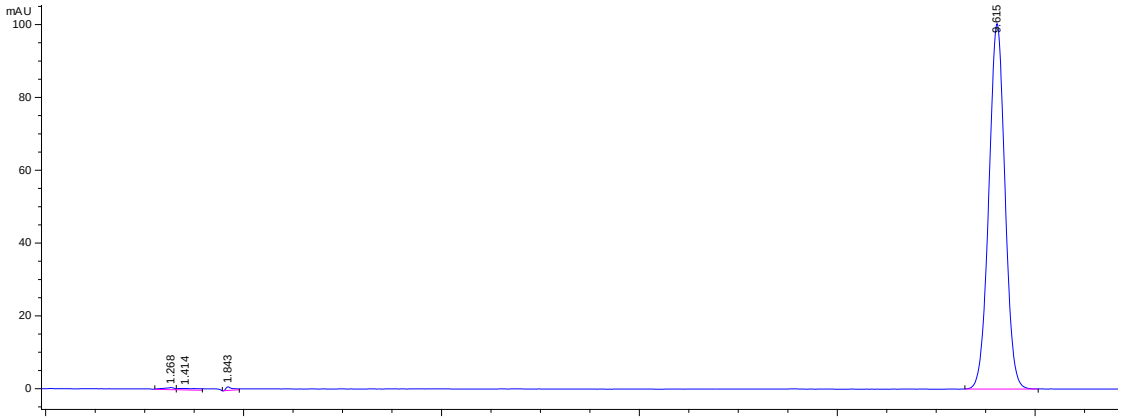


Figure A.3 Chromatogram of a standard sample used for optimisation and validation of HPLC method for nicotinamide assay

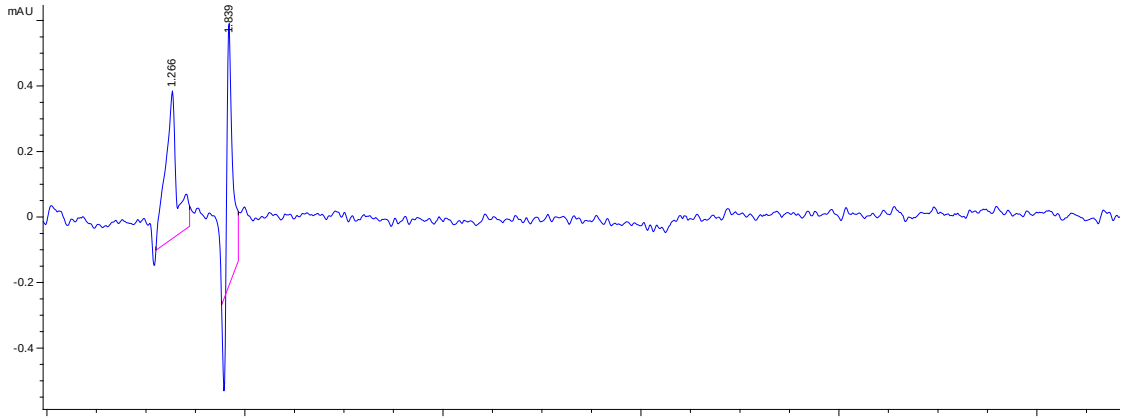


Figure A.4 Chromatogram of a blank sample (no drug added) used for optimisation and validation of HPLC method for nicotinamide assay

A.1.2.3 Accuracy

Accuracy measures the closeness of the experimental value to the 'true' value and should be established across the specified range of the analytical method. According to both the EMEA and FDA guidelines accuracy should be assessed using a minimum of 3 sample concentration, each analysed in triplicate. For the purpose of this validation accuracy has been established for all the standards analysed. Each standard was analysed in triplicate on the same day. Data from the HPLC chromatograms used for the purpose of accuracy determination are shown in Table A.6.

Table A.6 Peak areas obtained from the chromatograms of nicotinamide standard samples

Injection	1	2	3	Average	SD	RSD [%]
Standard concentration [µg/mL]	Peak area					
200	2260.1	2264	2275.2	2266.4	7.84	0.35
100	1139.3	1137	1136.4	1137.6	1.53	0.13
50	569.7	566.2	567.2	567.7	1.80	0.32
20	227.1	228.5	225.9	227.2	1.30	0.57
10	114.7	115.1	114.7	114.8	0.23	0.20
2	23	22.5	22.7	22.7	0.25	1.11
0.2	2.1	2.3	2.3	2.2	0.12	5.17

The calculated recovery values from the calibration curve obtained on the day of analysis (calibration curve 1) and on the next day (calibration curve 2) are listed in Table A.7 and Table A.8 and plotted in Figure A.5 and Figure A.6 respectively.

Table A.7 On-the-day accuracy of HPLC method for nicotinamide assay

Injection	1	2	3	Average	SD	RSD [%]
Standard concentration [µg/mL]	Calculated concentration [µg/mL]					
200	199.30	199.65	200.64	199.86	0.69	0.35
100	100.43	100.23	100.17	100.28	0.14	0.13
50	50.18	49.87	49.96	50.00	0.16	0.32
20	19.96	20.08	19.85	19.96	0.11	0.58
10	10.04	10.08	10.04	10.05	0.02	0.20
2	1.95	1.91	1.92	1.93	0.02	1.15
0.2	0.11	0.12	0.12	0.12	0.01	8.63

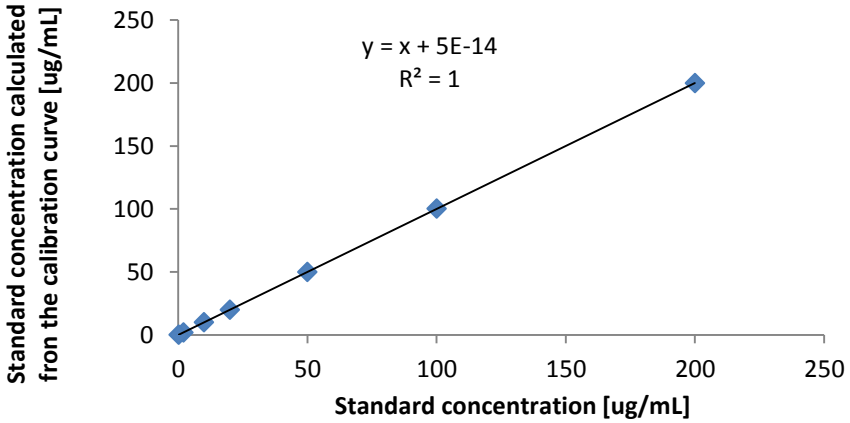


Figure A.5 On-the-day accuracy of HPLC method for nicotinamide assay

Table A.8 Between-days accuracy of HPLC method for nicotinamide assay

Injection	1	2	3	Average	SD	RSD [%]
Standard concentration [µg/mL]	Calculated concentration [µg/mL]					
200	201.86	202.21	203.21	202.42	0.70	0.35
100	101.73	101.52	101.47	101.57	0.14	0.13
50	50.84	50.53	50.62	50.66	0.16	0.32
20	20.23	20.36	20.12	20.24	0.12	0.57
10	10.19	10.23	10.19	10.20	0.02	0.20
2	2.00	1.95	1.97	1.97	0.02	1.14
0.2	0.13	0.15	0.15	0.14	0.01	7.27

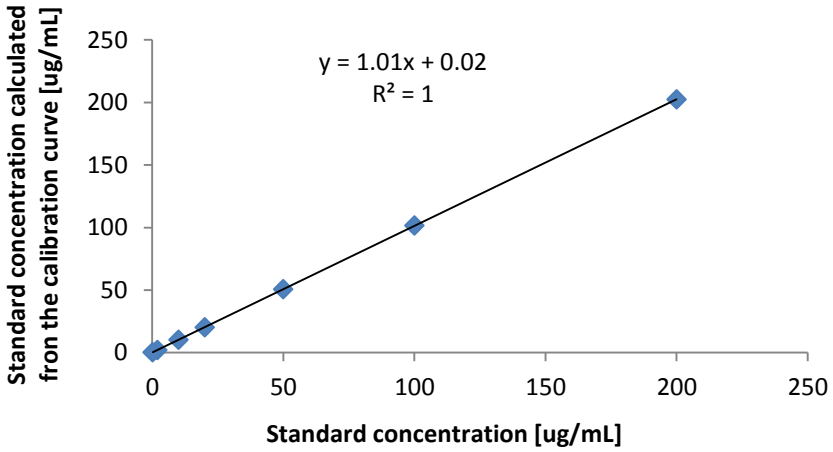


Figure A.6 Between-days accuracy of HPLC method for nicotinamide assay

An ideal accuracy curve (an example of which is shown in Figure A.5) should follow the linear equation $y = ax + b$ and the slope (a) value should be as close to 1 as possible with the intercept value (b) approaching 0. The kinearity of the curve is expressed as the R^2 value, which should be close to 1. Figure A.5 and Figure A.6 illustrate that the validated HPLC method complies very well with these criteria. Accuracy can be expressed as the difference between the mean and the accepted true value. It provides information on the systematic error of the method. For the whole range of concentrations the difference should not be higher than 10% (at the same day) or 15% (between days).

$$\%\ Accuracy = \frac{C_x - C_{st}}{C_{st}}$$

Equation A.6

Where:

C_x - Concentration calculated from the calibration curve

C_{st} - True concentration of a standard

The accuracy of the HPLC method subject to validation was calculated with the equation above and summarised in Table A.9 and Table A.10.

Table A.9 Summary of on-the-day accuracy of the HPLC method for nicotinamide assay

Injection	1	2	3	Average	SD	RSD [%]
Standard concentration [µg/mL]	% Accuracy					
200	-0.35	-0.18	0.32	-0.07	0.35	-506.10
100	0.43	0.23	0.17	0.28	0.14	48.93
50	0.36	-0.26	-0.08	0.01	0.32	5383.22
20	-0.22	0.40	-0.75	-0.19	0.57	-297.97
10	0.40	0.75	0.40	0.52	0.20	39.52
2	-2.49	-4.70	-3.82	-3.67	1.11	-30.24
0.2	-46.83	-38.01	-38.01	-40.95	5.09	-12.44

Table A.10 Summary of between-days accuracy of the HPLC method for nicotinamide assay

Injection	1	2	3	Average	SD	RSD [%]
Standard concentration [µg/mL]	% Accuracy					
200	0.93	1.10	1.60	1.21	0.35	28.90
100	1.73	1.52	1.47	1.57	0.14	8.70
50	1.68	1.05	1.23	1.32	0.32	24.40
20	1.16	1.78	0.62	1.19	0.58	49.00
10	1.90	2.25	1.90	2.01	0.21	10.24
2	-0.14	-2.37	-1.48	-1.33	1.12	-84.41
0.2	-35.00	-26.07	-26.07	-29.04	5.16	-17.76

The majority of the ranges of concentrations complies with the recommended limits. Only the readings for the lowest concentrations give higher percentage of recovery, when calculated from the calibration curve, which is typical for HPLC methods. This concentration is considered as a detection limit for the method.

A.1.2.4 Precision

Precision represents the closeness of the values obtained for a series of measurements of the same sample under the same conditions. In other words, it presents the degree of scatter between the readings. The more similar the results obtained, the more precise is the method. Precision increases with the concentration of the analyte, thus it provides information on the random errors. Usually the precision of a method is expressed as variance, standard deviation (SD) or relative standard deviation in % (RDS)

The European and United States guidelines divide the term of precision into three different levels:

- Repeatability - i.e. precision under the same conditions and during the same day of analysis, also termed intra-assay precision or same-day precision.
- Intermediate precision - evaluates variations within the same laboratory: different days, different equipment, and different analysts. Determination of this characteristic of the method assures that the results obtained with this method will be the same for the similar samples after method is developed.
- Reproducibility - is the precision between laboratories and if intermediate precision is established it is not necessary to include this characteristic in the method validation.

For the purpose of this validation repeatability and intermediate precision were established on the basis of EMEA and FDA recommendations.

Table A.11 Peak areas obtained from chromatograms of three standards analysed in triplicate used to determine repeatability of HPLC method expressed as SD and RSD

Injection	1	2	3	Average	SD	RSD [%]
Standard concentration [µg/mL]	Peak area					
200	2260.1	2264	2275.2	2266.4	7.84	0.35
50	569.7	566.2	567.2	567.7	1.80	0.32
10	114.7	115.1	114.7	114.8	0.23	0.20

Repeatability was assessed by injection of three standards of high, medium and low concentration and each standard was analysed in triplicate. For the whole range of concentrations RSD should not be higher than 10%. Table A.11 summarises the results of repeatability determination. All the values meet the criteria stated in the guidelines.

Intermediate precision was assessed for two separate occasions: between two consecutive days and between two HPLC chromatographs of the same brand. Three standards of high,

medium and low concentrations were analysed in triplicate and the precision of the readings from each assay was compared in terms of SD and RSD [%]. Table A.12 and Table A.13 summarise the results, which are in agreement with recommendations, i.e. RSD should not exceed 15%.

Table A.12 Intermediate precision between days (the same chromatograph, different day)

Injection	1	2	3	Average	SD	RSD [%]
Standard concentration [µg/mL]	Peak area					
200	2230.9	2238.6	2242.1	2237.2	5.73	0.26
50	561.3	563.2	562.2	562.2	0.95	0.17
10	112.1	111.5	111.9	111.8	0.31	0.27

Table A.13 Intermediate precision between instruments (different HPLC chromatograph)

Injection	1	2	3	Average	SD	RSD [%]
Standard concentration [µg/mL]	Peak area					
200	2390.6	2414.3	2413	2406.0	13.32	0.55
50	600.8	601.4	601.2	601.1	0.31	0.05
10	117	117.3	117.2	117.2	0.15	0.13

A.1.2.5 Linearity

Linearity is the ability of the analytical method (in the defined range) to give a response proportional to the concentration of the analyte in the samples. It is the range of the calibration curve, where the signal exhibits a linear correlation with the concentrations of the standards. If the correlation is linear the results could be calculated with the use of statistical methods such as the least squares regression. The linear range of HPLC methods with UV detection established according to Beer's Law is dependent on the analyte and the detector. All the samples analysed should fall in the linear range, hence the linearity of the method is established by the analysis of at least 5 standards of different concentrations, which represent the range of 50-150% (sometimes 0-200%) of the expected values of the samples to be analysed [3]. To establish linearity of the method three calibration curves obtained during validation process were plotted by calculation of a regression lines by the method of least squares. Data from the regression lines were used to assess the linearity. The squared correlation coefficients (R^2), y-intercept (b) and slope (a) of each regression line were compared. The slope of calibration curve is the measure of method sensitivity - the higher the

value of the slope the higher the sensitivity of the method. RSD of the slope values for calibration curves should not be higher than 15%. In practice R^2 should not be less than 0.999. These data are summarised in Table A.14 and a representative calibration curve is shown in Figure A.7. In general, all the calibration curves have low SD and RSD values for determined slopes and the R^2 value falls within the recommended limit.

Table A.14 Summary of linearity evaluation of the method

	Slope	Intercept	R^2
Calibration curve 1	11.34	0.89	1.00
Calibration curve 2	11.19	0.64	1.00
Calibration curve 3	11.29	-0.75	1.00
Average	11.27	0.26	1.00
SD	0.07	0.89	0.00
RSD [%]	0.64	337.34	0.00

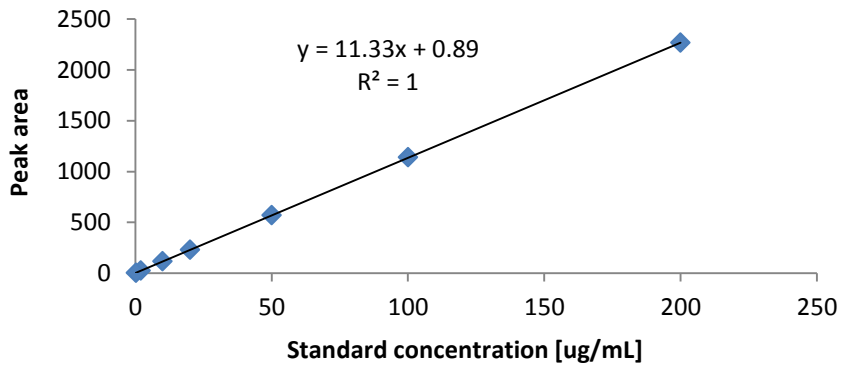


Figure A.7 Representative calibration curve for linearity of the method evaluation

A.1.2.6 Range

The range of the analytical method is defined as the interval between the upper and lower concentration of the sample for which the method is validated. This parameter is derived from the linearity of the method. It depends on the purpose of the assay and is established by confirming that the analytical method is linear, accurate and precise within the specified range.

The working range of the validated HPLC method for NA assay was established to be 2-200µg/mL (16.35-1635.32nmol/mL) and the linearity in this range is illustrated in Figure A.7.

A.1.2.7 Detection limit

The detection limit is the lowest amount of a compound of interest in a sample which can be detected, but not necessarily quantified. There are several ways for determination of this parameter and for the purpose of this validation one was chosen. Based on the visual evaluation of the chromatograms and the ability of the integrator to quantify the signal, the lowest concentration (0.2µg/mL) was taken as the detection limit for the validated method. A relevant chromatogram illustrating the detection limit of the method is presented in Figure A.8.

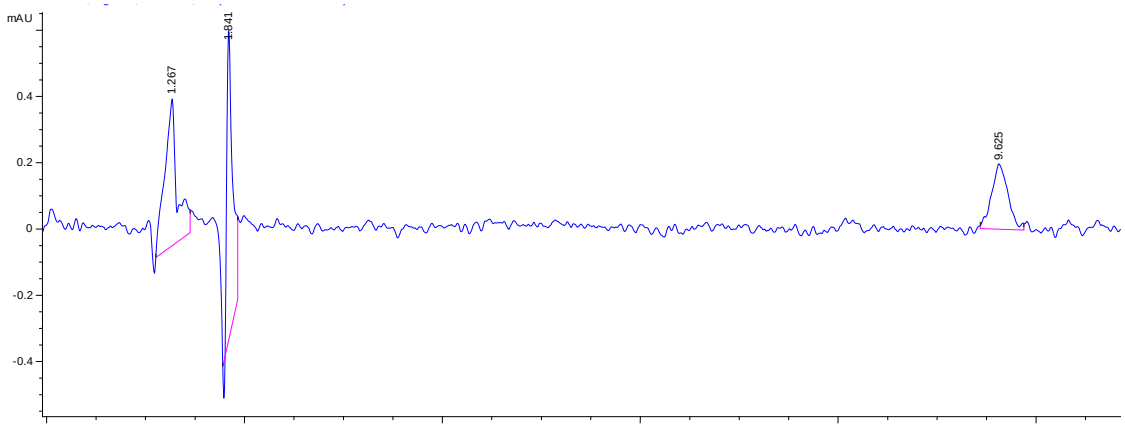


Figure A.8 Representative chromatogram of a standard sample of a concentration of 0.2µg/mL of nicotinamide

A.1.2.8 Quantitation limit

The quantitation limit is the lowest amount of a compound of interest in a sample which can be quantified with suitable precision and accuracy. As with the detection limit, there are several ways of determination of this characteristic and for the purpose of this validation one was chosen. Based on the visual evaluation of the chromatograms and the data obtained during the whole validation process a concentration of 2µg/mL was taken as a quantitation limit for the validated method. An example of a relevant chromatogram is presented in Figure A.9.

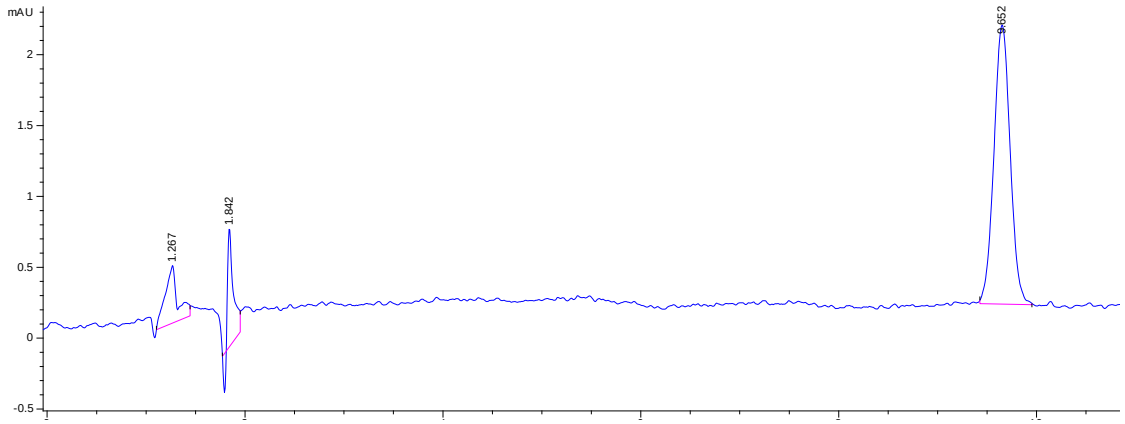


Figure A.9 Representative chromatogram of a standard sample of a concentration of 2µg/mL of nicotinamide

A.1.2.9 Robustness

The ability of a method to remain unaffected by small variations in analytical parameters is very important. Robustness indicates that the method is reliable during its normal use. For this work the robustness of the validated HPLC method was assured by good system suitability specifications. For the purpose of this validation the robustness of the method was checked using two parameters:

- Sample solution stability (samples in PBS),
- Change of solvent used to prepare the samples (samples in the mobile phase).

Sample solution stability was evaluated as an important parameter for the future use of the method. Three standards of high, medium and low concentrations were prepared on the first day of validation and samples of them were kept in two different environmental conditions (in the fridge in 4°C and in the room temperature) and analysed on the day of preparation, after 1, 2, 4, 7 and 14 days. The concentration of each sample was calculated using a calibration curve prepared on the day of analysis. The calculated concentration values obtained after different sample treatments are summarised in Table A.15 and Table A.16.

Table A.15 Results of stability of the standard solutions studies for the samples kept at 4°C

Day after standard preparation	**0**	**1**	**5**	**7**	**14**
Nominal standard concentration [µg/mL]	**% of initial standard concentration**				
200	99.91	99.05	99.47	101.80	97.26
50	100.34	99.57	99.97	101.94	98.22
10	99.33	100.15	100.59	101.51	98.72

Table A.16 Results of stability of the standard solutions studies for the samples kept at room temperature

Day after standard preparation	0	1	5	7	14
Nominal standard concentration [µg/mL]	% of initial standard concentration				
200	99.91	99.47	98.23	99.22	65.58
50	100.34	99.37	96.81	93.90	25.91
10	99.33	99.36	83.83	64.59	0.00

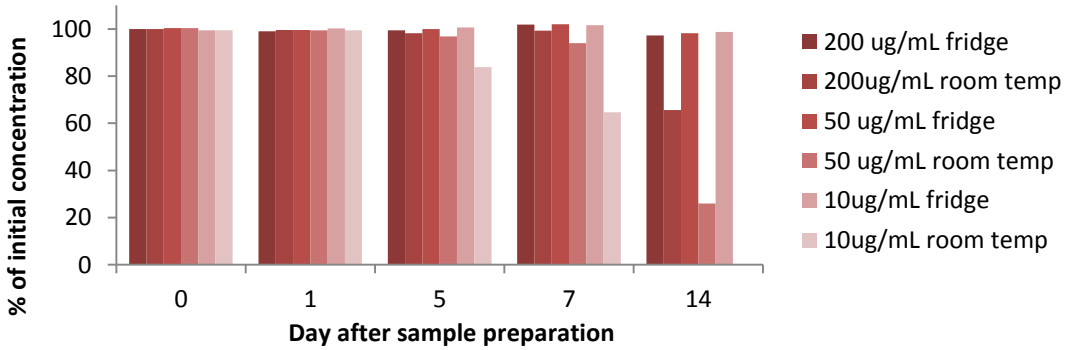


Figure A.10 Stability of standards in PBS - % of the initial sample concentration for 200, 50 and 10µg/mL standards kept at room temperature (~25°C) and in the fridge (~4°C)

The results shown above confirm that the samples are stable in the solvent used for their preparation for 14 days when kept at 4°C but not stable at room temperature (approximately 25°C), which implies, that samples should be analysed on the day of their acquisition or kept in the fridge for no longer than 14 days.

The method robustness with respect to the change of solvent used to prepare standard and samples was evaluated by analysing three standards of high, medium and low concentrations prepared in the mobile phase. Standard concentration values were determined using a calibration curve prepared in PBS on the day of analysis. The results are shown in Table A.17 and

Table A.18 details the method robustness to the change of solvent used to prepare samples.

Table A.17 Peak areas obtained from the assay of the standards prepared in the mobile phase used for determination of method robustness

Injection	1	2	3	Average	SD	RSD [%]
Standard concentration [µg/mL]	Peak area					
200	2262.2	2260	2270.3	2264.17	5.42	0.24
50	561.5	561.4	557.8	560.23	2.11	0.38
10	112.4	111.8	112.1	112.10	0.30	0.27

Table A.18 Concentrations calculated for the peak areas shown in Table A.17 from the calibration curve obtained on the same day

Injection	1	2	3	Average	SD	RSD [%]
Standard concentration [µg/mL]	Calculated concentration [µg/mL]					
200	202.05	201.85	202.77	202.22	0.48	0.24
50	50.11	50.10	49.78	49.99	0.19	0.38
10	9.98	9.93	9.96	9.96	0.03	0.27

The stability of the samples prepared in the mobile phase was evaluated in the same manner as the stability of the samples in PBS. Figure A.11 summarises the results of the stability testing. The results indicate that samples prepared in the mobile phase are stable both at room temperature and at 4°C for 14 days after sample preparation.

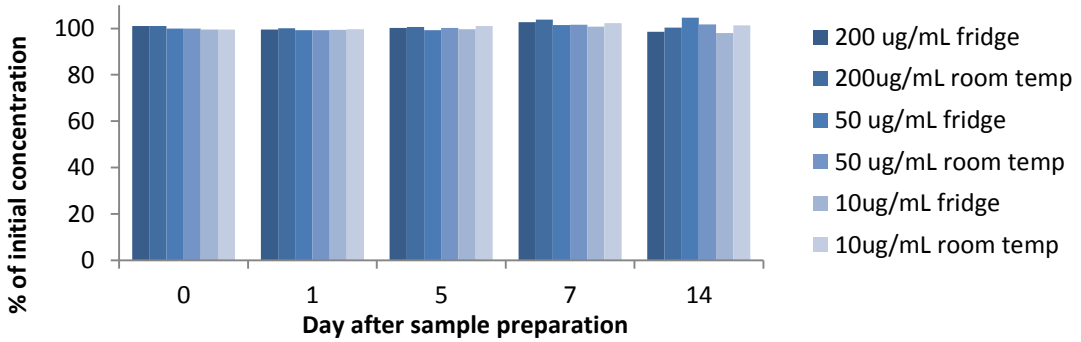


Figure A.11 Stability of standards in mobile phase - % of the initial sample concentration for 200, 50 and 10µg/mL standards kept at room temperature (~25°C) and in the fridge (~4°C)

A.1.2.10 Summary of the validation procedure

According to the recommendations utilised as guidelines for performing the validation of the analytical procedure of NA analysis with HPLC, the validated method meets the requirements of EU and US legislation and may be utilised for the purpose of the experimental work in this thesis. The validation characteristics are listed below.

Table A.19 Summary of the validation characteristics for the HPLC method for nicotinamide assay

Parameter	Value	Recommendation
Accuracy [%] on the day	< 10% *excluding 0.2µg/mL standard	< 10%
Accuracy [%] between days	< 15% *excluding 0.2µg/mL standard	< 15%
Repeatability (RSD)	< 10%	< 10%
Intermediate precision (RSD)	< 15%	< 15%
Linearity (Average R^2 value)	0.9999	> 0.999
Linearity (RSD of slopes values)	1.05%	< 15%
Range	2 - 200µg/mL	-
Detection limit	0.2µg/mL	-
Quantitation limit	2µg/mL	-
Robustness	Sample stable for 14 days at 4°C; Samples robust to the change of solvent	

A.2 Optimisation and validation of GC methods for solvents assay

GC was chosen as an appropriate analytical method for solvent analysis. The GC system parameters, analysis conditions and equipment used aredetailed in Table A.20.

Table A.20 Parameters of the GC methods for solvent quantification

Column	Agilent 19091J-413; 325°C; 30mx320µmx0.25µm
GC System	Agilent Technologies 7890A GC system with FID detector
Software for chromatograms analysis	OpenLAB software; ChemStation Edition for GC Systems, Copyright© Agilent Technologies 2001-2012,
Injection	Volume: 1 µL, Sample Pumps: 6, Injection type: L1 air gap 0.2 µL
Inlet	Heater: 250°C, Septum purge flow 3mL/min, Split 25:1, gas: nitrogen
Flow	1.2 mL/min (for PG 3.5mL/min)
Owen temperature	Initial 100°C, hold 0min; Ramp rate 30°C/min; Final 280°C, hold 2 min (for PG initial hold 2 min, final 0 min)
Detector	FID, Heater 300°C, hydrogen flow 35 mL/min, air flow 350 mL/min, makeup flow (nitrogen) 30mL/min
Retention time	IPM ~5.6, DPPG ~7.3, PGML ~5.7, PGMC ~4.9, PG ~3.8 min
Analysis time	8min
Standards	0.005-1 µL/mL (6 standards of the concentrations of 0.005; 0.01; 0.05; 0.1; 0.5; 1 µL/mL of solvents prepared from a stock solution of a concentration of 10µL/mL by dilution with methanol)
Derivatisation (for PG)	Internal standard (IS) (1:1 with the sample) 0.6µmol 1,2-butanediol in methanol (retention time ~4.3 min) Derivatising agent (1:1 with the sample/IS mixture) 40 mmol phenylboronic acid (forms complexes with diols) in acetone

A.2.1 Optimisation of the analysis conditions - system suitability testing

The summary of system suitability testing is shown in Table A.21. The method was optimised according to the CDER recommendations [2]. The representative chromatograms of standard solvent samples are shown in Figure A.12.

Table A.21 System suitability testing of GC methods for solvent assay

Parameter	Value calculated for the standard chromatogram					CDER recommended value
	IPM	DPPG	PGML	PGMC	PG	
Capacity factor (k')	1.7	2.5	1.7	3.9	2.8	k' > 2
Peak area repeatability (% RSD) n=6	0.8	1.7	2.8	1.4	0.3	RSD ≤ 1%
Retention time repeatability (% RSD) n=6	0.1	0.0	0.0	0.0	0.0	RSD ≤ 1%
Tailing factor (T)	1.0	1.0	1.5	1.5	1.7	T ≤ 2
Theoretical number of plates (N)	200704	54569	10268	38416	41074	N > 2000

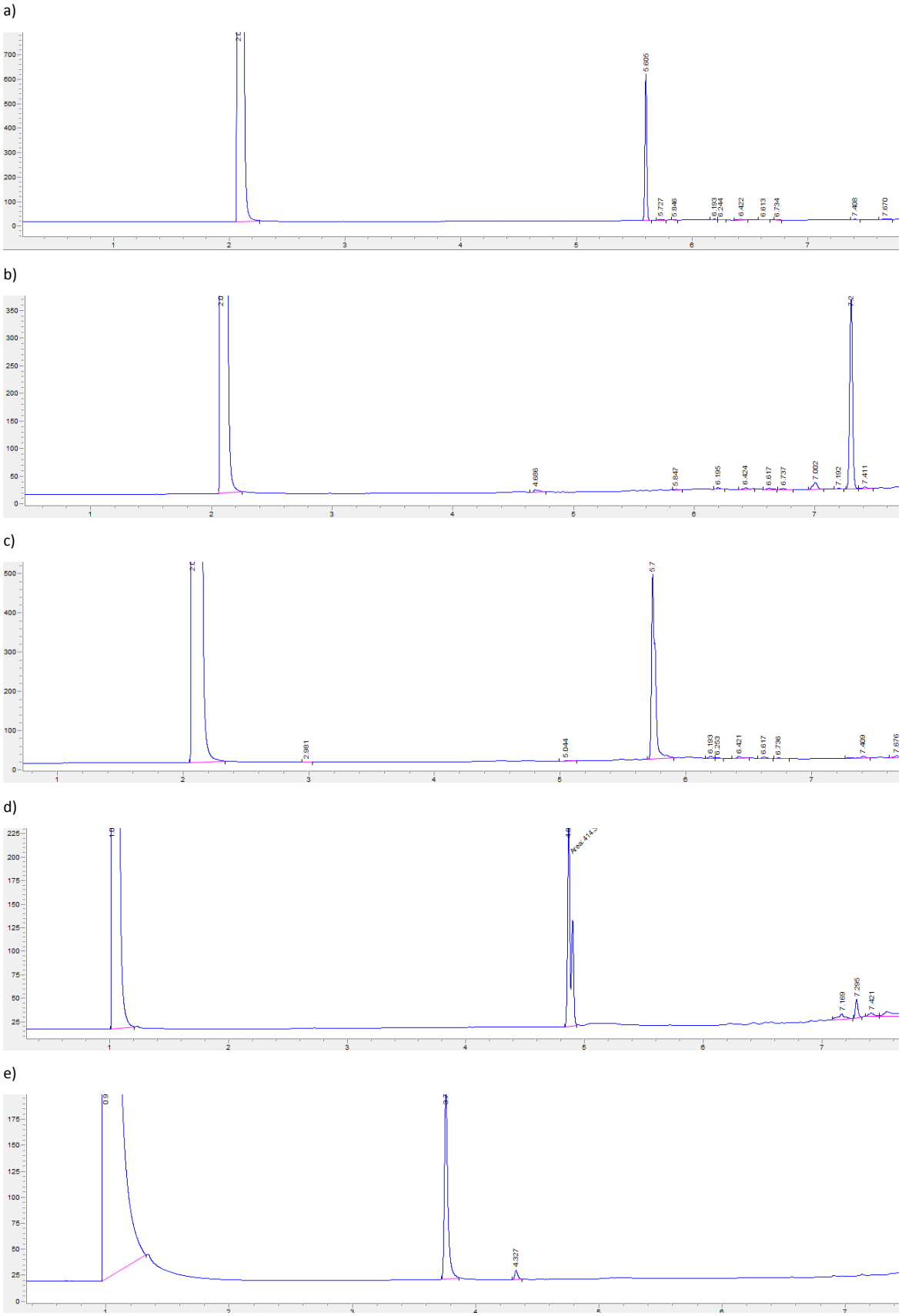


Figure A.12 Representative chromatograms of standard samples used for system suitability testing of GC methods for solvents assay a) IPM, b) DPPG, c) PGML, d) PGMC, e) PG

A.2.2 Validation of the GC methods for solvent assay

A.2.2.1 Specificity

The chromatograms of a blank sample of the receptor fluid and the blank skin extraction sample are shown in Figure A.13 and Figure A.14. No interfering peaks were found.

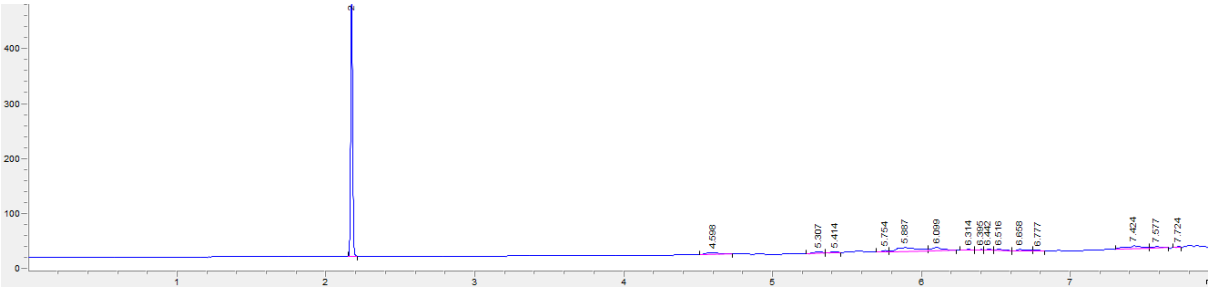


Figure A.13 Chromatogram of a blank sample of the receptor fluid

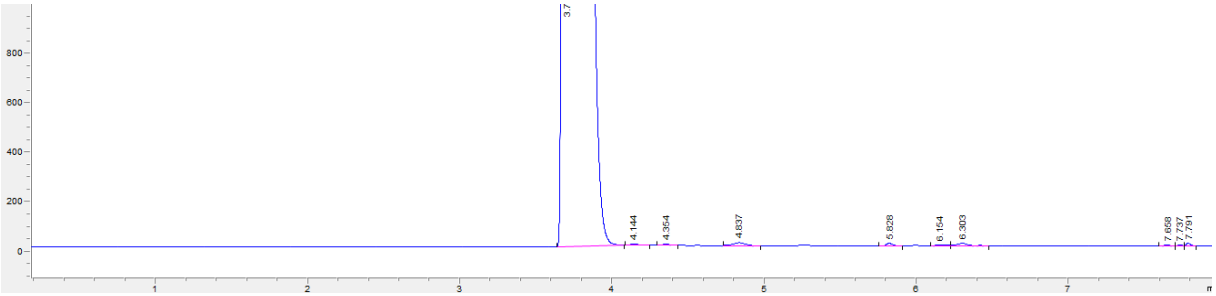
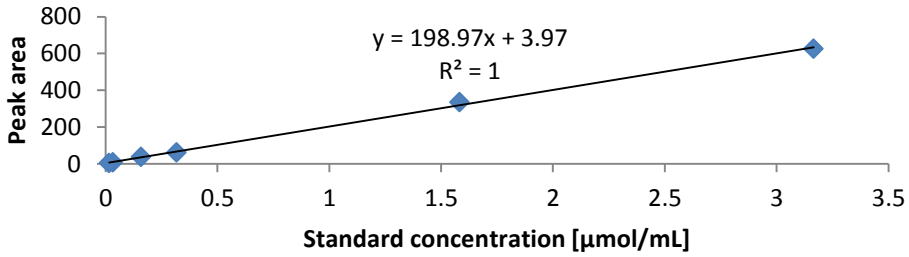


Figure A.14 Chromatogram of the blank skin extraction sample

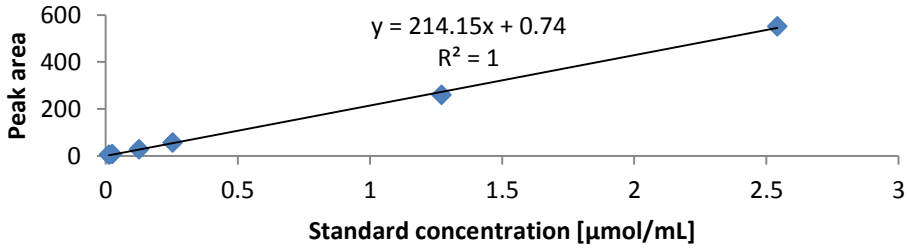
A.2.2.2 Calibration curves

The representative calibration curves for the GC analysis of the solvents are shown below.

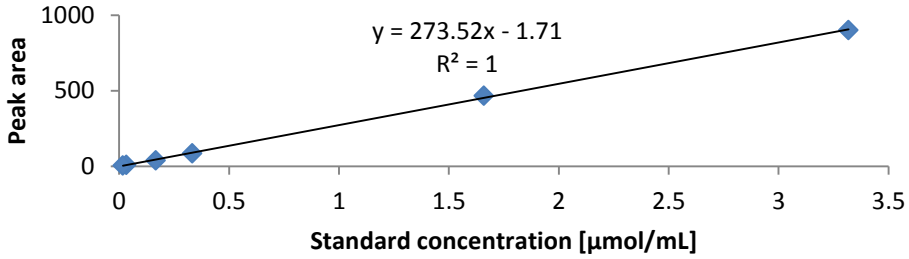
a)



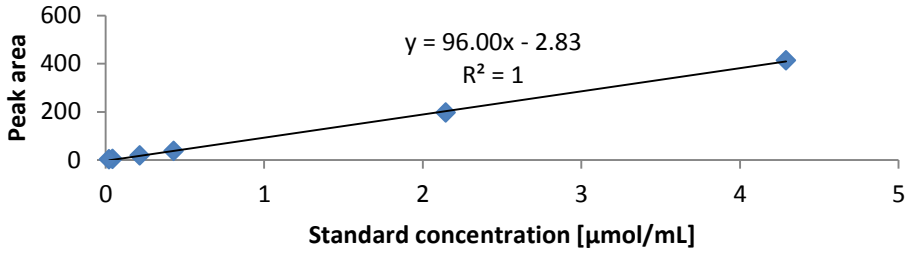
b)



c)



d)



e)

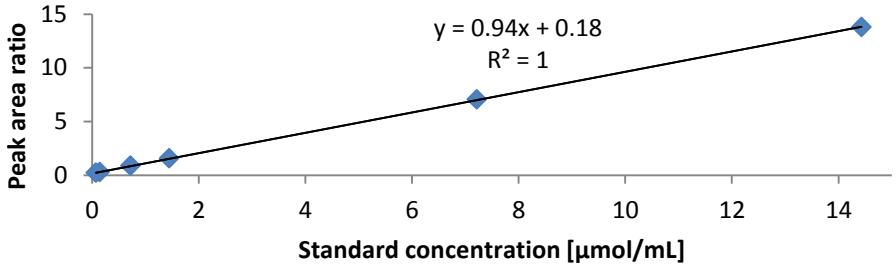


Figure A.15 Representative calibration curves for solvents GC analysis: a) IPM, b) DPPG, c) PGML, d) PGMC, e) PG

A.2.2.3 Summary of the validation procedure

The validation parameters obtained for each of the solvents are listed in the tables below.

According to the recommendations [1, 2, 4], the methods for solvent analysis meet the requirements of EU and US legislation and can be used for the purpose of the experimental work conducted during the project.

Table A.22 Validation characteristics of the GC methods for solvent assay

Parameter	IPM	DPPG	PGML	PGMC	PG	Recommended
Accuracy [%]	< 10% for the range of 0.16-3.17 µmol/mL	< 10% for the range of 0.17-3.35 µmol/mL	< 10% for the range of 0.03-3.39 µmol/mL	< 10% for the range of 0.21-4.29 µmol/mL	< 10% for the range of 0.67-13.39 µmol/mL	< 10%
Repeatability (RSD)	< 15%	< 10%	< 15%	< 10%	< 10%	< 10%
Intermediate precision (RSD)	< 15%	< 15%	< 15%	< 10%	< 10%	< 15%
Linearity (Average r^2)	0.9989	0.9995	0.9991	0.9994	0.9996	> 0.999
Linearity (RSD of slope)	0.00%	0.04%	0.05%	0.01%	0.06%	< 15%
Range	0.03 - 3.17 µmol/mL	0.03 - 3.35 µmol/mL	0.03 - 3.17 µmol/mL	0.04 - 4.29 µmol/mL	0.13 - 13.39 µmol/mL	-
Detection limit	0.02 µmol/mL	0.02 µmol/mL	0.02 µmol/mL	0.02 µmol/mL	0.07 µmol/mL	-
Quantitation limit	0.03 µmol/mL	0.03 µmol/mL	0.03 µmol/mL	0.04 µmol/mL	0.13 µmol/mL	-

A.3 Choice of receptor phase for *in vitro* permeation studies

For lipophilic substances, such as the solvents tested, addition of appropriate solubilisers into the hydrophilic receptor phase is necessary to perform a permeation study [5]. The recommended solubilisers are ethanol (used as 1:1 mixture with water) or non-ionic surfactants, such as polyoxyethylene (20) oleyl ether (PE(20)OE) typically added to the receptor phase at 6% w/v concentration [6]. According to the OECD guidelines, the solubility of tested compounds in the receptor fluid should be adequate, so that sink conditions are maintained throughout the study [7].

The solubility of chosen solvents in a 1:1 ethanol:water mixture and 6% PE(20)OE in phosphate buffered saline (PBS) was tested after 48h equilibration at 32°C and quantified by GC. 6% PE(20)OE in PBS was chosen as an appropriate receptor phase for the permeation studies because of its superior solubilising properties for the majority of the solvents tested. The comparison between the two receptor phases tested is shown in Figure A.16.

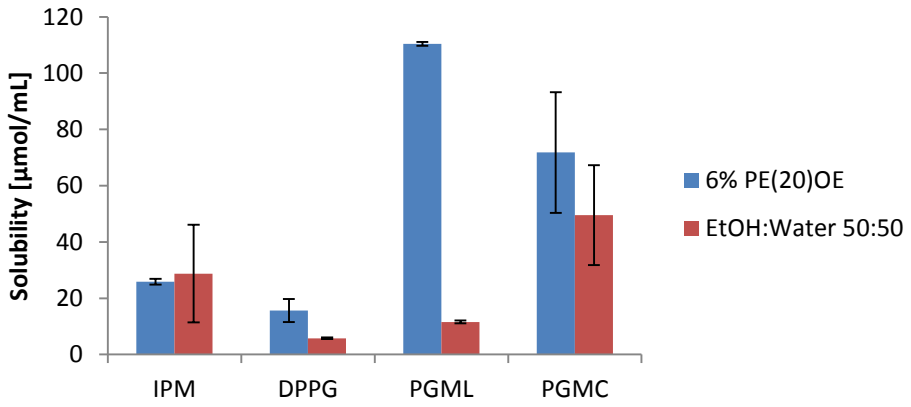


Figure A.16 Solubility of chosen solvents in 6% polyoxyethylene (20) oleyl ether and in 1:1 ethanol:water mixture, 32 ± 1 °C, mean ± SD, n=3

For all of the solvents tested, the solubility in 6% PE(20)OE was high enough to maintain sink conditions throughout the permeation study [8]. Moreover, addition of 50% ethanol into the receptor phase may lead to a decrease in barrier properties of the membranes tested [6]. Thus, 6% PE(20)OE in PBS was used as a receptor phase in the permeation studies.

A.4 Mass balance protocol validation

A.4.1 Mass balance protocol for finite dose silicone permeation study - prototype formulations

Prior to cell disassembly, the membrane was washed with 1mL of 6% polyoxyethylene (20) oleyl ether (PE(20)OE) and rubbed thoroughly with a cotton bud dipped in the washing solution. The cotton bud was then added into the washing fraction and extracted for 1h at 45°C. Thereafter, the cells were disassembled. The membrane was placed in an Eppendorf vial and extracted with 1mL of purified water for 1h at 45°C in an orbital incubator (Stuart Scientific, UK). Appropriate aliquots of the washing and extraction samples were taken for dilution and further HPLC analysis.

The mass balance procedure was validated prior to the studies. The experiment was performed in triplicate. For the membrane washing validation, a known amount of the DENI formulation was applied to the donor compartment of the assembled Franz cells. Immediately after the application (before the drug partition into the membrane) the membrane was washed with 1mL of 6% PE(20)OE and rubbed thoroughly with a cotton bud dipped in the washing solution. The cotton bud was then added into the washings and extracted for 1h at 45°C. The recovery obtained in the washing procedure validation was 104.03±4.62% (n=3).

For the membrane extraction validation, a known amount of the DENI formulation was applied to the donor compartment of the assembled Franz cells (without the receptor fluid) and left for 2 hours allowing drug partitioning into the membrane. Thereafter, the cells were disassembled. The membrane was placed in an Eppendorf vial and extracted with 1mL of purified water for 1h at 45°C. The recovery obtained for the extraction procedure validation was 102.09±1.56% (n=3).

The validation results indicate that the recovery is good for both washing and extraction procedures and is in line with the recommended range [7]. Thus, this method of membrane washing and extraction can be used in further studies for the assessment of the drug mass balance in the receptor phase, the donor compartment and the silicone membrane.

A.4.2 Mass balance protocol for finite dose pig ear permeation study - prototype formulations

The membrane washing and extraction procedures were performed using a protocol similar to the mass balance study described in section 4.2.8.1. The membrane was washed with 1mL of 6% PE(20)OE and rubbed thoroughly with a cotton bud dipped in the washing solution. The cotton bud was added to the washing solution and extracted for 1h at 45°C. Cells were

disassembled and the full thickness pig ear skin was separated into epidermis and dermis prior to the extraction procedure. This was done to enable assessment of NA retention in different skin layers. The epidermis was separated from the dermis using a dry heat separation method described by previous authors [9-11]. Skin samples were subject to a hot air treatment at a temperature of 50°C for 30 seconds followed by the removal of the epidermis with a surgical scalpel. The temperature and the time of the hot air treatment were controlled (Digitron™-22 Thermometer, RS Components, UK and Traceable® Extra - Extra Loud Timer, Fisher Scientific, UK). The epidermis and the dermis were placed in pre-weighed Eppendorf vials and the mass of the membrane was determined. This was followed by epidermis and dermis extraction with 1mL of purified water for 1h at 45°C. Appropriate aliquots of the washing and extraction samples were taken for dilution and further HPLC analysis.

The mass balance procedure was validated prior to the studies. The experiment was performed in triplicate. A blank sample, where no drug was applied to the skin, was included in the validation. For the skin washing validation, a known amount of the DENI formulation was applied to the donor compartment of the assembled Franz cells. Immediately after the application, the membrane was washed 3 times (W1, W2, W3) with 1mL of 6% PE(20)OE and rubbed thoroughly with a cotton bud dipped in the washing solution. The cotton bud was then extracted in 1 mL of the washing solution for 1h at 45°C. The recovery for the first wash obtained in the validation procedure was 116.60±10.91% (n=3). Thus, in later studies skin was washed once with 1mL of 6% PE(20)OE. No drug was found in the extracts from the cotton bud. Nevertheless, this step of the washing procedure was included in the further studies to ensure that no drug remains on the skin surface before extraction. No NA was found in the blank samples.

For the membrane extraction validation, the epidermis and the dermis samples were spiked with a known amount of NA. The drug was applied to the separated membranes in an aqueous solution. The solution was allowed to dry entirely prior to the extraction. Samples were extracted with 1mL of purified water for 1h at 45°C. Appropriate aliquots of the extraction samples were taken for dilution and further HPLC analysis. The experiment was performed in triplicate for three different amounts of NA applied to the skin. High recovery values were obtained for the epidermis extraction and lower values were found for the dermis extraction (Table A.23). However, further experiments showed that the total recovery of the mass balance procedure is satisfactory.

Two different formulations were applied to the skin surface: 4% aqueous NA solution and 4% DENI formulation (n=3 for both formulations). After 2 hours, the mass balance procedure was

performed. The total recovery obtained was 87.59±5.81% and 84.35±10.15%, for the solution and DENI formulation respectively. The total recovery is in the recommended range [12]. Thus, this method of membrane washing and extraction can be used in further studies for the assessment of the drug mass balance in the receptor phase, the donor compartment, the epidermis and the dermis.

Table A.23 Skin extraction validation (mean ± SD, n=3)

Amount of NA applied [µg]	Epidermis recovery [%] ± SD	Dermis recovery [%] ± SD
40	99.18 ± 3.31	83.40 ± 8.70
100	97.93 ± 1.19	76.24 ± 6.44
200	96.70 ± 1.02	75.09 ± 7.07

A.4.3 Mass balance protocol for finite dose silicone permeation study - simple solvent systems

Prior to cell disassembly, the membrane was washed with 1mL of 6% PE(20)OE and rubbed thoroughly with a cotton bud dipped in the washing solution. The cotton bud was then added into the washing fraction and extracted for 1h at 45°C. Thereafter, the cells were disassembled. The membrane was placed in the Eppendorf vial and extracted with 1mL of methanol for 1h at 45°C in an orbital incubator (Stuart Scientific, UK). Appropriate aliquots of the washing and extraction samples were taken for dilution and further HPLC analysis.

The mass balance procedure was validated prior to the studies. The experiment was performed in triplicate. For the membrane (pig ear skin) washing validation, 10 µL of saturated NA solution in PG was applied to the donor compartment of the assembled Franz cells. Immediately after the application the membrane was washed with 1mL of 6% PE(20)OE and rubbed thoroughly with a cotton bud dipped in the washing solution. The cotton bud was then added into the washings and extracted for 1h at 45°C. The recovery obtained in the washing procedure validation was 97.29 ± 5.34% (n=3).

PG was chosen as the solvent in which the solubility of NA is the highest from the solvents tested. Thus, high recovery obtained using this solvent should indicate that the mass balance protocol was valid for solvents with less drug dissolved. For the membrane (pig ear skin) extraction validation, 10 µL of saturated NA solution in PG was applied to the donor compartment of the assembled Franz cells (without the receptor fluid) and left for 2 hours to allow for drug partitioning into the membrane. Thereafter, the membrane was washed with 1mL of 6% PE(20)OE and rubbed thoroughly with a cotton bud dipped in the washing solution. The cotton bud was then added into the washings and extracted for 1h at 45°C. The cells were

disassembled and the membrane was placed in the Eppendorf vial and extracted with 1mL of methanol for 1h at 45°C. The recovery obtained in the extraction procedure validation was 100.53 ± 3.88 % (n=3).

The validation results indicate that the recovery is good for both washing and extraction procedures and is in line with the recommended range [7]. Thus, this method of membrane washing and extraction may be used in further studies for the assessment of the drug mass balance between the receptor phase, the donor compartment and a model membrane.

A.5 Optimisation and validation of HPLC method for AMC assay

HPLC was chosen as an appropriate analytical method for the aminomethyl coumarin (AMC) assay in the samples obtained during studies performed for the purpose of this project. The HPLC system parameters, optimal analysis conditions and equipment used in analysis are listed in Table A.24.

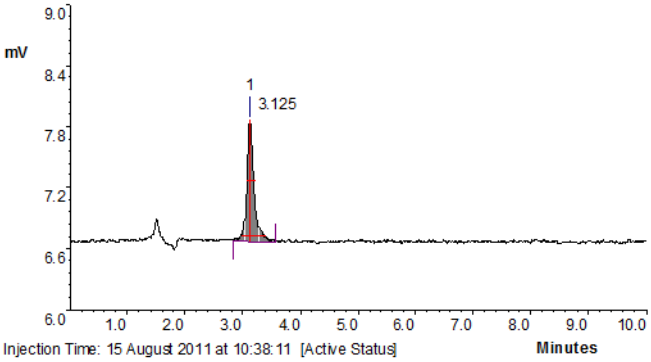
Table A.24 Parameters of the HPLC method for AMC quantification

Column	**Symmetry Shield RP18, 5µm, 4.6x150mm** (Waters, Milford, MA, USA)
Guard column	**SecurityGuard Cartridge System with C18 (ODS, Octadecyl) 4mmLx3mm filter** (Phenomenex, USA)
HPLC System	**HP Series** 1050 quaternary pump, HP Series 1050 autosampler, HP series 1100 degasser, Waters 470 Fluorescence Detector
Software for chromatograms analysis	**PRIME software** Ver.4.2.0., HPLC Technology Co. Ltd, UK
Mobile phase 1	Acetonitrile:Water:Trifluoroacetic acid (30:70:0.1, v/v/v) KLK5
Mobile phase 2	Acetonitrile:Water:Trifluoroacetic acid (25:75:0.1, v/v/v) TRY, PLA
Mobile phase 3	Acetonitrile:Water:Trifluoroacetic acid (20:80:0.1, v/v/v) KLK7
Injection volume	20µL
Flow rate	1 mL/min
Temperature control	40deg.C
Fluorescence detection wavelength	442nm for emission 354nm for excitation
Retention time of AMC	~3min
Analysis time	~6min
Calibration curve range	1-10ng/mL (5 standards of the concentrations of 1; 2,5; 5; 7,5; 10ng/mL of AMC prepared from a stock solution of a concentration of 1000ng/mL by dilution with reaction buffer)

A.5.1 Optimisation of the analysis conditions - system suitability testing

The representative chromatograms of a standard sample are shown in Figure A.12 and the terms determined from this chromatogram are listed in Table A.25.

a)



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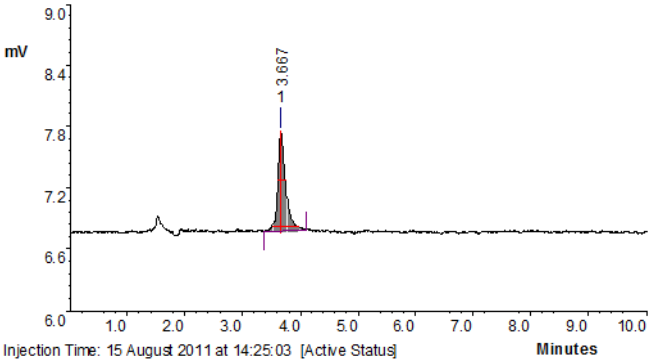


c)

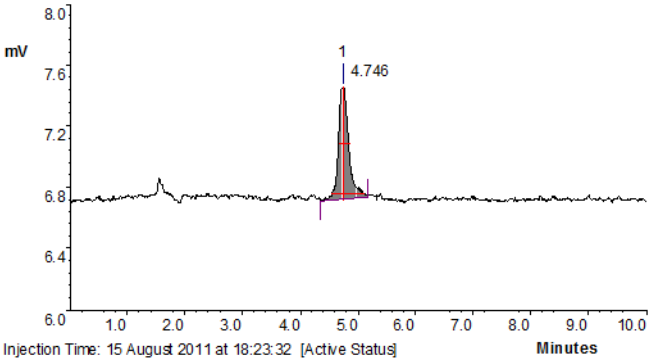


Figure A.17 Representative chromatograms of a standard sample used for the HPLC method for the AMC assay (standard prepared in reaction buffer at a concentration of 1ng/mL with the use of reference standard AMC); a) mobile phase 1, b) mobile phase 2, c) mobile phase 3

Table A.25 The terms for system suitability parameter calculations defined from the chromatograms of a standard sample of AMC

Term	Value [min]		
	Mobile phase 1	Mobile phase 2	Mobile phase 3
$W_{0.05}$	0.46	0.54	0.62
$W_{0.1}$	0.38	0.42	0.54
$W_{0.5}$	0.15	0.15	0.15
$f_{0.1}$	0.23	0.27	0.35
t_0	1.46	1.54	1.54
t_R	3.13	3.67	4.73
t_W	0.27	0.23	0.35

Table A.26 Injection repeatability for a standard sample of a concentration of 1ng/mL of AMC; a) mobile phase 1, b) mobile phase 2, c) mobile phase 3

a)

Injection	1	2	3	Average	SD	RSD [%]
Peak area	10.83	11.05	10.77	10.88	0.14	1.33
Retention time	3.13	3.13	3.13	3.13	0.00	0.00

b)

Injection	1	2	3	Average	SD	RSD [%]
Peak area	9.65	9.73	10.22	9.87	0.31	3.09
Retention time	3.67	3.68	3.68	3.67	0.00	0.13

c)

Injection	1	2	3	Average	SD	RSD [%]
Peak area	8.97	8.98	8.92	8.96	0.03	0.36
Retention time	4.74	4.75	4.74	4.74	0.00	0.10

A.5.1.1 Summary of the system suitability testing procedure

The summary of system suitability test parameters is shown in Table A.21.

Table A.27 Summary of system suitability test parameters for HPLC method for AMC assay

Parameter	Value calculated for the standard chromatogram			CDER recommended value
	Mobile phase 1	Mobile phase 2	Mobile phase 3	
Capacity factor (k')	1.14	1.38	2.07	k' > 2
Injection repeatability (RSD) n=3				
Peak area	1.33	3.09	0.36	RSD ≤ 1%
Retention time	0.00	0.13	0.10	RSD ≤ 1%
Tailing factor (T) from $W_{0.5}$	1.00	1.00	1.00	T ≤ 2
Theoretical number of plates (N)	2156	4040	2986	N > 2000

A.5.2 Validation of the HPLC method for AMC assay

A.5.2.1 Calibration curves

During the validation process calibration curves were obtained on each day of validation by HPLC analysis of each standard performed at least in triplicate. The average areas of the peaks obtained for each standard were plotted against the concentrations of the standards and the calibration curve equation was calculated with the method of least squares. Table A.28 contains the representative data used to plot the calibration curves, which is shown in Figure A.18.

Table A.28 Data obtained from HPLC analysis of the AMC standards, used to calculate the calibration curve equations; a) mobile phase 1, b) mobile phase 2, c) mobile phase 3

a)

Injection	**1**	**2**	**3**	**Average**	**SD**	**RSD [%]**
Standard concentration [ng/mL]	**Peak area**					
10	110.52	105.70	104.17	106.80	3.32	3.11
7.5	78.98	78.81	79.35	79.05	0.27	0.35
5	54.24	54.27	53.94	54.15	0.18	0.34
2.5	26.38	25.70	25.89	25.99	0.35	1.35
1	10.83	11.05	10.77	10.88	0.14	1.33

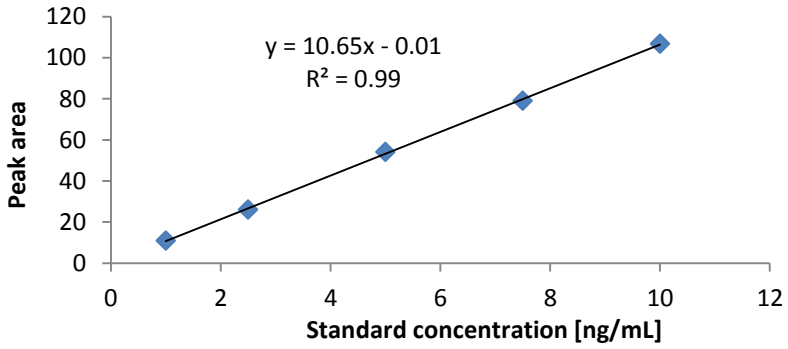
b)

Injection	**1**	**2**	**3**	**Average**	**SD**	**RSD [%]**
Standard concentration [ng/mL]	**Peak area**					
10	96.52	10	96.52	96.32	0.35	0.37
7.5	74.38	7.5	74.38	73.79	0.55	0.74
5	49.86	5	49.86	50.62	0.68	1.34
2.5	24.19	2.5	24.19	23.87	0.28	1.16
1	9.65	1	9.65	9.87	0.31	3.09

c)

Injection	**1**	**2**	**3**	**Average**	**SD**	**RSD [%]**
Standard concentration [ng/mL]	**Peak area**					
10	96.61	10	96.61	94.15	2.29	2.43
7.5	71.08	7.5	71.08	70.14	0.82	1.16
5	48.46	5	48.46	48.63	0.70	1.44
2.5	23.25	2.5	23.25	22.93	0.67	2.94
1	8.97	1	8.97	8.96	0.03	0.36

a)



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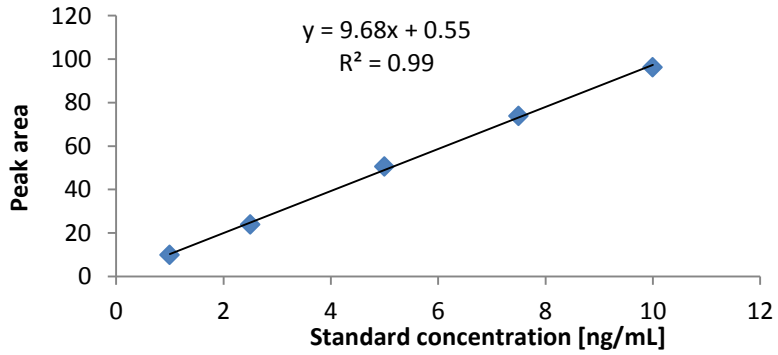


c)

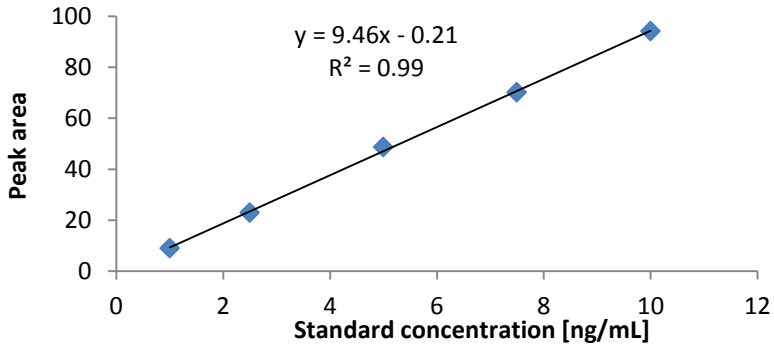


Figure A.18 Calibration curve for AMC standards plotted from the data listed in Table A.28; a) mobile phase 1, b) mobile phase 2, c) mobile phase

A.5.2.2 Specificity

To assure specificity of the HPLC method to be validated two representative chromatograms were compared. No interference is expected in the analysis from the matrix present in the sample, since the blank sample gives a steady baseline reading throughout the analysis time,

with no peaks visible at the retention time of AMC. Blank sample was prepared in the same manner as the samples for protease activity measurements with the exception of no substrate addition to the reaction mixture. The discrimination between the blank and standard sample was easy to perform. The analyte peak had no interference from other sample components.

A.5.2.3 Accuracy

For the purpose of this validation accuracy has been established for all the standards analysed. Each standard was analysed in triplicate on the same day. The calculated recovery values from the calibration curve obtained on the day of analysis are listed in Table A.29 and plotted in Figure A.19, which confirms that the validated HPLC method falls very well into these criteria. The accuracy of the HPLC method subject to validation is summarised in Table A.30. For the whole range of concentrations accuracy complies with the recommended limits.

Table A.29 On-the-day accuracy of HPLC method for AMC; a) mobile phase 1, b) mobile phase 2, c) mobile phase 3

a)

Injection	1	2	3	Average	SD	RSD [%]
Standard concentration [ng/mL]	Calculated concentration [ng/mL]					
10	9.99	9.90	9.93	9.94	0.04	0.43
7.5	7.55	7.54	7.58	7.56	0.02	0.30
5	4.92	5.15	5.09	5.05	0.12	2.44
2.5	2.51	2.48	2.44	2.48	0.04	1.41
1	0.99	1.03	0.90	0.97	0.07	7.20

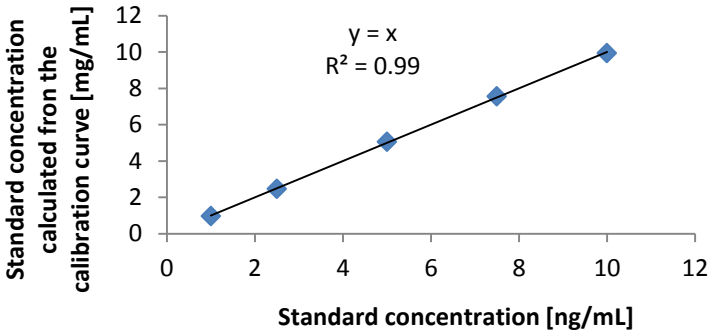
b)

Injection	1	2	3	Average	SD	RSD [%]
Standard concentration [ng/mL]	Calculated concentration [ng/mL]					
10	9.91	9.85	9.91	9.89	0.04	0.37
7.5	7.63	7.55	7.51	7.56	0.06	0.74
5	5.09	5.23	5.20	5.17	0.07	1.36
2.5	2.44	2.39	2.39	2.41	0.03	1.18
1	0.94	0.95	1.00	0.96	0.03	3.28

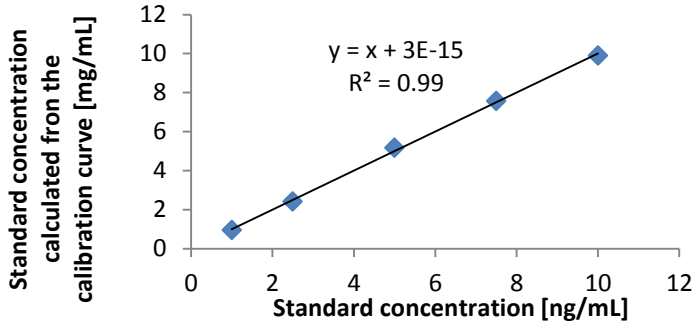
c)

Injection	1	2	3	Average	SD	RSD [%]
Standard concentration [ng/mL]	Calculated concentration [ng/mL]					
10	10.24	9.94	9.76	9.98	0.24	2.43
7.5	7.54	7.38	7.40	7.44	0.09	1.16
5	5.15	5.25	5.10	5.17	0.07	1.44
2.5	2.48	2.49	2.36	2.45	0.07	2.92
1	0.97	0.97	0.97	0.97	0.00	0.35

a)



b)



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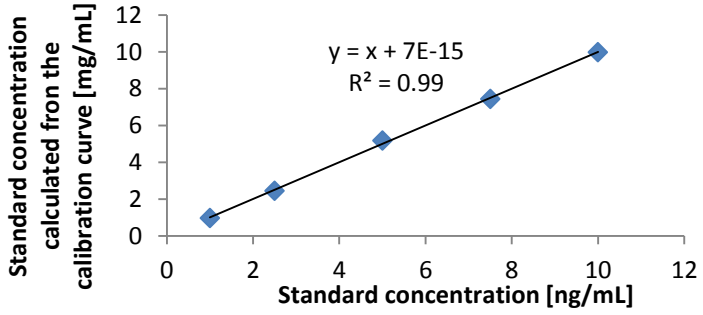


Figure A.19 On-the-day accuracy of HPLC method for AMC; a) mobile phase 1, b) mobile phase 2, c) mobile phase 3

Table A.30 Summary of on-the-day accuracy of the AMC quantification HPLC method; a) mobile phase 1, b) mobile phase 2, c) mobile phase 3

a)

Injection	1	2	3	Average	SD	RSD [%]
Standard concentration [ng/mL]	% Accuracy					
10	-0.12	-0.95	-0.74	-0.61	0.43	-71.33
7.5	0.71	0.47	1.06	0.75	0.30	39.69
5	-1.68	3.07	1.83	1.07	2.46	229.65
2.5	0.33	-0.71	-2.44	-0.94	1.40	-149.25
1	-0.55	3.18	-10.39	-2.59	7.01	-270.88

b)

Injection	1	2	3	Average	SD	RSD [%]
Standard concentration [ng/mL]	% Accuracy					
10	-0.87	-1.50	-0.86	-1.08	0.37	-33.86
7.5	1.68	0.71	0.20	0.86	0.75	87.18
5	1.86	4.54	3.92	3.44	1.40	40.77
2.5	-2.33	-4.32	-4.30	-3.65	1.14	-31.25
1	-5.97	-5.12	-0.13	-3.74	3.15	-84.32

c)

Injection	1	2	3	Average	SD	RSD [%]
Standard concentration [ng/mL]	% Accuracy					
10	2.39	-0.61	-2.41	-0.21	2.42	-1152.91
7.5	0.52	-1.60	-1.32	-0.80	1.15	-143.21
5	2.94	4.94	2.03	3.30	1.49	44.97
2.5	-0.76	-0.22	-5.41	-2.13	2.85	-133.87
1	-2.91	-2.82	-3.45	-3.06	0.34	-11.18

A.5.2.4 Precision

Repeatability was assessed throughout the concentration range of the calibration curves for all three mobile phases and each standard was analysed in triplicate. For the whole range of concentrations the RSD should not be higher than 10%. All the calculated values meet the criteria stated in the guidelines.

The intermediate precision of the readings for samples analysed in triplicate was evaluated in terms of SD and RSD [%]. The results are in agreement with recommendations, i.e. RSD did not exceed 15% for all three mobile phases.

A.5.2.5 Linearity

The data for linearity evaluation are summarised in Table A.31 and a representative calibration curve is shown in Figure A.20. In general, all the calibration curves have low SD and RSD values of the determined slopes and the R^2 value falls within the recommended limit.

Table A.31 Summary of linearity evaluation of the method

	Slope	Intercept	R^2
Calibration curve 1	10.27	0.86	0.9998
Calibration curve 2	10.23	1.02	0.9991
Calibration curve 3	10.65	-0.01	0.9997
Average	10.38	0.62	0.9995
SD	0.23	0.56	0.0004
RSD [%]	2.23	89.49	0.0379

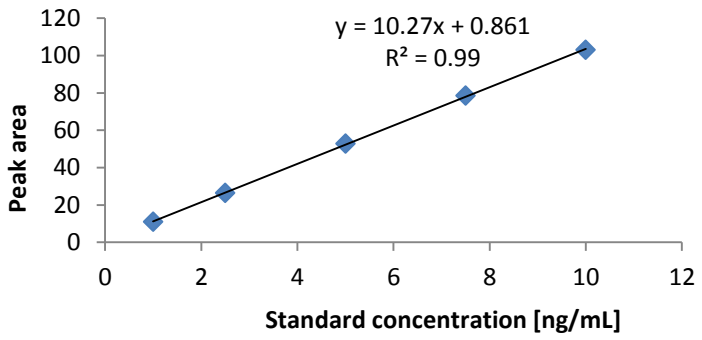


Figure A.20 Representative calibration curve for evaluation of the linearity of the method

A.5.2.6 Range

The working range of the validated HPLC method for the AMC assay was established to be 1-10ng/mL.

A.5.2.7 Quantitation limit

Based on the visual evaluation of the chromatograms and the data obtained during the whole validation process a concentration of 1ng/mL was taken as the quantitation limit for the validated method. Relevant chromatograms are presented in Figure A.12.

A.5.2.8 Robustness

For the purpose of this validation the robustness of the method was checked for the influence of two parameters:

- Standard solution stability (standards kept at room temperature),
- Sample solution stability (samples from protease activity measurements kept at 4°C).

The standard solution stability was evaluated as an important parameter for the future use of the method. Two standards of high and low concentrations were prepared on the first day of validation. They were kept at room temperature and analysed on the day of preparation, after 1, 2, 4, 7 and 14 days. The concentration of each sample was calculated with use of the calibration curve plotted on the day of analysis. The results are presented in Table A.32 and Figure A.21.

Table A.32 Results of stability of the standard solutions studies for the samples kept in the room temperature

Day after standard preparation	0	1	4	7	14
Nominal standard concentration [ng/mL]	% of nominal standard concentration				
5	101.07	103.33	101.70	102.72	103.06
1	97.41	95.78	102.30	91.85	100.44

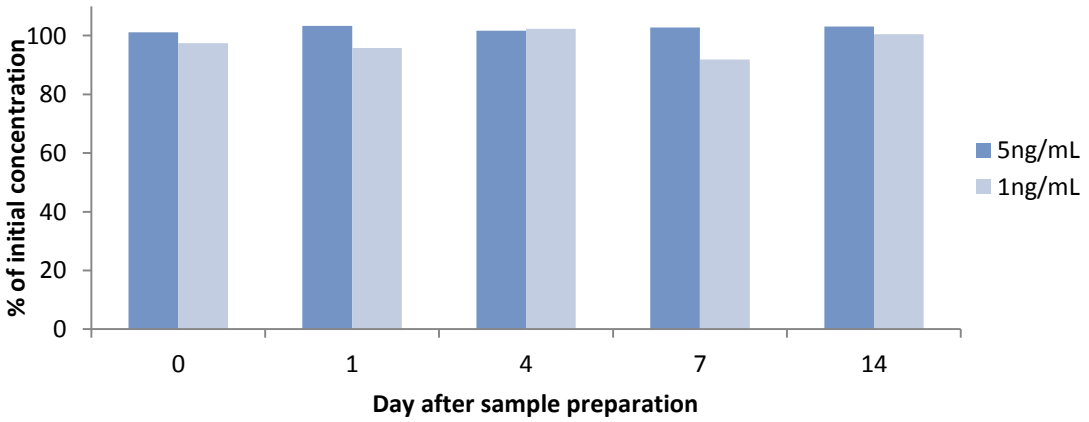


Figure A.21 Stability of AMC standards - % of the initial standard concentration for 10, 5 and 1ng/mL standards kept at room temperature (~25°C)

The results shown above confirm that the standards are stable in the solvent used for their preparation for 14 days when kept at room temperature (approximately 25°C).

Sample solution stability was evaluated for samples from KLK5 protease activity measurements kept at 4°C analysed on several days after preparation. The results shown in Table A.33 indicate that the samples are stable for a period of 6 days.

Table A.33 Results of stability of the sample solutions studies for the samples kept at a temperature of 4°C

Day after sample preparation	0	3	6	13
Sample from KLK5 protease activity measurements	**% of initial standard concentration**			
1	100.00	91.46	94.56	87.39
2	100.00	92.17	94.64	86.35
3	100.00	93.04	100.07	93.67

A.5.2.9 Summary of the validation procedure

According to the recommended guidelines for performing the validation of the analytical procedure of AMC analysis with HPLC, the validated method meets the requirements of EU and US legislation and may be utilised for the purpose of the experimental work, which will be conducted during the project. The validation characteristics are listed in Table A.34.

Table A.34 Summary of the validation characteristics for the HPLC method for AMC assay

Parameter	Value	Recommendation
Accuracy [%] on the day	< 10%	< 10%
Repeatability (RSD)	< 10%	< 10%
Intermediate precision (RSD)	< 15%	< 15%
Linearity (Average R^2 value)	0.9995	> 0.999
Linearity (RSD of slopes values)	2.23%	< 15%
Range	1 - 10ng/mL	-
Quantitation limit	1ng/mL	-
Robustness	Standards stable for 14 days at room temperature and samples stable for 6 days at 4°C	

A.6 Ethical approval documentation

Participant Information Leaflet

Ref. No A01/2011

Title: The influence of topical formulation composition on test compound transport into the skin and on skin condition

You are being invited to take part in a research study. This research has been reviewed by the National Research Ethics Committee. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully. Talk to others about the study if you wish.

Part 1 tells you the purpose of this study and what will happen to you if you take part.

Part 2 gives you more detailed information about the conduct of the study.

Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

Part one

What is the purpose of the Study?

This research is to investigate the influence of formulation composition on test compound permeation through the skin. The study will provide insight into our understanding of the ways in which formulation excipients act within the skin to aid permeation. The research will also explore ways of assessing associated changes in skin condition, brought about by application of these formulations, which may be of future interest when designing improved topically administered products developed for a variety of cosmetic and possibly medicinal uses.

Do I have to take part?

No. It is up to you to decide whether or not to take part. If you do, you will be given this information sheet to keep and asked to sign a consent form. You are still free to withdraw at any time and without giving a reason.

If I agree to take part what will I have to do?

Before testing can begin all participants must have completed Informed Consent Form, Form I and Form II. You will be asked to write your name, address and email address in Form I. You will be asked to record your gender, ethnic group, age range and skin condition in Form II.

You will have 4 application sites measuring approximately 25cm2 demarcated using a laminated template and a surgical marker on the left and right volar (underneath) surface of your forearms. Normally there will be 2 sites for each arm.

You will be given four test formulations and asked to apply 50µL of each of them separately to the four application sites 3 times each day for a treatment period of 21 days (total of 63 applications). 1mL syringes (without needles) will be provided for you to measure the amount of formulation to apply. You will have to be very careful to make sure you apply each formulation to the correct site, and use a different finger to gently massage each formulation into the skin to avoid cross contamination.

You will be asked to complete a Participant Diary, where you will record the dates and times of the applications. The Participant Diary is attached to this Participant Information Leaflet. The completed Diary will be collected by the investigator on the last day of the study (measurements day).

If you agree to participate you will be asked to refrain from applying any topical products to your arms 24 hours prior to the study and throughout the study. There will be no other restrictions regarding your normal day to day activities, including the clothing you wear, washing, bathing, showering, sports participation etc.

Measurements day

After treating the application sites with the test formulations for the 21 day period, a standard, semi-invasive technique called skin tape stripping will be used to repeatedly sample layers of the outer dead skin which can then be assayed in the laboratory to determine a) the quantities of test compound and particular excipients permeated, and b) a variety of biochemical markers and/or physical characteristics indicative of skin condition. The latter include measuring: protease activity (an enzyme system involved in skin cell turnover); protein content (which provides a very sensitive way of estimating the amount of skin cells removed which is too small to weigh); and skin cell maturity and size (which provide further indication of the quality of the skin). In addition, by non-invasively measuring transepidermal water loss (TEWL) at the sites before formulation treatment and at various intervals during stripping, it will be possible to obtain more information relating to the integrity and barrier properties of the skin.

***In vivo* tape stripping**

This is a routine and minimally invasive procedure that involves the investigator repeatedly and gently pressing pieces of adhesive tape on the skin sites and then gently removing them. The adhesive tapes remove successive layers of cells from the outermost dead layer of the skin (the stratum corneum). Using this procedure the skin at each application site and an untreated

control site (5 sites altogether) will be repeatedly stripped by the investigator up to a maximum of 20 times.

TEWL measurements

This is a routine and non-invasive procedure that involves the investigator repeatedly and gently placing a probe on the test sites for approximately 15 seconds. This will measure water evaporation from the skin and provide a measure of skin permeability.

These TEWL measurements will be performed at the five test sites after the 21 day treatment regimen and will be repeated, during the tape stripping procedure, after every fourth stripping.

What are the potential benefits and disadvantages of taking part in this investigation?

There are no specific benefits or particular disadvantages to individuals taking part in this work. However, the results of the study may lead to future improvements in products designed for cosmetic and/or medicinal use.

All of the techniques used in this study have been used routinely in previous studies. The tape stripping technique can feel slightly uncomfortable and subjects will have the opportunity to restrict the number taken to less than 20 if they so wish, although this has not been necessary in previous work of this sort undertaken in our group. Only ingredients found in commonly used products that can be purchased over the counter by the general public will be used in the test formulations. Individuals with known sensitivity to any of the ingredients will be excluded from taking part.Participants will not be exposed to significant risk. In the unlikely case of negligent or non-negligent harm occurring to a participant during the course of the study, the participant will be covered by the School of Pharmacy's insurance policy.

In the unlikely event of any adverse events occurring at treatment sites, full details will be recorded and appropriate medical advice/treatment arranged. Participants will be able to withdraw from the study at any time by giving an oral or written notice to the investigator.

What if there is a problem?

Any complaint about the way you have been dealt with during the study or any possible harm you might suffer will be addressed. If you have a concern about any aspect of this study, you should ask to speak with the investigator who will do their best to answer your questions (Anna Duszyńska Tel. 02077535800 ext. 4870). If you remain unhappy and wish to complain formally, you can do this through the Head of the Department of Pharmaceutics at the School of Pharmacy (Professor Kevin Taylor).

Will my taking part in the study be confidential?

Yes. All the information about your participation in this study will be kept confidential. Further details about this aspect are included in Part 2.

Exclusion criteria

Anyone with any previous or ongoing skin disease will be excluded from the study. Anyone who has known sensitivity to any of the formulation ingredients will be excluded, as will anyone who is taking or using medication purchased over the counter in pharmacies or prescribed by a healthcare practitioner which may have an effect on the skin. This especially applies to medicines that are listed by the World Health Organisation (WHO) as affecting skin differentiation and proliferation which include Steroids (topical or systemic), Benzoyl peroxide, Coal tar, Dithranol, Fluorouracil, Podophyllum resin, Salicylic acid and Urea. Individuals with any skin abnormalities (tattoos, birth marks, scars etc) at the test sites will also be excluded from taking part.

Contact Details:

You are free to ask any further questions to Anna Duszyńska (investigator) before or after you decide to take part (Tel. 02077535800 ext. 4870; e-mail: a.duszynska@live.pharmacy.ac.uk).

This completes Part 1 of the Information Sheet

If the information in Part 1 has interested you and you are considering participation, please continue to read the additional information in Part 2 before making any decision.

Part two

Will my taking part in the study be kept confidential?

Any personal information about participants (e.g. as disclosed on their signed consent forms) will be kept confidential but may be disclosed to appropriately authorised individuals including the regulatory authorities and quality assurance/audit personnel.

Data will be recorded, saved, analysed and reported under participants' unique study identification numbers so that the data remain anonymous.

Publications

Presentations/publications resulting from his work will not disclose any personal data.

Confidentiality policy for research

The personal information gained in this study will remain confidential and will not be passed on to other individuals outside of the research team.

What will happen to the results of the research study?

Data obtained from this study will be studied in order to further our understanding of the research area. The results will also be published in scientific journals. If you would like a summary of the final results, please contact Anna Duszyńska (contact details on Part one of this Participant Information Leaflet).

Consent Form

Ref. No A01/2011

Title of project: The influence of topical formulation composition on test compound transport into the skin and on skin condition

Participant's Identification number.....

Please tick

1. I confirm that I have read and understand the information sheet for the above study (Participant Information Leaflet, version number 1.0). I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily. ☐
2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected. ☐
3. I agree of my own free will to take part in the above study. I agree to comply with the study requirements. ☐
4. I confirm that the information I give to the study personnel will be correct to the best of my knowledge. ☐
5. I understand that sections of any of my study records may be looked at by responsible individuals from the School of Pharmacy, the sponsoring company or from regulatory authorities where it is relevant to my taking part in research. I give permission for these individuals to have access to my records. ☐
6. I agree to waive ownership of all data collected including the right to withdraw data from the study, and I give permission for the data to be transferred to countries outside the EU. ☐

…… …… ……..

Participant name Signature Date

…… …… ……..

Chief investigator name Signature Date

Participant's Diary

Ref. No A01/2011

Participant's Identification number .....

Day of study	Date	Application (tick the box if you applied the formulations)		
		Morning	**Afternoon**	**Evening**
0		Don't apply anything!		
1				
2				
3				
4				
5				
6				
7				
8				
9				
10				
11				
12				
13				
14				
15				

16				
17				
18				
19				
20				
21				
22		**Wash-out period: Don't apply anything**		
23		**Measurements day!** **We're meeting in the lab 322** **The School of Pharmacy, University of London, 29-39 Brunswick Square, WC1N 1AX London**		

Any questions?

Contact Anna Duszynska at +447593270619 or a.duszynska@live.pharmacy.ac.uk

How to apply?

- Take the **laminated template** and rubber bands provided
- Align the template on your left forearm and secure with rubber bands according to the instructions on the template (wrist side towards your wrist, elbow side towards your elbow)
- Take one of the syringes provided
- Withdraw **0.05mL** of **Formulation1** from its container (0.05mL is marked as half of the first unit on the 1mL syringe)
- Apply the formulation to the **appropriate site as marked in the picture**
- Gently massage the formulation into the area of your skin demarcated by the template
- Take **another syringe** and apply **Formulation2** in the same manner as described above to the appropriate site on your left forearm
- Take off the template from your left forearm and align it on your right forearm as before
- Apply **Formulation3** and **Formulation4** to the designated sites in the same manner as described above
- After application **wait for about 2 minutes** before rolling your sleeves down or putting on long sleeved clothes to allow the formulation to absorb

Apply:

- **4 formulations**
- **3 times a day**
- **for 3 weeks (21 days)**

Please always take special care to apply each formulation to the correct site

Use a different finger to gently massage each formulation into the skin to avoid cross contamination and remember to wash your hands before and after application of formulations

Don't apply anything on your forearms 24 hours before the first application

Don't apply any other products on your forearms while you are applying the test formulations

Don't apply anything on your forearms on the 22nd and 23rd day of the study (this is the wash out period necessary for the measurements to be performed)

Don't exchange formulations with other volunteers

Meet me in the lab 322 on the day 23rd for the measurements (please allow about 2 hours time for the meeting)

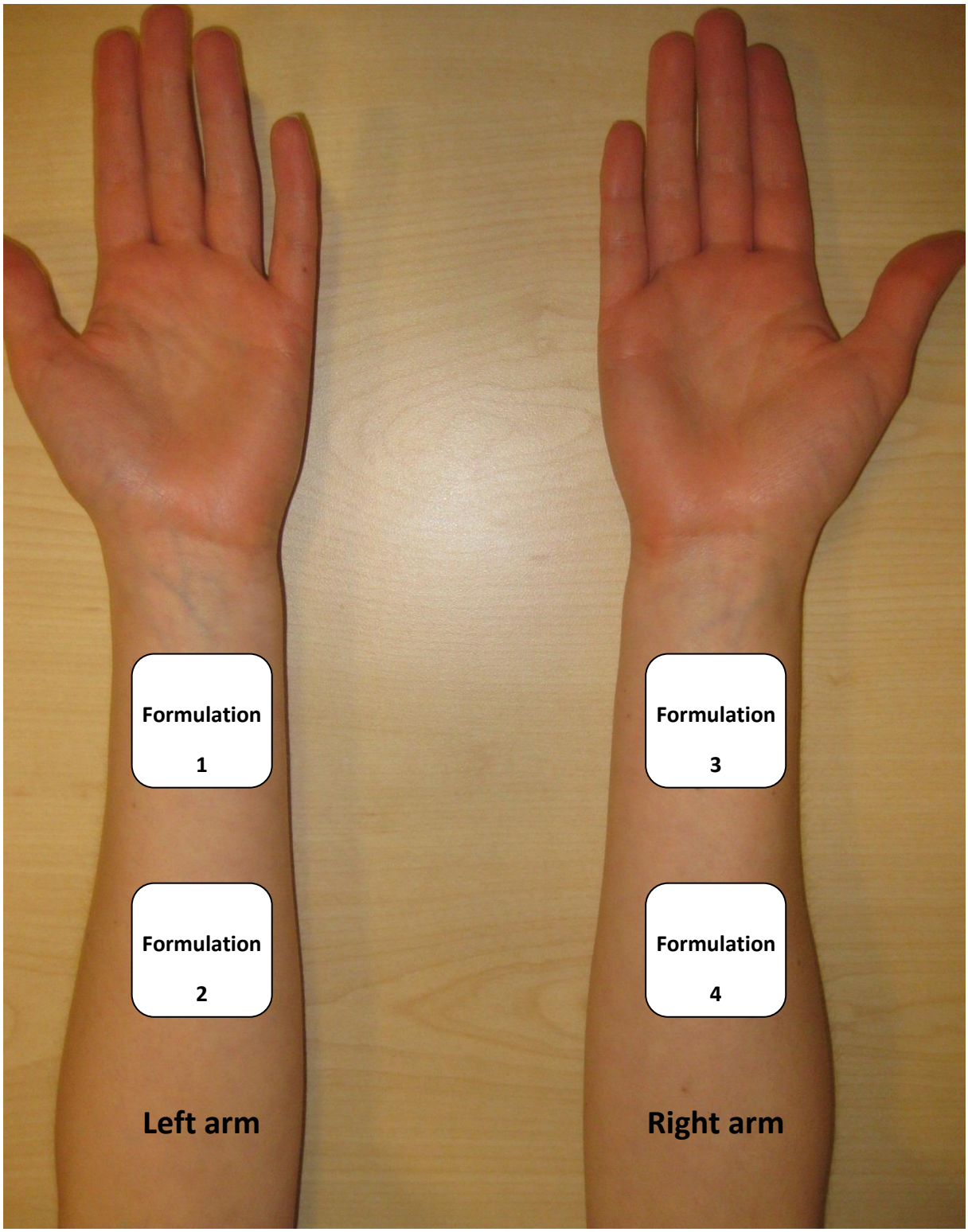
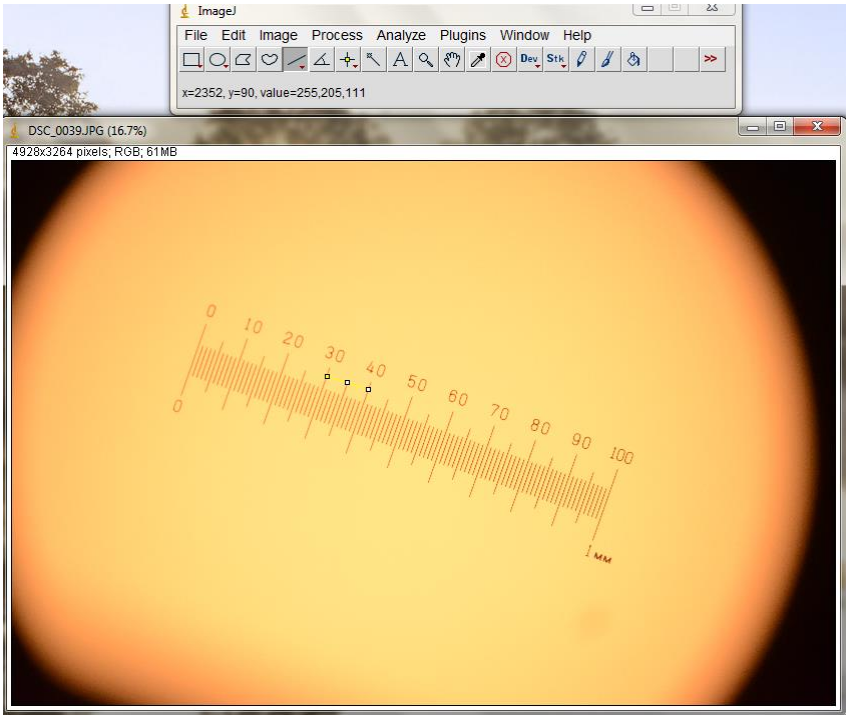


Figure A.22 Application sites

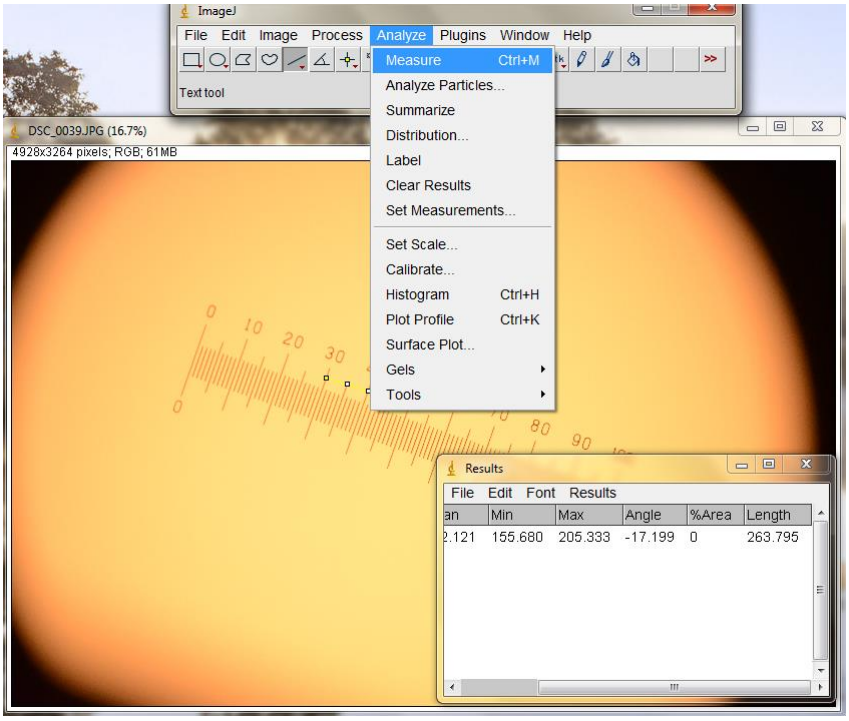
A.7 Corneocyte size measurement with ImageJ® procedure

The following steps were taken to calculate the average area of cells:

1. Calculate the pixel/µm conversion by analysing an image of stage micrometer (1000µm):
    a. Open ImageJ® software and open an image of the stage micrometer and draw a line of a known length (for example 100µm) (File → Open → choose image),

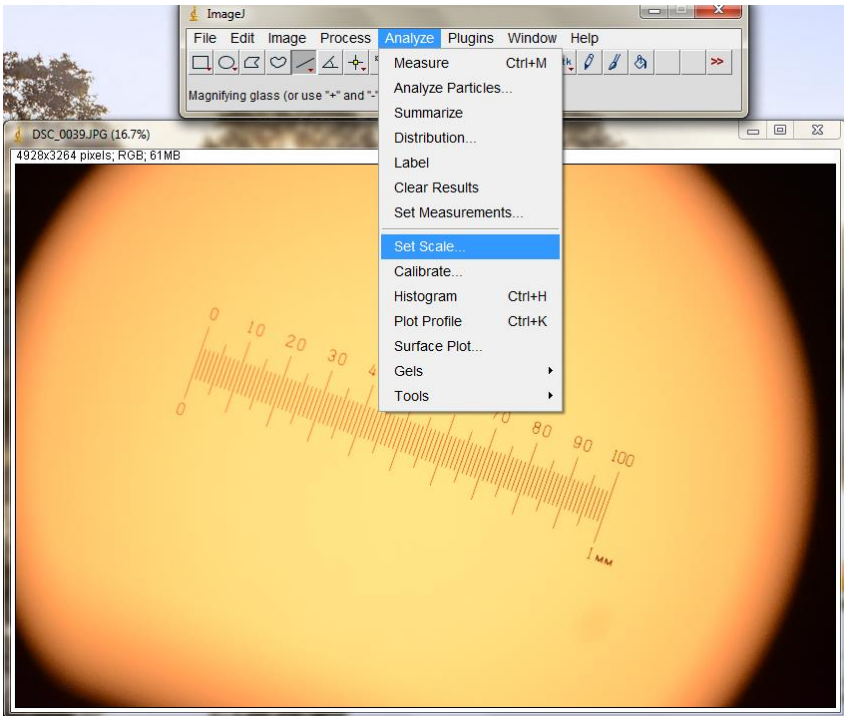


b. Measure the length of the line in pixels,

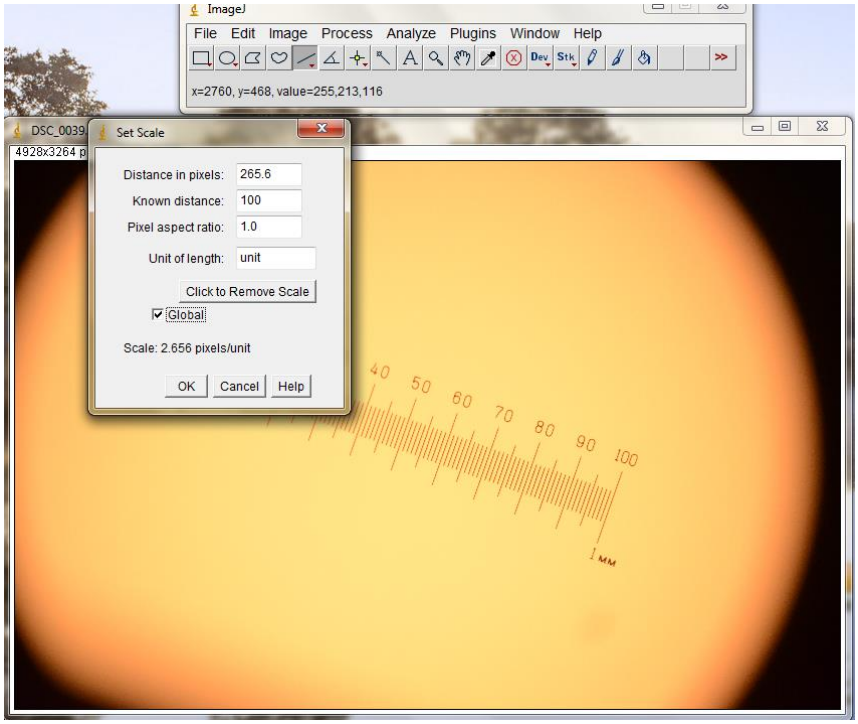


c. Note the value,

d. Set the scale (Analyse → Set Scale),

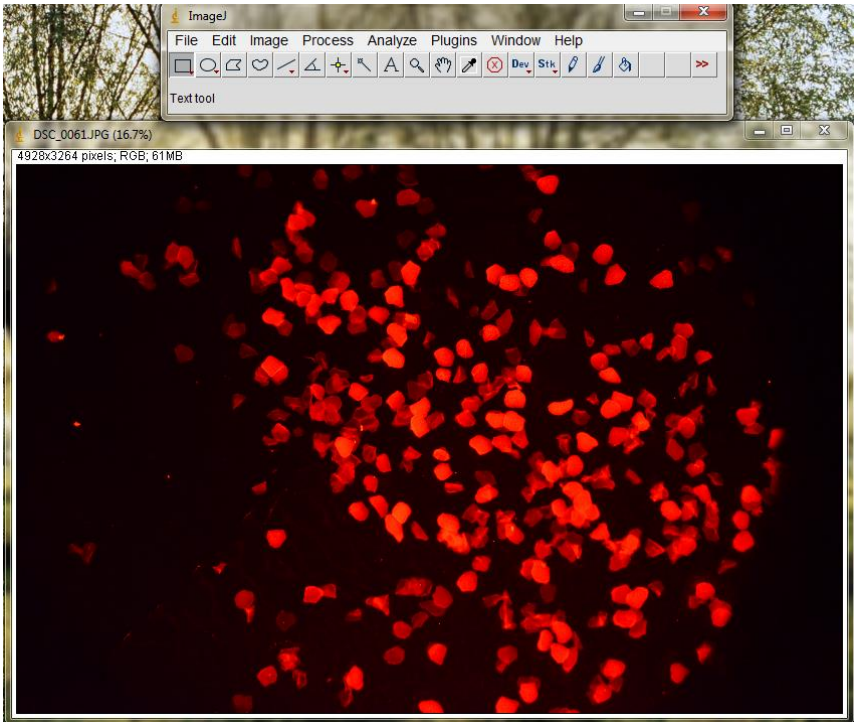


e. Enter the value of pixels counted previously and the distance in µm.

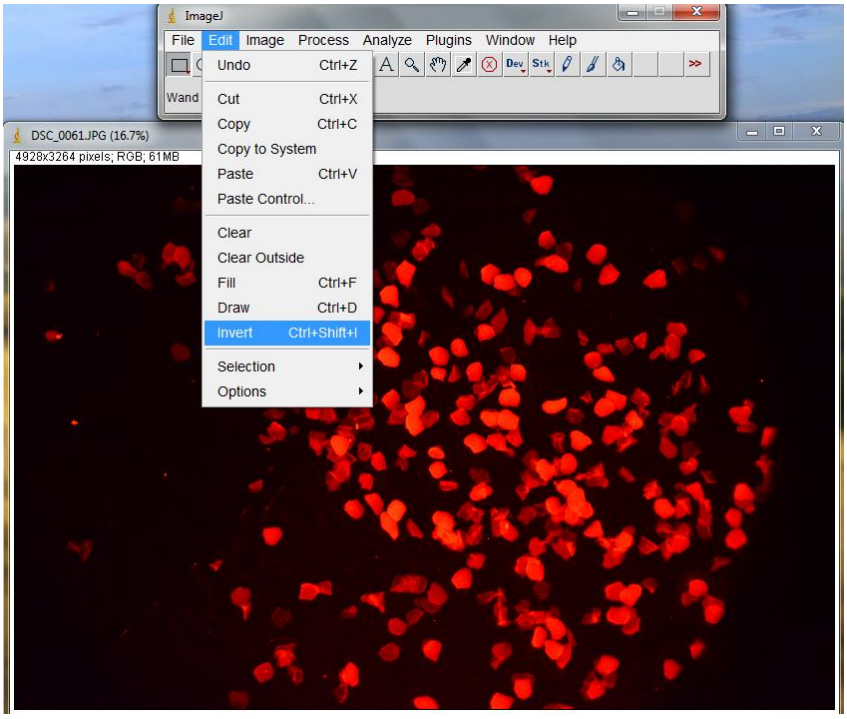


2. Analyse cell size (area)

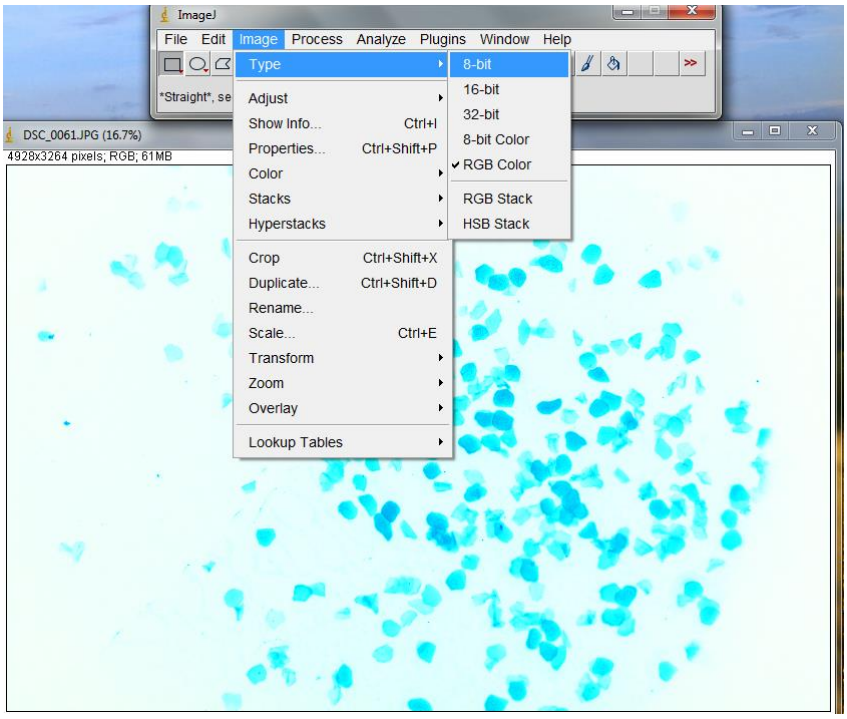
a. Open red fluorescence image of the microscopic slide (File → Open → choose file to open),



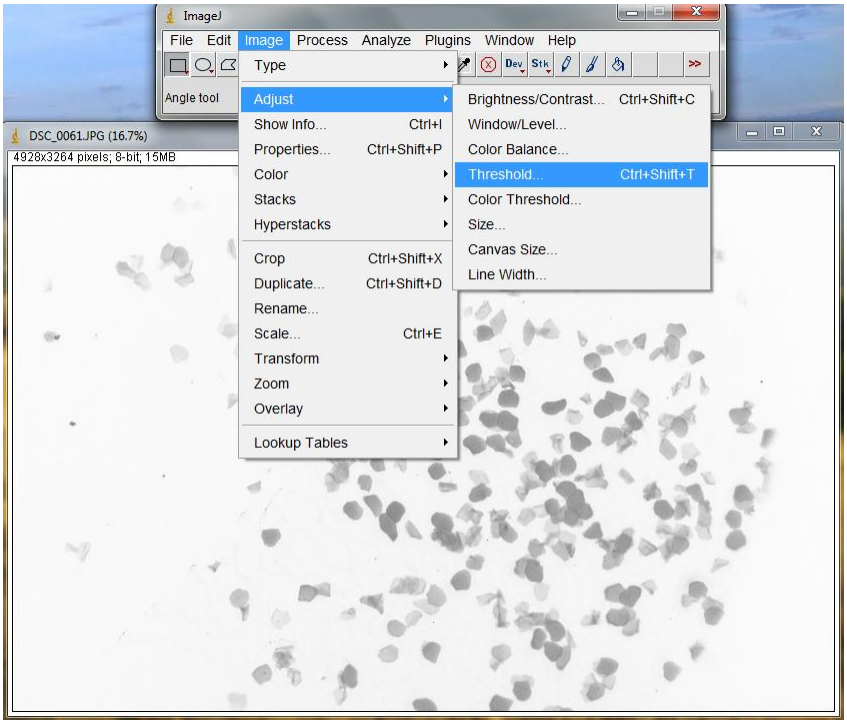
b. Invert the colours of the image (Edit → Invert),



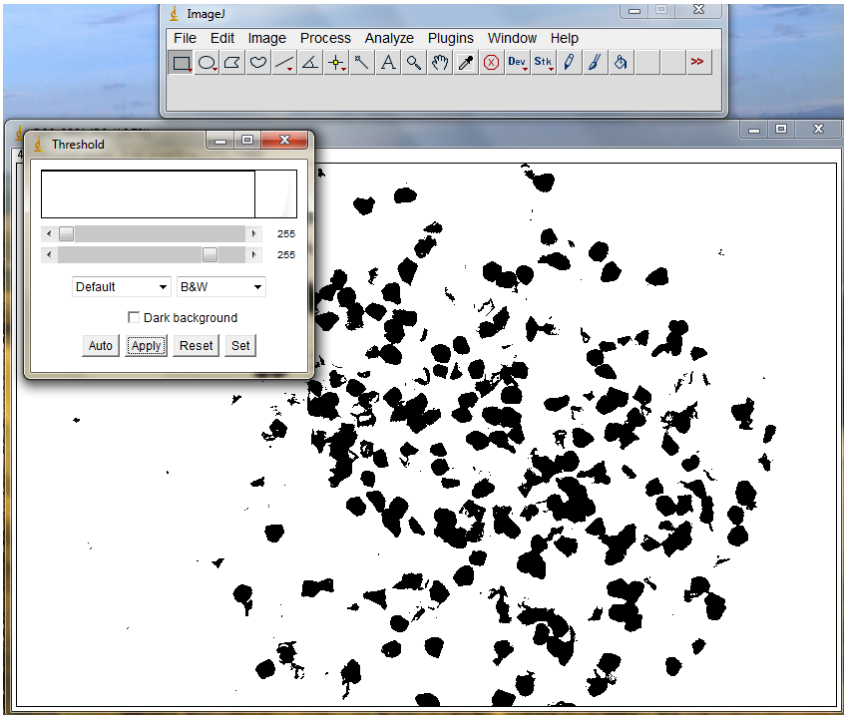
c. Change the image type to 8-bit (Image → Type → 8-bit),



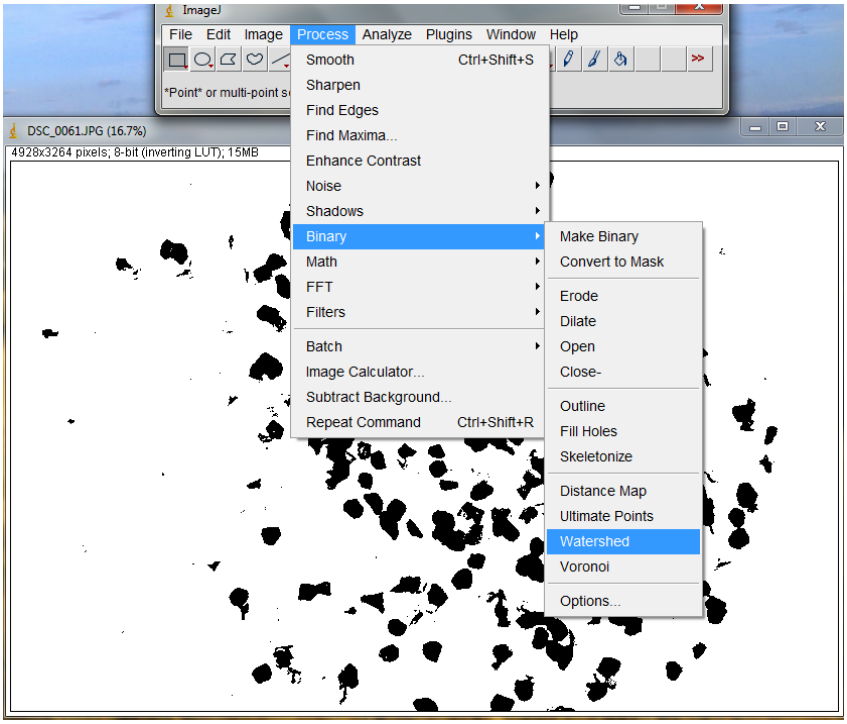
d. Adjust threshold (Image → Adjust → Threshold),



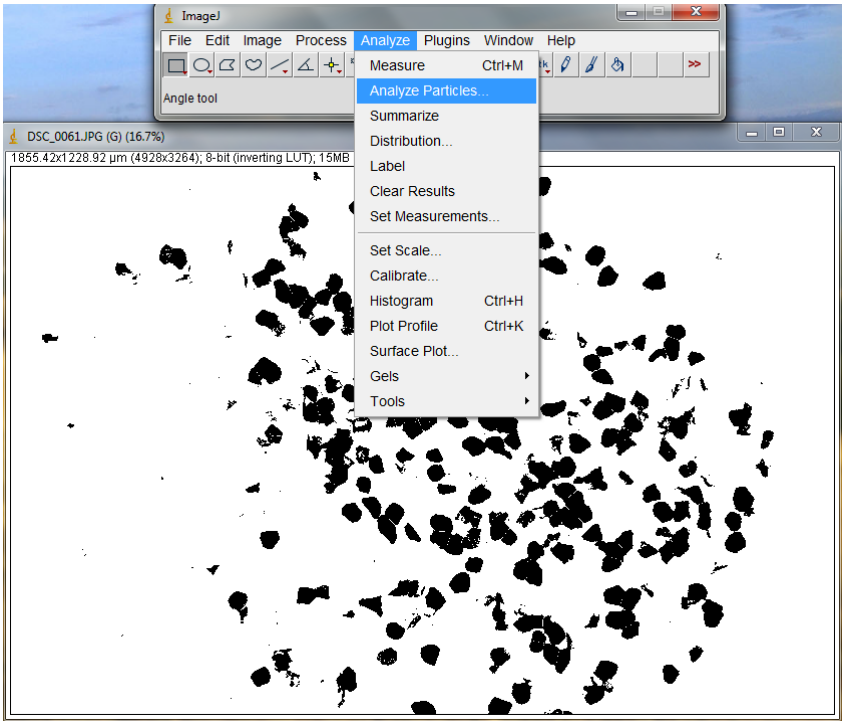
e. Apply threshold by clicking 'Apply' in the 'Threshold' box that appeared,



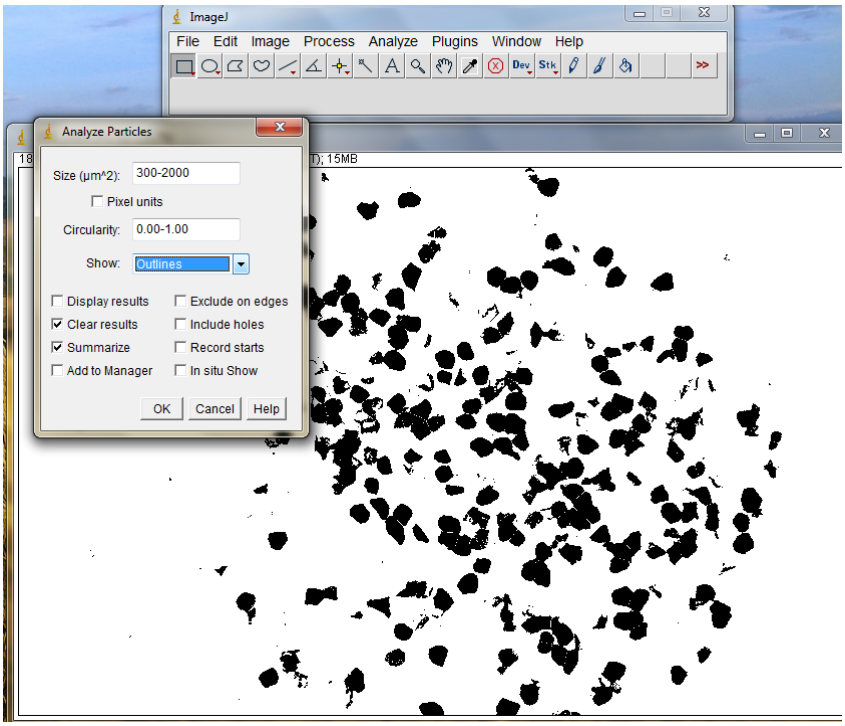
f. Process the image by applying watershed (Process → Binary → Watershed)



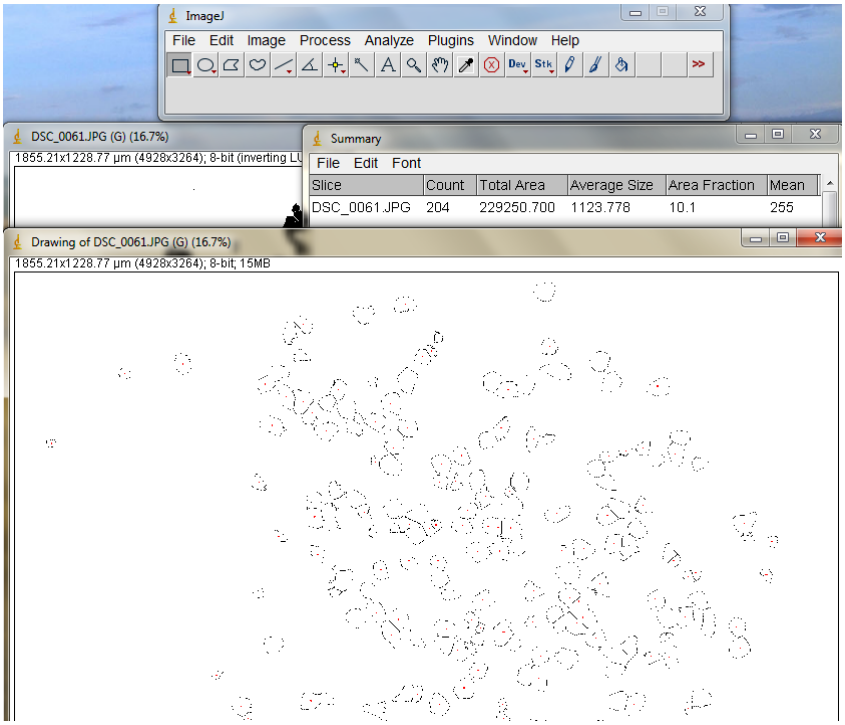
g. Analyse particles (Analyse → Analyse particles)



h. Set the size of particles you want to analyse in the 'Analyse Particles' box; for this study particles of the area between 300 and 2000µm^2 were taken into account,



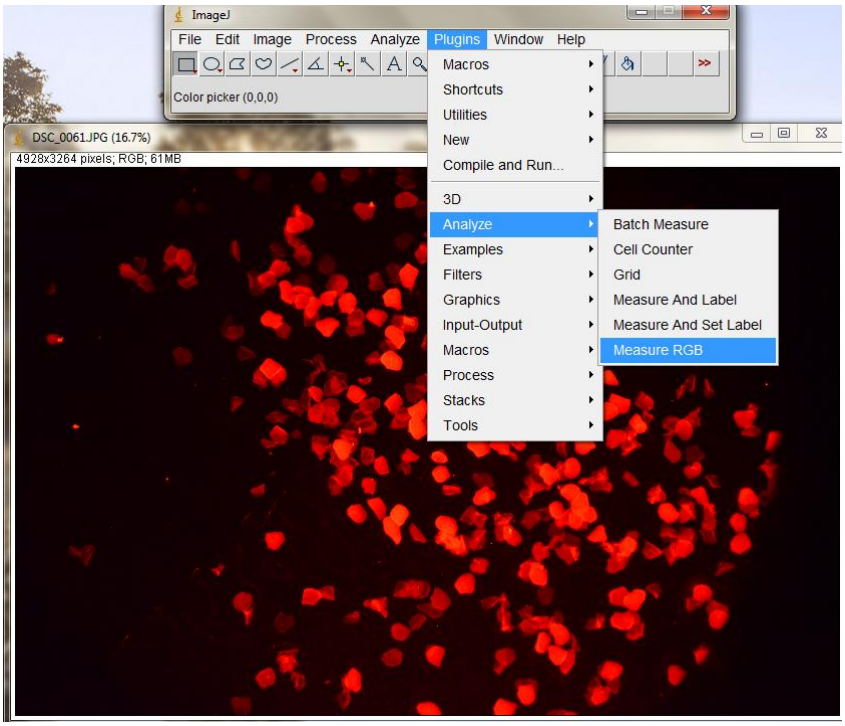
i. Click OK. Make note of the value for 'Mean' size that appear in the 'Summary' box.



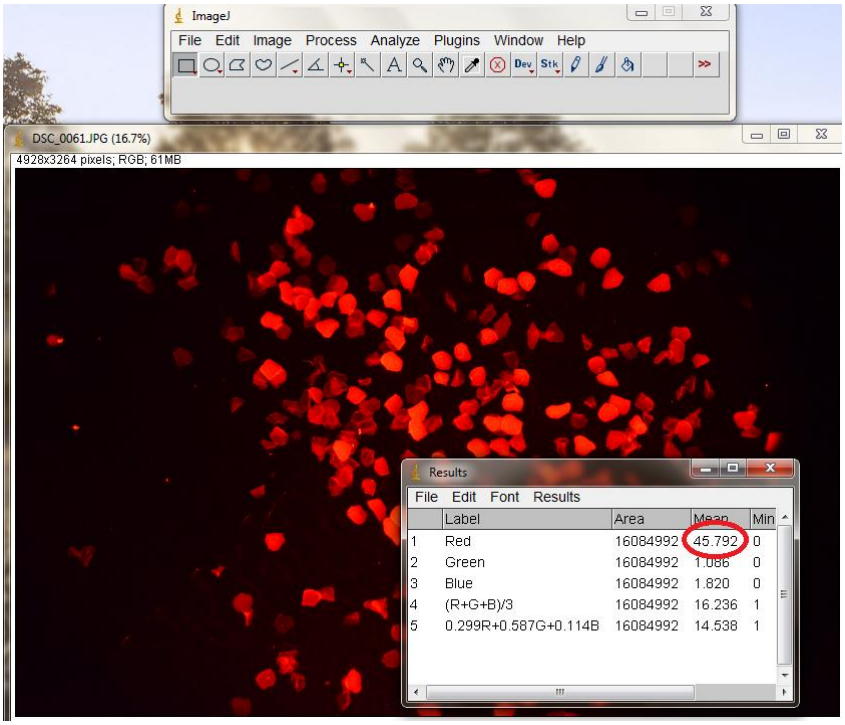
A.8 Corneocyte maturity measurement procedures

The following steps were taken to estimate the maturity of cells with ImageJ®:

1. Open red fluorescence image of microscopic slide (File → Open → choose image),
2. Measure RGB pixels (Plugins → Analyse → Measure RGB),

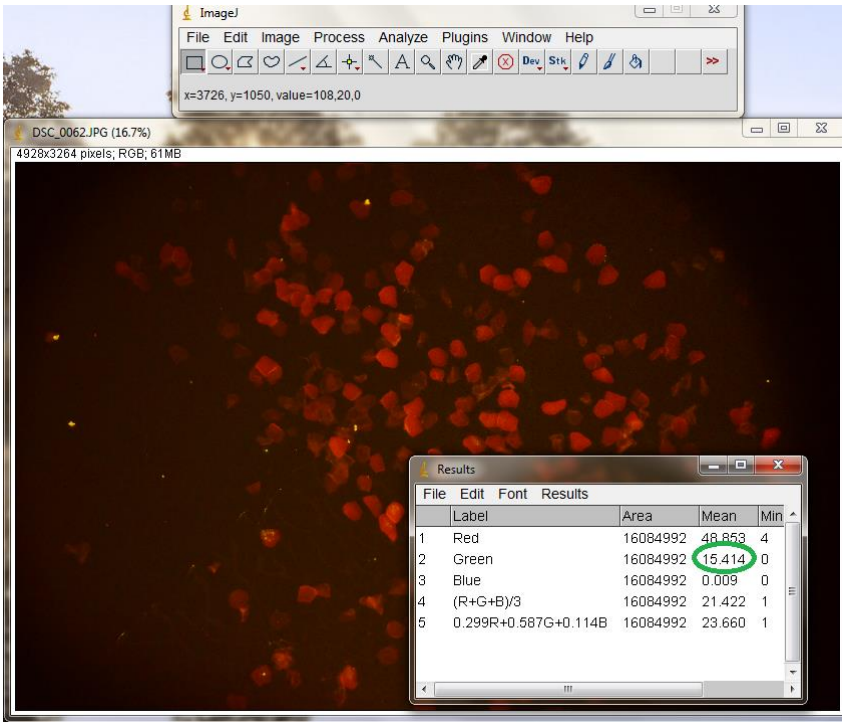


3. Take note of the 'Mean' value for red pixels



4. Open green fluorescence image of microscopic slide (File → Open → choose image),

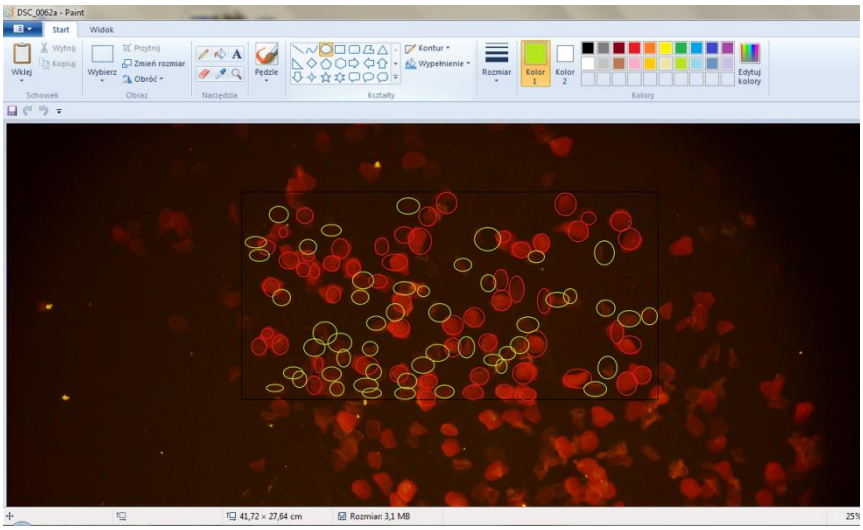
5. As previously measure RGB pixels (Plugins → Analyse → Measure RGB) and take note of the 'Mean' value for green pixels



6. Calculate the red/green ratio for the noted values

The following steps were taken to estimate the maturity of cells with manual cell counting

1. Open green fluorescence image of microscopic slide with Microsoft Paint,
2. Mark a rectangular area (19.99x10.00cm) in a place representative for the whole picture with square drawing tool,
3. Mark with red circle, count bright red cells in the designated area, note the value,
4. Mark with green circle drawing tool, count pale green cells in the area, note the value,
5. Calculate red/green cell ratio.



A.9 Pig ear preparation methods

Pig ears obtained from local abattoir were kept at -20°C before preparation of the membrane.

Full thickness skin was prepared based on the methods of pig ear skin preparation summarised in Table A.35 [9, 10, 13-21]. The ears were cleaned under cold running tap water. The full thickness skin was removed from the underlying cartilage with a scalpel. Circles of a similar size, big enough to cover the opening of a Franz cell receptor were cut with surgical scissors. Hair was trimmed with surgical scissors. The isolated skin samples were stored on the aluminium foil in the freezer at -20°C until required for permeation studies.

Table A.35 Summary of the literature methods for pig ear skin preparation

Reference	Bounoure et al., 2008	Caon et al., 2010	Davison et al., 2009	Dick and Scott, 1992	Kanikkannan et al., 2001
Source of the skin	Pig ears; slaughterhouse	Pig ears from young animals; local slaughterhouse	Pig ears from single animal; local abattoir	Outer region of pig ears (Cheshire White Pigs), local abbatoir	Dorsal surface of the ears from 5-6 months old pigs; prior to scalding; local slaughterhouse
Cleaning procedure	-	Tap water	Sterile water, patted dry	Cold running water	Water and blotted dry with Kim wipes
Type of skin preparation	FT for the permeation studies; SE for FT-IR studies	FT	-	Epidermis	ST
Praparation method	FT skin removed leaving the fat behind; Epidermis peeled off after 12h soaking in 2M potassium bromide at 37°C and dried for 3 days in desiccator	Hair and subcutaneous fat removed	Shaved with electric clippers	Shaved with callipers, whole skin membrane removed from the underlying cartilage, epidermis isolated by soaking the whole skin in water at 60°C for 70s, followed by blunt dissection of the epidermal membrane and floating of the membrane onto aluminium foil	Hair on the dorsal surface removed with surgical scissors; Dermatomed to 0.5mm
Thickness	FT <1.30mm	FT 1.00±0.05mm	-	- (Epidermal membrane)	ST 0.5mm
Storage conditions	-	-80°C, max 2 months; thawed at room temp. before permeation experiments	2-4°C until the following day	Up to 7 days at -20°C	-
Utilisation of the skin samples	Evaluation of iontophoresis and penetration enhancers on metopimazine transdermal absorption with permeation and FT-IR methods	Evaluation of the transdermal permeation of different paraben combinations	Control of microbial contamination of Franz diffusion cell receptor phase	Comparison of the permeability of pig ear skin with human abdominal skin	Percutaneous permeation of JP-8+100 jet fuel

Summary of the literature methods for pig ear skin preparation (continued)

Reference	Padula et al., 2008	Schmook et al, 2001	Senygit et al., 2009	Singh et al., 2002	Vallet at al., 2007
Source of the skin	Pig ears collected immediately after the death of the animal; local slaughterhouse	Skin of domestic pigs (Landrasse Edelschwein, females, aged 5 months) ;local breeder; was dissected after euthanasia with Ketavet:T-61	Inner part of pig ears excised after sacrifice, local slaughterhouse	Outer region of pig ears, local slaughterhouse	Fresh pig ears obtained from local abattoir immediately after animals were killed
Cleaning procedure	-	-	-	Cold running water	Cold running water
Type of skin preparation	FT	ST	-	ST for permeation studies; SC for binding studies	FT and ST for permeation comparison studies
Praparation method	Skin excised from outer region of the ears with a surgical blade; hair trimmed with scissors; subcutaneous fat removed	Dermatomed to 0.6mm with Aesculap dermatome	-	Shaved with clippers; dermatomed to 0.5mm; SC isolated by incubation for 4h at 37°C in 0.5% tripsin in PBS	FT skin removed from the underlying cartilage; ST dermatomed with electric dermatome (Zimmer® Inc., Dover, Ohio, USA)
Thickness	-	ST 0.6mm	-	ST 0.5mm	FT 1.90±0.41mm ST 0.61±0.14mm
Storage conditions	-20°C until used; wrapped in aluminium foil	-	Up to 3 days at 2-5°C	ST skin up to 5 days at 4°C in Eagle's minimum essential medium to preserve viability; SC in desiccator	4°C when transported from the abattoir; - 20°C, max one year
Utilisation of the skin samples	HPLC method validation for determination of retinoids in pig skin layers	Comparison of human skin or epidermis models with human and animal skin in in-vitro percutaneous absorption of model drugs: salicylic acid, hydrocortisone, clotrimasole, terbinafine	Skin accumulation of topical corticosteroids	*In vitro* permeability and binding of hydrocarbons in pig ear and human abdominal skin	*In vitro* percutaneous penetration of organophosphorus compounds using full-thickness and split-thickness pig and human skin

A.10 Nicotinamide extraction method from the tapes

NA content in the tapes was determined using the HPLC method described in section A.1. The extraction method was validated prior to the study. Blank samples were prepared by tape stripping of the non-treated skin according to the method described in Chapter 5. The tape was placed in a separate Eppendorf tube and extracted with 750µL of buffer at pH 8.0 containing 0.1M Tris-HCl and 0.5% TritonX-100, 15 min., 25°C, 1000rpm vortex (Orbital incubator). The experiment was performed in triplicate. No interfering peak was found in blank samples.

Validation samples were obtained by spiking the tapes with a known amount of NA and performing the extraction procedure as above. The recovery values for three different amounts of NA were above 90% and are summarised in Table A.36.

Table A.36 Validation of nicotinamide quantification in the tape strips (% recovery)

Tape spiking [µg/mL]	% of nicotinamide recovery from the tapestrips			Average	SD	% RSD
	1	2	3			
0 (blank)	0	0	0	0	0	0
1	96.22	99.85	96.22	97.43	2.09	2.15
2	97.09	101.18	99.81	99.36	2.08	2.09
4	95.72	95.72	96.62	96.02	0.52	0.55

The stability of the extraction samples was investigated. Samples are stable over the period of 14 days at 4°C and at room temperature (recovery above 90%). The results are summarised in Table A.37 and Table A.38.

Table A.37 Room temperature stability of nicotinamide samples extracted from tapes

Day after sample preparation	0	5	12	14
Sample concentration [µg/mL]	**% of initial sample concentration**			
1	100.00	103.93	95.68	103.04
2	100.00	95.00	95.04	99.97
4	100.00	98.59	91.17	100.99

Table A.38 Stability of nicotinamide extraction samples kept at 4°C

Day after sample preparation	0	5	12	14
Sample concentration [µg/mL]	% of initial sample concentration			
1	100.00	109.25	94.79	108.37
2	100.00	96.36	96.38	100.86
4	100.00	98.83	99.22	100.76

A.11 Validation of time needed to achieve saturation in the solubility studies

Preliminary saturated solubility studies in single solvents were performed to assess the time necessary to achieve saturation. The summary of the results is shown below.

Table A.39 Summary of the solubility studies of nicotinamide in chosen single solvents (results shown as mean ± SD, n=3)

Solvent	Solubility [mg/mL]		
	24h	48h	72h
MO*	-	-	0.0057 ± 0.001
IPM	0.68 ± 0.02	0.67 ± 0.008	0.68 ± 0.005
DPPG	1.57±0.01	1.62±0.008	1.69±0.01
PGML	21.97±0.22	22.15±0.35	22.73±3.42
PGMC	38.70±0.74	38.17±1.25	39.31±0.76
EtOH	134.86 ± 2.93	149.82 ± 0.64	138.63 ± 8.65
GLY	198.09 ± 24.89	232.17 ± 10.97	238.66 ± 9.02
PG	229.74±13.09	243.37±8.76	263.97±11.68
H_2O	555.58 ± 2.81	552.96 ± 8.63	561.23 ± 5.51

*The solubility of NA in MO is very low and thus the results after 72 hours are assumed to be the closest approximation of the drug solubility in this solvent.

No significant differences were found between the results obtained at each time point. However some literature data indicate that the equilibration time for saturated solutions should be at least 48h. Thus, this equilibration time was chosen for further experiments [22, 23].

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