Topical delivery of niacinamide, encapsulated in niosome and proniosome carriers

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“Work hard in silence. Let your success be your noise”-Frank Ocean

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Abstract

Niacinamide, better known as vitamin B_3, is water soluble and takes part in oxidation-reduction reactions in the mitochondria of the skin (Bissett, 2009:438). This active possesses anti-inflammatory action, decreases transepidermal water loss (TEWL) and prevents the loss of dermal collagen (Mohammed *et al*., 2013:192). For this reason niacinamide is an ideal product to be applied topically for the treatment of ageing, hyperpigmentation, melasma and rosacea (Gehring, 2004:88).

Considering niacinamide for topical application, the physicochemical characteristics of the active, as well as the physicochemical properties of the skin must be brought into account (Barry, 2007:566). Since the skin is the outermost organ of the human body; it protects the body by acting as a barrier against the penetration of agents from the external environment (Bolzinger *et al*., 2012:156). This barrier function of the skin resides in the stratum corneum, which is the outermost layer of the epidermis (Feingold & Denda, 2012:263). Thus, for this reason, the barrier function of the stratum corneum makes the delivery through the skin a challenge.

For an active to penetrate through the skin and concentrate in the underlying layers; the molecular size, solubility, partition coefficient and ionisation is of importance (Benson, 2012). According to Barry (2001:102), the molecular size must be less than 500 Dalton in order to penetrate the skin. The molecular size of niacinamide is 122.1 Dalton (Dollery, 1999:N62), indicating that the size of the active will be ideal for topical penetration. The solubility of niacinamide was determined in the study as 112.70 mg/ml, which according to Naik *et al*. (2000:319), is the ideal solubility, since it is higher than 1 mg/ml. The partition coefficient was e -0.43. This value indicates that the active is not ideal for topical penetration; since it does not possess both lipophilic and hydrophilic properties (Donnelly & Sing, 2015:13). For the active to penetrate the skin the ideal partition coefficient must be between one and three. When considering the ionisation of the active, the ionised form of a molecule is less likely to penetrate the skin than the unionised form (Donnelly and Sing, 2015:15). Niacinamide is 99.99% ionised at a pH of 7.4, causing the active to pass through the stratum corneum with difficulty. For these reasons niacinamide was encapsulated into a vesicle system in order to “mask” the physicochemical characteristics which cause a challenge to penetration through the skin.

Vesicle systems are hollow particles composed of a protective layer consisting of a hydrophilic head and a hydrophobic tail (Honeywell-Nguyen & Bouwstra, 2008:206). These systems are ideal for topical delivery of actives, since they can entrap both hydrophilic and hydrophobic substances. They deliver the entrapped substance at a specific location and are non-toxic

(Honeywell-Nguyen & Bouwstra, 2008:206). These systems are known as niosomes and proniosomes and the difference between them is the method of preparation (Seleci *et al*., 2016:4). Proniosomes are prepared to form a dry powder in order to have a longer storage life than the niosomes (Yeom *et al.,* 2014:83), consequently, proniosomes are hydrated (just before use) to form niosomes. Although both the niosomes as well as the proniosome systems present with excellent properties, their low viscosity can be a drawback when trying to apply the dosage form.

During this study, the viscosity was determined as 4.036 cP and 3.616 cP for the niosomes and proniosomes, respectively. According to Barry (2007:595), designing a product for maximum topical effect is important, but designing a product to be acceptable to the consumer is of more importance. Consumers prefer a product that can be transferred from the container easily, spreads readily and is not difficult to remove from the skin, thus, a product with poor appearance will lead to non-patient compliance (Barry, 2007:595). In order to increase the viscosity and enhance compliance only the niosomes were incorporated into a gel and a cream, since proniosomes become niosomes once hydrated.

Franz cell diffusion studies were performed on the pre-formulations (niosomes and proniosomes) and on the formulations (niosome gel and niosome cream). First, the membrane diffusion studies were performed to determine if any release of the active occurred from the formulation. The preparation which presented with the highest median flux were the proniosomes (1 163.58 µg/cm^2.h), followed by the niosomes (1 162.58 µg/cm^2.h), then the niosome gel (1 042.08 µg/cm^2.h) and lastly, the niosome cream (469.72 µg/cm^2.h). During the skin diffusion studies, all the preparations presented with niacinamide in the receptor phase. The preparation with the highest median flux value in the skin diffusion studies were niosomes (0.844 µg/cm^2.h), followed by the niosome gel (0.692 µg/cm^2.h), the proniosomes (0.462 µg/cm^2.h) and lastly, the niosome cream (0.172 µg/cm^2.h). All the preparations presented with niacinamide in the stratum corneum-epidermis (SCE) and epidermis-dermis (ED). The preparation with the highest median concentration in the SCE was the niosome (2.13 µg/ml), followed by the niosome gel (1.94 µg/ml), the niosome cream (1.05 µg/ml) and lastly, the proniosomes (0.71 µg/ml). The niosomes (5.02 µg/ml) were the dispersion with the highest median concentration in the ED, followed by the niosome cream (2.86 µg/ml), the proniosomes (2.04 µg/ml) and lastly, the niosome gel (1.94 µg/ml). Since the aim of this study was to deliver the active topically, all four the preparations were successful.

Stability tests were performed on the niosome gel and niosome cream for a period of three months. According to ICH (2006:3) guidelines, the formulations were stored at ± 25° C/60% RH (relative humidity), ± 30° C/65% RH and ± 40° C/75% RH to determine if any change in the physicochemical properties of the formulations occurred. The chemical stability (concentration of

the active) as well as physical properties (pH, conductivity, viscosity, light microscopy changes, visual changes, mass loss and zeta-potential) were determined initially (t=0) as well as after 1, 2 and 3 months respectively. Both the formulations were stable regarding the stability of niacinamide, the pH, mass loss (only the niosome gel), light microscopy, visual appearances and zeta-potential.

Keywords: Niacinamide, Vesicle systems, Transdermal diffusion studies, accelerated stability tests, semi-solid dosage forms.

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Uittreksel

Niasienamied beter bekend as vitamien B_3 is wateroplosbaar en neem deel aan oksidasie-reduksie reaksies in die mitochondria van die vel (Bissett, 2009:438). As gevolg van die deelname aan die reaksies besit die aktief anti-inflammatoriese eienskappe, verminder dit transdermale waterverlies (TEWL) en voorkom dit die verlies van dermale kollageen (Mohammed *et al*., 2013:192). Vir dié rede is niasienamied die ideale produk om topikaal aan te wend vir die behandeling van veroudering, hiperpigmentasie, melasma en rosacea (Gehring, 2004:88).

Omdat niasienamied gebruik gaan word vir topikale aanwending moet die fisies-chemiese eienskappe van die aktief asook die fisiese eienskappe van die vel in ag geneem word (Barry, 2007:566). Omdat die vel die buitenste orgaan van die menslike liggaam is, beskerm dit die liggaam deur op te tree as 'n skans teen penetrasie van molekules vanuit die eksterne omgewing (Bolzinger *et al*., 2012:156). Hierdie beskermingsfunksie van die vel vind plaas in die stratum corneum wat die uiterlike gedeelte van die epidermis is (Feingold & Denda, 2012:263). Dus maak hierdie skans funksies van die stratum corneum die aflewering deur die vel amper onmoontlik.

Vir die aktief om deur die vel te penetreer en in die onderliggende lae van die vel te konsentreer moet die molekulêre gewig, oplosbaarheid, verdelingskoeffisiënt en ionisasie in ag geneem word (Benson, 2012). Volgens Barry (2001:102) moet die molekulêre gewig minder wees as 600 Dalton om die vel te penetreer. Die molekulêre gewig van niasienamied is 122.1 Dalton (Dollery, 1999:N62) wat beteken dat dit ideaal sal wees vir topikale aanwending.

Die oplosbaarheid van niasienamied soos bepaal in die studie was 122.70 mg/ml. Volgens Naik *et al*. (2000:319), is die ideale oplosbaarheid hoër as 1 mg/ml en daarom beteken dit dat die aktief ideaal is vir topikale aflewering. Die verdelingskoëffisiënt van niasienamied is bepaal as -0.43. Die waarde gee 'n aanduiding dat die aktief nie ideaal is vir topikale penetrasie nie omdat dit nie beide lipofiele en hidrofiele eienskappe besit nie (Donnelly & Sing, 2015:13). Vir die aktief om die vel te penetreer is die ideale verdelingskoëffisiënt dus tussen 1 en 3. Wanneer die ionisasie in ag geneem word sal die geïoniseerde vorm van 'n molekule die vel moeiliker penetreer as die ongeïoniseerde vorm (Donnelly & Sing, 2015:15). Niasienamied is 99.99 % geïoniseerd by 'n pH van 7.4 wat daartoe lei dat die aktief nie deur die stratum corneum sal beweeg nie. Vir die redes was niasienamied in 'n vesikelsisteem geïnkorporeer om die fisies-chemiese eienskappe wat die afname in penetrasie veroorsaak te maskeer.

Vesikel sisteme is hol partikels wat 'n hidrofiliese kop en 'n hidrofobiese stert besit (Honeywell-Nguyen & Bouwstra, 2008:206). Hierdie sisteme is ideaal vir topikale aflewering van die aktief

omdat hulle beide hidrofiliese en hidrofobiese verbindings kan bevat, hulle die geïnkorporeerde verbinding by ’n spesifieke area kan aflewer en hulle nie toksies is nie (Honeywell-Nguyen & Bouwstra, 2008:206).

Hierdie sisteme word geken as niosome en proniosome. Die enigste verskil tussen niosome en proniosome is die metode van bereiding (Selecie*t al*., 2016:4). Die proniosome word berei om ’n droë poeier te vorm sodat dit ’n langer rakleeftyd het teenoor die niosome (Yeom *et al.,* 2014:83). Proniosome word net voor gebruik gehidreer om dan weer niosome te vorm. Al besit hierdie sisteme baie goeie eienskappe is daar een ding wat nie in hulle guns tel nie en dit is hulle lae viskositeit.

Tydens die studie was die viskositeit van die niosome en die proniosome onder andere 4.036 cP en 3.616 cP. Volgens Barry (2007:595), is die ontwikkeling van ’n produk vir maksimale topikale effek baie belangrik, maar die ontwikkeling van ’n produk wat aanvaarbaar is vir die kliënt van nog meer belang. Pasiënte verkies ’n produk wat maklik uit die houer uitgehaal kan word, eweredig oor die vel kan versprei en weer maklik van die vel verwyder kan word. Dus sal ’n produk wat ’n swak voorkoms het swak pasiënt meewerkendheid tot gevolg hê (Barry, 2007:595). Vir die rede was die niosome geïnkorporeer in ’n jel en ’n room om die viskositeit te verhoog om sodoende dan die pasiënt meewerkendheid te bevorder.

Franz sel diffusie studies was uitgevoer op die preformulering (niosome en proniosome) en op die formulerings (niosoom jel en niosoom room). Eerstens was membraan diffusie studies uitgevoer om te bepaal of die aktief wel vrygestel word uit die formulering. Die bereiding wat die hoogste mediaan vloed waarde gehad het was die proniosome (1 163.58 µg/cm^2.h), gevolg deur die niosome (1 162.58 µg/cm^2.h), dan die niosoom jel (1 042.08 µg/cm^2.h) en laastens die niosoom room (469.72 µg/cm^2.h). Tydens die vel diffusie studie het al die bereidings niasienamied in die reseptor fase afgelewer. Die bereiding met die hoogste mediaan vloed waarde was die niosome (0.844 µg/cm^2.h), gevolg deur die niosoom jel (0.692 µg/cm^2.h), die proniosome (0.462 µg/cm^2.h) en laastens die niosoom room (.172 µg/cm^2.h). Al die bereidings het ook niasienamied in die stratum corneum epidermis (SCE) en in die epidermis-dermis (ED) afgelewer. Die bereiding met die hoogste mediaan konsentrasie in die SCE, was die niosoome (2.13 µg/ml), gevolg deur die niosoom jel (1.94 µg/ml), dan die niosoom room (1.05 µg/ml), en die proniosome (0.71 µg/ml). Die niosome (5.02 µg/ml) was die bereiding met die hoogste mediaan konsentrasie in die ED gevolg deur niosome room (2.86 µg/ml), die proniosome (2.04 µg/ml) en die niosoom jel (1.94 µg/ml). Omdat die doel van die studie was om die aktief topikaal af te lewer, is dit veilig om te se dat al vier die bereidings suksesvol was.

Stabiliteitstoetse was op die niosoom jel en niosoom room uitgevoer oor ’n tydperk van drie maande. Volgens die ICH (2006:3) was die formulerings was gestoor by 25° C / 60 %RH (relatiewe humiditeit), ± 30° C /65 % RH en ± 40° C/75 % RH om te bepaal of enige verandering in die fisies-chemiese eienskappe plaas gevind het. Die chemiese stabiliteit (konsentrasie van die aktief) sowel die fisiese eienskappe (pH, konduktiwiteit , viskositeit, ligmikroskoop veranderinge, visuele veranderinge, massa verlies en zetapotensiaal) was bepaal op die aanvanklike (t=0) sowel na maand 1,2 en 3. Albei die formulerings was stabiel na aanduiding van die konsentrasie van niacinamide, pH, massa verlies (net die niosoom jel), lig mikroskopie, visuele verandering en zetapotensiaal.

Sleutel woorde: Niasienamied, Vesikel sisteem, transdermale diffusie studies, versnelde stabiliteit toetse, semi-soliede doseervorm.

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$$D = \frac{kT}{(6 \pi r \eta)}$$ **Equation 2.1**

Chapter 3

EE% = (Concentration (t) – Concentration (f)/Concentration (t)) x100 **(1)**

Appendix A

$$y = mx + c$$ **Equation A.1**

Appendix B

$$EE\% = \frac{\text{Concentration (t) - Concentration (f)}}{\text{Concentration (t)}} \times 100$$ **Equation B.1**

Appendix C

$$V = \frac{2 r^2 (\rho_1 - \rho_2) g}{9 \eta}$$ **Equation C.1**

Appendix E

Log D = Concentration in *n*-octanol/Concentration in PBS **Equation E.1**

Chapter 1

Introduction and problem statement

1.1 Introduction

Niacinamide, an amide form of vitamin B_3, is water soluble and possesses a wide range of mechanisms of action. This active is not naturally found in the body and may be obtained through the intake of foods, such as chicken, beef, pork, fish, nuts, mushrooms and grain products (Chen & Damian, 2014:169). Niacinamide is an anti-oxidant that participates in biological oxidation-reduction reactions and furthermore possesses anti-inflammatory action (Mohammed *et al*., 2013:192). In addition to its anti-inflammatory properties, this active also causes a decrease in transepidermal water loss (TEWL) by stimulating ceramide synthesis, which normally decreases during aging (Lupo, 2001:468). According to Bissett (2009:435), niacinamide has demonstrated the prevention of photocarcinogenesis, which prevents the loss of dermal collagen and accompanies photo-aging. Niacinamide has also shown anti-carcinogenic action by acting as a protease inhibitor that stimulates the repair of deoxyribonucleic acid (DNA) (Lupo, 2001:468). Due to the working mechanisms of niacinamide, it can be used to treat a wide range of dermatological conditions that include wrinkling (anti-aging), hyperpigmentation and red blotchiness (Chen & Damian, 2014:169).

According to Flynn (2008:261), wrinkles occur when the skin loses the ability to return to its original form after having been stretched. Due to niacinamide causing a decrease in TEWL by inhibiting keratinocytes, it can be used as an anti-wrinkle substance.

Hyperpigmentation is a skin disorder caused by an acute inflammatory response, such as sunburn, or by exposure to ultraviolet (UV) rays (Hakozaki *et al*., 2010:496). According to Rossi and Perez (2011:313), the inflammatory response causes an over stimulation of melanin production, which leads to the skin becoming increasingly pigmented or coloured. Hyperpigmentation can be treated with drugs and actives that possess anti-inflammatory action, such as niacinamide (Chen & Damian, 2014:169).

Red blotchiness may be treated with niacinamide since one of its functions is to improve the skin's barrier function by increasing the lipid and protein components of the stratum corneum. This enhanced skin barrier function results in a reduction in TEWL and an increase in the skin's resistance to possible harm, which would mean less irritation and redness (Bissett *el al.,* 2004:236).

The skin is the largest organ of the human body and acts as a chemical and physical barrier between the body and the external environment (Venus *et al.,* 2011:471). According to Williams (2003:2), the skin can be divided into four main layers:

- the innermost, subcortical fat layer (hypodermis),
- the overlying dermis,
- the viable epidermis, and
- the outermost layer, the stratum corneum that is a non-viable epidermal layer.

The hypodermis, the overlying dermis and the viable epidermis differ from the stratum corneum, because of the differences in polarity of these layers (Honeywell-Nguyen & Bouwstra, 2008:410). Since the stratum corneum consists of corneocytes that are embedded in a lipid matrix, the barrier function of the skin is located in the stratum corneum (Honeywell-Nguyen & Bouwstra, 2008:410). The hypodermis, the overlying dermis and the viable epidermis are predominantly hydrophilic (Honeywell-Nguyen & Bouwstra, 2008:410), which means that any substance being applied to the stratum corneum must possess both lipophilic and hydrophilic properties in order for it to cross this skin layer.

Given that niacinamide will be used for cosmetic action during this study, it must be formulated for topical delivery to the skin, as the target-site will be the dermis. Since this active is hydrophilic (log D = - 0.32), it will not cross the stratum corneum for topical delivery easily, unless the formulation is adjusted to compensate for this characteristic. Another formulation challenge of niacinamide will be to avoid hydrolysis, as it could lead to severe skin reddening or a flushing response (Bissett, 2009:437). Hydrolysis can be prevented by formulating this active at a pH range of 4 – 7 (Bissett, 2009:437). According to Benson (2012), the physicochemical characteristics that require consideration during formulation include the partition coefficient, molecular size and solubility of the drug. With an optimal octanol-water partition coefficient (log P) of 2 – 3, molecules with an intermediate lipophilicity can penetrate both the hydrophilic and lipophilic layers. Solubility indirectly influences the diffusion coefficient within the stratum corneum, which means that for ideal skin penetration properties, the active must be both lipid soluble and water soluble; the melting point can be used as a predictor of the aqueous solubility of the active. The ideal size for excellent penetration is less than 500 Da (Benson, 2012). Niacinamide consists of a molecular weight of 122.12 Da, a melting point of 128 – 131 °C and is soluble in water. The solubility of niacinamide, in a phosphate buffered solution (PBS) at a pH of 7.4 at 32° C, is 212.95 mg/ml, and its octanol-buffer distribution coefficient (log D) value is 0.32 (Jacobs, 2009:ii). Due to its solubility in PBS and its log D value, niacinamide will be formulated into a vesicle system, i.e. niosomes and proniosomes, to attempt its successful topical delivery.

A vesicle system is a hollow colloidal particle that consists of a polar hydrophilic head and a hydrophobic tail to form amphiphilic molecules. Due to its amphiphilic property, a vesicle can entrap both water soluble and water insoluble substances, which makes it ideal for entrapping the active, niacinamide (Honeywell-Nguyen & Bouwstra, 2008:206). The hydrophilic niacinamide active will be entrapped within the internal aqueous compartment of the vesicles. Vesicles are mostly prepared using phospholipids, or non-ionic surfactants and the composition can influence the physical and chemical properties of the active, which can increase the effectiveness of delivery to the skin (Honeywell-Nguyen & Bouwstra, 2008:206).

According to Honeywell-Nguyen and Bouwstra, 2008:206, a vesicle should improve the topical delivery of the active, because:

- A vesicle acts as a delivery system for the entrapped active at a specific location. In this study, the drug target-site will be the dermis. For this reason, a vesicle system will be used to improve the concentration of the active in the dermis and to reduce systemic uptake of the active.
- A vesicle acts as a carrier. This should improve drug delivery, as will be attempted during this study, because of the lipophilic character of the stratum corneum and the hydrophilic character of the active, both of which are not conducive to drug transport across the skin.
- Vesicles have little toxicity and are biodegradable. The importance thereof to this study stems from the fact that the active will be in direct contact with the human skin.
- Vesicles also have cosmetic properties, such as smoothing of the skin and an increase in the moisture of the skin.

Niosomes are vesicles that are non-ionic surfactants and are formed through self-assembly of the non-ionic amphiphiles in an aqueous medium. These non-ionic surfactant vesicles have more advantages, compared to liposomes, such as a higher chemical stability, they show better skin penetration and lower costs are involved during manufacturing (Uchegbu & Suresh, 1998:33). According to Carafa *et al.* (2013:74), the characterisation of niosomes is important for the quality control of the resultant product. The characterisation of the vesicle system would indirectly affect the stability of the product, as well as its development and clinical applications. Such characterisation includes morphology, size, number of lamellae, zeta-potential, bilayer fluidity, composition, pH and entrapment efficiency. The entrapped efficiency of a vesicle measures the amount of entrapped active that has been delivered by the niosome to the target-area and can be determined through spectrophotometry. Another important characterisation, as stated above, is the pH of the vesicle system. The pH of the vesicle system must be thoroughly studied because

of the effect of its pH on its physical stability, therefore, the pH of the system must be adjusted with PBS buffers to a pH of 7.4 or at 5.5.

The difference between niosomes and proniosomes is that proniosomes are a dry formulation which transforms into niosomes when the dry powder is hydrated (Yeom *et al.,* 2014:83), whereas the niosome is coated with a water soluble carrier in non-ionic surfactants. Proniosomes are formulated to be hydrated before use and have many advantages compared to niosomes, such as lower aggregation of the particles, fusion and leakage (Yeom *et al.,* 2014:83).

Since vesicle systems are ideal for topical delivery of the active, further formulation of a suitable semi-solid dosage form such as gels and creams must be brought into consideration. Niosomes are formulations with a very low viscosity, causing topical application to be a problem. By incorporating niosomes into a gel and a cream ease application and may improve the therapeutic efficacy and prolonged shelf–life (Verma & Pathak, 2012:489).

Gel formulations are colloids which consist of 99% liquid and are designed for topical delivery (Gupta *et al*., 2010:250). Gel formulations are used for topical application because they provide a suitable delivery system since they are less greasy and may be easily removed from the skin (Patel *et al*., 2010:156). According to Barry *et al*. (2002:594), creams are semi-solid emulsions which may be used for application to the skin and may be in the form of oil-in-water (o/w) or water-in-oil (w/o). Most patients prefer the o/w cream as it spreads more rapidly, tends to be less greasy and smooths the inflamed tissue because of the water that evaporates (Barry *et al*., 2002:594).

1.2 Research problem

The topical delivery of niacinamide is a problem since its physicochemical properties are not ideal. Niacinamide is extremely hydrophilic with an aqueous solubility of 212.95 mg/ml (Jacobs, 2009:ii), making topical delivery quite difficult, as the hydrophilic niacinamide will struggle to cross the stratum corneum.

1.3 Aim and objectives

The aim of this study is to deliver niacinamide topically by formulating it into a vesicle system, i.e. niosomes and proniosomes. To achieve this aim, the objectives were to:

- Validate a high performance liquid chromatographic (HPLC) method to determine the concentration of niacinamide.
- Determine the aqueous solubility and log D of niacinamide at pH 7.4.
- Formulate niosomes and proniosomes, containing niacinamide in a chloroform mixture.
- Characterise the vesicle system by performing the following tests, i.e. pH sensitivity, entrapment efficiency, morphology, zeta-potential, surface properties, viscosity and vesicle size.
- Formulate a gel and cream that contains niosomes for cosmetic action.
- Perform membrane studies to determine the release of niacinamide from the vesicle system, cream and the gel.
- Perform transdermal diffusion studies with vertical Franz cells, as well as tape stripping, by using dermatomed, Caucasian skin to determine the concentration of niacinamide being delivered through and into the skin, respectively.
- Accelerated stability testing on the cream and gel will be performed at 0, 1, 2 and 3 months. The conditions will consist of three different temperatures at a specific relative humidity (RH), i.e. 25° C/60% RH; 30° C/60% RH and 40° C/75%RH.

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Chapter 2

Formulation of niosomes and proniosomes containing niacinamide into a semi-solid dosage form

2.1 Introduction

According to Manela-Azulay *et al.* (2009:496), the term cosmeceutical was created 25 years ago by Albert Kligman as any substance that cannot be considered as a drug. It can be seen as a logical concept leading to advances in skin anatomy and physiology. These active substances are found in many forms including peptides, growth factors and vitamins, hence, vitamins that are topically applied show promising effects on the human skin.

Niacinamide (vitamin B_3) is a water-soluble vitamin that is converted to coenzymes, such as NAD (nicotinamide adenine dinucleotide) and NADP (nicotinamide adenine dinucleotide phosphate) (Dollery, 1999:N62). Since niacinamide is not naturally stored in the body, it can be obtained by the intake of vitamin B_3 and tryptophan (Chen & Damian, 2014:169). Vitamin B_3 can be found in meats, seafood, fruits (such as apples and bananas) and dairy products (Chen & Damian, 2014:169). Niacinamide is a white odourless crystalline powder with a bitter taste and since its working mechanism is to regulate enzymes, which plays a role in deoxyribonucleic acid (DNA) repair as well as the expression of inflammatory cytokines, it can be used topically as an anti-inflammatory, anti-aging and moisturising agent (Chen & Damian, 2014:169). Therefore, the active may be used for the treatment of conditions such as hyperpigmentation, rosacea, anti-aging and melasma (Gehring, 2004:88).

For the treatment of these conditions, the active should be applied to the skin and enter through the outermost layer, known as the stratum corneum (Jepps *et al*., 2013:153) there after it will pass through the underlying layers, namely the epidermis, dermis and the hypodermis in order to reach the target site. According to Roman *et al*. (2004:1818), topical delivery is difficult to achieve due to the excellent composition and barrier function of the skin. Only a few actives are able to diffuse passively through the lipid stratum corneum to the aqueous domains.

When using niacinamide in a cosmetic product for topical delivery, its physicochemical properties should be considered. Since vitamin B_3 is highly soluble in water (log D = -0.32) (Dollery, 1999:N62) and it has to pass through the lipophilic stratum corneum, topical delivery of niacinamide might be a challenge. Consequently, niacinamide can be incorporated into a vesicle system, i.e. niosomes and proniosomes, to improve penetration through the skin (Azeem *et al*.,

2009:671). Niosomes are surfactants formed by self-assembly of non-ionic surfactants and are capable to encapsulate both lipid soluble and water-soluble drugs (Ruckmani & Sankar, 2010:1119). Proniosomes are a dry white powder which forms niosomes after hydration. This is only a different method for manufacturing niosomes and is used to extend the storage period (Yeom *et al.,* 2014:83).

According to Mahato (2007:183), semi-solid preparations are lotions, creams, gels and pastes containing dissolved actives and may be used for topical delivery. A product designed for topical delivery is used for local effect to treat dermal disorders (Mahato, 2007:183). Pharmaceutical gels are semi-solid two-compartment systems, which are rich in liquid (Barry, 2007:593). According to Kurian and Barankin (2011:4), gels lead to a high patient satisfaction due to their cooling effect, have fast onset of action and high water content. Conversely, creams are semi-solid emulsions with a creamy appearance and can be used as either an oil-in-water (o/w), or water-in-oil (w/o) emulsion (Mahato, 2007:187).

2.2 Niacinamide

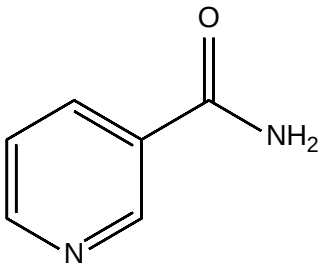
Niacinamide, an amide form of vitamin B_3, is chemically known as pytidine-3-carboxamide, which was isolated for the first time in 1935 from the erythrocytes of horses (Thomas *et al*., 2012:86). This vitamin is water-soluble and can be found in meat, fish and vegetables. Niacinamide is an important component that is involved in hydrogen transfers and is converted to NAD and NADP (Gehring, 2004:88). This reduced form of the molecule acts as co-enzymes for many cellular oxidation-reduction reactions (Bissett, 2009:438), therefore, the intake of niacinamide is very important. This means a deficiency of niacinamide can lead to symptoms such as pellagra, which is characterised by diarrhoea, dermatitis and dementia. Topical pellagra symptoms usually occur on the skin areas most frequently exposed to sunlight. First, it causes sunburn on the applied area, leading to a thickening of the skin with flaking; the skin also takes on a brown-blue shade, while blisters may appear leading to a strong itching sensation.

Although the deficiency of niacinamide sounds serious, the intake per day exceeding 14 mg/kg for a female and 16 mg/kg for a male may cause side-effects (Sinthupoom *et al*., 2015:11) such as flushing and gastrointestinal symptoms. The flushing-effect of niacinamide is caused by the stimulation of the GPR 109A receptors, which leads to a dilatation of the cutaneous blood vessels causing a rise in facial erythema, tangling and itching.

When considering the physicochemical characteristics of niacinamide, it is a heterocyclic amide and the stability can be influenced by factors that may include temperature, moisture, light, pH and oxygen. Exposure of the active to these conditions may result in a decrease in stability of

the active and the effect thereof (Elaine *et al*., 2009:161). The physicochemical characteristics of niacinamide are given in Table 2.1.

Table 2.1: The physicochemical characteristics of niacinamide (Jacobs, 2009:11).

Chemical name	3-Pyridinecarboxamide; nicotinic acid amide
Generic name	Niacinamide; nicotinamide; vitamin B3; vitamin PP
pKa	3.3
Molecular weight	122.13 g/mol
Melting point	128 – 131 °C
Appearance	White crystal powder
Taste	Bitter and salty
Density	1.44 g/cm^3
pH	pH 3.0 – 3.5
Log D	- 0.32
Solubility	212.95 mg/ml
Chemical structure	

As mentioned before, niacinamide is converted to NAD and NADP, which takes part in oxidation-reduction reactions. Playing a part in this reactions niacinamide may be considered to be applied topically for cosmetic action. When applying the active topically, it shows a stabilising effect on epidermal barrier function that causes a reduction in the transepidermal water loss (TEWL) and an improvement in the moisture content of the horny layer (Gehring, 2004:88). Niacinamide also leads to an increase in protein synthesis,such as keratin, has a stimulating effect on ceramide synthesis and speeds up the differentiation of keratinocytes. It may act as an antioxidant and may possess anti-inflammatory properties, which leads to improving the surface structure of the skin. It inhibits photocarcinogenesis and smooths wrinkles and therefore can be used to treat aging (Gehring, 2004:88). Due to these verifiable effects, niacinamide could be suitable for use in disorders such as aging, hyperpigmentation, melasma rosacea, acne and wound healing (Gehring, 2004:88).

2.3 Aging

According to Batisse *et al*. (2002:148), the skin is transformed in terms of its composition, structural organisation and size during aging. The aging process of human skin can be commonly associated with an increase in wrinkles, laxity and sagging (Jenkins 2002:801). Wrinkling is a phenomenon common to human skin. As the skin ages, its ability to return to its original shape diminishes and this leads to permanent wrinkles developing over time (Flynn & McCormack 2008:261). Skin aging has both intrinsic and extrinsic causes, and although these types differ, the effect is synergistic and leads to an aged look to the skin.

2.3.1 Intrinsic aging

Intrinsic aging occurs as a natural consequence of physiological changes over time and can also be called chronological aging. In the case of intrinsic aging, the genetics of an individual are responsible for the effect, although other factors can also be present (Ramos-e-Silva *et al*., 2013:751). The stratum corneum stays relatively unchanged, where the dermis and the epidermis becomes thin leaving the dermal-epidermal junctions flattened. In the papillary dermis there is a progressive disappearance of elastic tissue and a reduction in the quantity and capacity of the fibroblasts (Jenkins 2002:801).

According to Ramos-e-Silva *et al*. (2013:751), multiple factors can lead to intrinsic aging and can include:

- Ethnicity: high levels of melanin can lead to a difference in the pigmentation of the skin.
- Anatomic variations: due to the skin around the eyelids being thinner than other areas, this causes aging to be more visible in these areas.
- Hormonal changes: during the time of menopause the levels of oestrogen decreases and influence the synthesis of collagen by fibroblasts, resulting in the synthesis of hyaluronic acid causing water retention and an increase in the extracellular matrix.

2.3.2 Extrinsic aging

Extrinsic aging takes place due to controllable factors and shows different degrees of intensity (Ramos-e-Silva *et al.,* 2013:751). This aging is primarily the result of exposure of the skin to ultraviolet (UV) light, which can also be called photodamaged skin. This photodamaged skin is characterised by the loss of elasticity, increase in roughness and dryness, irregular pigmentation and deep wrinkles. The changes that occur in the epidermis of this photodamaged skin include a decrease in the thickness of the epidermis and loss of epidermal polarity (Jenkins 2002:801).

The factors related to extrinsic aging (Ramos-e-Silva et al. 2013:751) are:

- Ambient conditions: High temperatures lead to an increase in water evaporation causing an inappropriate formation in the structure proteins and lipids in the skin.
- Drugs: Causing and inducing xeroderma and desquamation.
- Smoking: Smoking causes a decrease in the blood flow in the capillary vessels, leading to the deprivation of nutrients in the cutaneous tissues.
- Sunlight exposure: Sunlight induces molecular and cellular changes that trigger a fast and dynamic disorder in the skin.

Although aging is a major concern, it can be treated with formulations containing acids, antioxidants and retinoids (Jenkins 2002:801).

2.3.3 The treatment of aging with niacinamide

According to Ramos-e-Silva *et al*. (2013:754), antioxidants can be used since they are responsible for reducing the damage caused by free radicals and can also offer protection against photodamage, as well as skin cancer by inhibiting inflammation. These antioxidants include: alpha-lipoic acid, ascorbic acid and niacinamide.

As stated above, niacinamide is a powerful and well-tolerated antioxidant that can be used to treat skin aging. Niacinamide causes an improvement in the lipid compound in the epidermal barrier and this leads to reduced TEWL. It acts as a melanosome transfer inhibitor that leads to improvement of hyperpigmentation and the skin's elasticity. According to Sinthupoom *et al*. (2015:6), the inhibition of oxidants is one of the best ways to prevent wrinkles, since potent antioxidants such as NAD and NADP inhibit collagenase and elastase breakdown. The co-enzymes of niacinamide are responsible for maintaining the moisture in the skin by increasing the ceramide levels.

2.4 Hyperpigmentation

In most regions of the world, having a light and even skin tone is highly valued, therefore hyperpigmentation skin disorders such as melasma, post-inflammatory hyperpigmentation and hypopigmentation are experienced as a major problem (Hakozaki *et al*., 2006:105). According to Fisk *et al*. (2014:353), hyperpigmentation results from an excessive deposition of melanin that leads to an increase in the pigment density in areas of unusual pigment dispersion. According to Nicolaidou *et al*. (2014:66), pigmentation disorders may include a number of heterogeneous conditions, which can be characterised by altered melanocyte density, melanin concentrations or both and result in pigmentation of the skin.

2.4.1 The treatment of hyperpigmentation with niacinamide

Since niacinamide functions as part of co-enzyme I (NAD), co-enzyme II (NADP) and are involved in over 200 enzymatic reactions, it causes inhibition of melanocyte transfer from keratinocytes to melanocytes and can be used to treat hyperpigmentation (Hakozaki *et al*., 2006:110). Thus niacinamide will improve the skin colour by reducing hyperpigmented spots and red blotchiness (Ramos-e-Silva *et al*., 2013:751).

2.5 Melasma

Melasma is a common dysfunction of the pigmentary system in the skin, which results in an irregular brown or greyish-brown hypermelanosis that mostly affects the areas of the skin exposed to the sun, such as the face, neck and forearms (Nicolaidou *et al*., 2014:66). According to Gupta *et al. (*2006:1048), this skin condition usually develops symmetrically and very slowly, lasting for many years, worsening in the summer and improving in the winter. During exposure to the sun, the UV irradiation causes melanogenesis and melanocyte proliferation, as well as cell migration causing the skin to change colour (Kang, 2014:292). The pathogenesis that can cause this disorder is pregnancy, exogenous hormones (oral contraceptives and hormonal replacement therapy), thyroid dysfunction, cosmetics and phototoxicity.

2.5.1 The treatment of melasma with niacinamide

Melasma treatment is challenging, requiring a specialised approach, especially in dark-skinned patients. Niacinamide can be used to treat melasma because it inhibits melanosome transfer to keratinocytes and thereby reduces the pigmentation (Arya, 2015:46).

2.6 Rosacea

Rosacea is a chronic inflammatory skin disease, which mostly affects the central region of the face that includes the nose, cheeks, chin, forehead and glabella. Rosacea is mostly characterised by signs of erythema, telangiectasia, papules and pustules (Barco & Alomar, 2008:244). In many individuals, rosacea may be worsened by factors that trigger flushing, such as exercise, emotion, menopause and alcohol (Yamasaki & Gallo, 2009:77). Rosacea mainly affects individuals between the ages of 30 and 50 and in Europe, between 1.5% and 10.0% of the population is diagnosed with this disease.

Although the pathophysiologic mechanisms that are responsible for this disease are not well understood, there are a number of possibilities, such as innate immunity, vascular changes, reactive oxygen species, UV radiation and microbes (Yamasaki & Gallo, 2009:77). By triggering the innate immune system, it can lead to a controlled increase of cytokines and anti-microbial

molecules in the skin. Cathelicidin is an anti-microbial molecule that is expressed in high levels in individuals with rosacea. These peptides are vasoactive and can cause a post inflammatory response.

This chronic inflammatory disease is classified into four subtypes (Crawford *et al.,* 2004:327):

- Erythematotelangiectatic type (ETR)
- Papulopustular rosacea (PPR)
- Phymatous rosacea
- Ocular rosacea

2.6.1 The treatment of rosacea with niacinamide

According to Wozniackz *et al*. (2005:632), the treatment of rosacea with niacinamide is used because of the interfering effect niacinamide has on the inflammatory process. 1-Methylnicotinamide (MNA) is one of the primary metabolites of Niacinamide, which possesses anti-inflammatory properties, hence, this causes the active to be used in dermatitis, burns and rosacea. The mechanism can be explained by the ability of MNA to stimulate the release of prostacyclin (PGI_2), causing a decrease in the response of the post-inflammatory cells to the surface of the vascular endothelium. Since the molecule MNA is ionic in character, it can bind to the glycosaminoglycans, which may have a beneficial effect (Wozniackz *et al*., 2005:634).

2.7 The skin

The skin is the largest organ of the human body and consists of almost 16% of the total body weight (Wickett & Visscher, 2006:98). The skin performs many vital functions, including protecting the body against external, physical, chemical and biological assailants, playing a role in thermoregulation and preventing excess water loss (Kolarsick *et al.,* 2011:204). As the epidermis contains anti-microbial peptides, Langerhans cells, as well as T-cells, the skin may act as a first-line defence for the human body.

The human skin is composed of several layers that differ in their composition and structure (Bolzinger *et al.,* 2012:156). The main layers of the human skin are:

- Stratum corneum
- Epidermis
- Dermis

2.7.1 The stratum corneum

According to Foldvari (2000:417), the stratum corneum is the outermost layer of the epidermis, highly hydrophobic and containing between 10 – 15 layers of corneocytes, which are embedded in a lipid matrix. The highly hydrophobic property of the stratum corneum is the key to the remarkable defence of the body against chemical and microbiological attack, as well as dehydration (Wickett & Visscher, 2006:99). Transport across the stratum corneum is usually by passive diffusion, whether via the intracellular pathway (restricted to the lipid matrix) or the transcellular pathway (through both the corneocytes and the lipid matrix) (Jepps *et al*., 2013:154).

2.7.2 The epidermis

The epidermis is an avascular environment that is populated with keratinocytes, melanocytes, Langerhans cells and Merkel cells, which consist approximately of 40% protein, 40% water and 10 – 20% lipids (Fore-Pfliger, 2004:417-418). The main role of the epidermis is to produce the stratum corneum and to act as a barrier to regulate the intracellular lipid channels (Mikesh *et al*., 2013:190). When investigating diffusion through the epidermis, it can be considered as diffusion through an aqueous medium that is hindered by the presence of proteins (Jepps *et al*., 2013:154).

2.7.3 The dermis

According to Venus *et al*. (2011:469), the dermis is a resilient layer that protects the body against mechanical injury. The dermis is the junction between the epidermis and the internal subcutaneous tissue, and because it is connective tissue, it contains cells and fibres. The cells are fibroblasts, which synthesise collagen and elastin fibres. Elastin that is formed by fibroblast provides the skin with a degree of elasticity, where the collagen is an important substance for skin aging.

2.7.4 Transport through the human skin

Transdermal delivery is the transport of a substance from the surface of the skin through the underlying layers into the blood circulation. Transdermal delivery contains many advantages over other delivery methods including, avoiding pain, excluding possible infection that may occur with injections, controlling the release of a substance over a period and avoiding the first-pass metabolism that takes place in the liver during oral administration (Jepps *et al*., 2013:153).

Since the skin acts as a barrier for chemical substances, the active has three potential pathways to move through to the viable tissue when applied topically. As seen in Figure 2.1 this can take place through the hair follicles with their associated sebaceous glands, through the sweat ducts or across the stratum corneum (Barry, 2001:101).

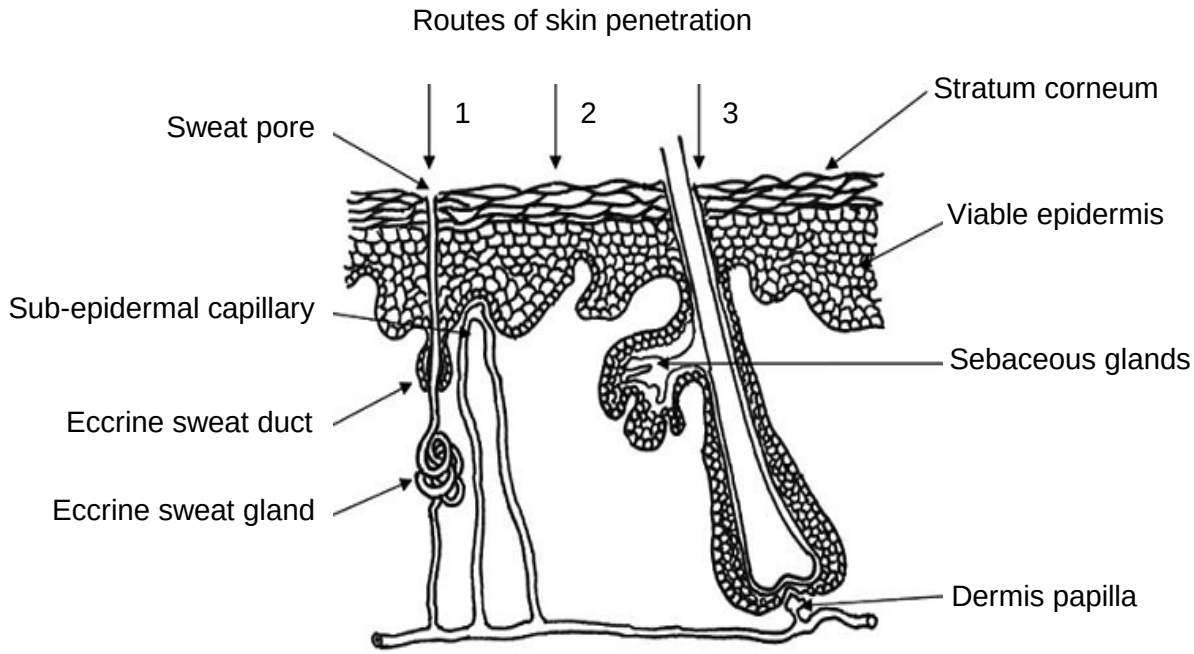


Figure 2.1: Skin penetration routes (Barry, 2001:101).

Different mechanisms exist by which the absorption through the skin may occur, namely the transepidermal route and transappendageal route.

2.7.4.1 The transepidermal route

The transepidermal route is divided into the transcellular and the paracellular route.

2.7.4.1.1 The transcellular route

According to Bhowmick and Sengodan (2013:637), this transport, also known as the intracellular route, takes place when molecules cross the epithelial membrane. It includes passive and active transport of ionic and polar compounds.

2.7.4.1.2 The paracellular route

The paracellular route or intercellular route takes place when molecules transport around or between the cells. In the intercellular route, tight junctions are normally found between the cells (Bhowmick & Sengodan, 2013:637).

2.7.4.2 Transappendageal diffusion

This type of diffusion is known as the 'random walk' of molecules through the structures within the skin and is one of the primary transports systems. This walk of molecules can take place by active transport (when the transport is facilitated by proteins) or convective transport (when the molecules are driven by the lymphatic or vascular systems or by the interstitial space) (Jepps *et al*., 2013:153). This route, known as the shunt route because it avoids penetration through the stratum corneum (Bhowmick & Sengodan, 2013:638), involves the transport through sweat glands, apocrine glands and hair follicles with its associated sebaceous glands. These processes are dependent on the permeability of each layer and the solubility of the active (Benson 2012).

2.8 Physicochemical properties

For an active to cross the skin barrier at an acceptable rate, the physicochemical properties must be brought into consideration (Barry, 2001:101). The speed at which the active moves through each environment, also known as the diffusion coefficient, is dependent on the molecular size, solubility, the partition coefficient of the molecule and ionisation.

2.8.1 Molecular size

Since the stratum corneum is the main barrier of the body, the shape and size of a molecule will influence diffusion through or into this layer. This leads to an inverse relationship between size and skin penetration, causing a large molecule to be poorly absorbed. The ideal size for skin penetration must be less than 500 Da (Barry, 2001:102). Niacinamide is a very small molecule with a molecular size of 122.1 Da (Dollery, 1999:N62) and therefore could be absorbed well through the skin.

2.8.2 Solubility

The solubility in context to the skin permeability refers to the aqueous solubility of the molecule. The solubility of the molecule plays a large role in determining the rate of penetration (Smith, 1990:25). As the solubility of an active will influence diffusion through the intercellular pathway, the active applied to the skin must be both lipid soluble as well as aqueous soluble. If a substance is too lipophilic, it will accumulate in the stratum corneum causing no transdermal absorption. Conversely, being too hydrophilic will influence the percutaneous penetration, leading to no diffusion through the main barrier of the skin (Benson 2012). Aqueous solubility should be > 1 mg/ml for topical application (Niak *et al*., 2000:319) and since niacinamide has an aqueous solubility of 212.95 mg/ml, meaning that it is extremely soluble in water the permeability through the lipophilic stratum corneum may be a problem (Jacobs, 2009:11).

2.8.3 Partition coefficient

The partition coefficient is the ability of a particle to partition between two immiscible phases in the skin (Donnelly & Sing, 2015:13). If a particle has a high partition coefficient, it is lipophilic and can therefore penetrate the lipid domains of the skin easily. Although lipophilic formulations will have a high solubility in the stratum corneum and the intercellular lipids, the partition through the aqueous viable epidermis will be limited (Benson 2012). For a formulation to penetrate the skin, an optimal log D value of 2 – 3 is required (Benson 2012). In practice, this means that for ideal topical penetration a particle must possess both lipophilic and hydrophilic properties (Donnelly & Sing, 2015:13). The log D value of niacinamide, according to Jacobs (2009:11), is 0.32 meaning the active is more soluble in the water phase than in the lipid phase. The active should therefore easily penetrate through the hydrophilic domains but might struggle to penetrate through the lipid domains.

2.8.4 Ionisation

Since the nature of the stratum corneum is lipophilic and the particle needs to move through a largely lipophilic pathway, the ionised form of the molecule is less likely to penetrate the skin than the unionised form (Donnelly & Sing, 2015:15). This can be explained by the lipophilic regions of the skin that act as a barrier to ionised species, causing this form to pass through pores instead. If niacinamide were 99.99% ionised at a pH of 7.4, the active would rather penetrate the skin through the pores than through the stratum corneum.

2.9 Biological factors that influences topical delivery

Physiological factors can also influence the delivery of the active into and through the skin. These physiological properties may include (Donnelly & Sing, 2015:18):

- Skin condition
- Skin hydration and occlusion
- Skin age
- Race
- Skin temperature

2.9.1 Skin condition

Since the stratum corneum acts as the first-line of defence, any disease that changes the continuity of this barrier can lead to physiological changes. These changes may include an increase in vasodilatation that causes an increase in the permeability of the skin. When the skin is damaged, as in the case of cuts and burns, it can result in a decrease in the barrier function of the skin and an increase in the absorption. According to Mills and Cross (2006:225), objects that lead to skin irritation such as alcohols, i.e. methanol and ethanol, cause the stratum corneum to disrupt because of the extraction of lipids thus affecting the absorption.

2.9.2 Skin hydration and occlusion

When the skin is hydrated, it causes rheological properties to change, leaving the skin more elastic and causing an increase in the rate of penetration of most molecules (Donnelly & Sing, 2015:17). According to Donnelly and Sing (2015:17), this can be explained by the diffusion coefficient. The diffusion coefficient of the stratum corneum increases with an increase in water content and may be influenced by some dosage forms applied to the skin, resulting in hydration and leading to an increased absorption through the skin.

2.9.3 Skin age

When the skin ages, the structure and appearance of the skin significantly changes. According to Donnelly and Sing (2015:18), the permeability of the barrier decreases with age. Hence, permeability of chemicals is higher in infants than in the elderly.

2.9.4 Race

Although there is no difference in skin penetration between different races, i.e. African, Asian and European, it has been proven that darker pigmented skin types have a better barrier that leads to the stratum corneum recovering slightly faster than races containing a light-pigmented skin (Donnelly & Sing 2015:19).

2.9.5 Skin temperature

Temperature can affect skin penetration in two ways; the first mechanism is by affecting the blood flow and the second mechanism is by increasing the metabolism in the skin. The effect of temperature on skin can be seen as directly proportional, meaning that when the temperature increases; the penetration into the skin will increase and when the temperature decreases, the penetration decreases (Donnelly & Sing, 2015:19). Passive transport through the skin is temperature dependant and is illustrated by the Stokes-Einstein equation:

$$D = \frac{kT}{(6 \pi r \eta)}$$ **Equation 2.1**

Where D is the diffusional constant, *k* is the Boltzmann constant, T is the temperature, r is the radius of the diffusing molecule and η the viscosity of the system (Donnelly & Sing, 2015:19).

2.10 Niosomes

According to Moghassemi and Hadjizadeh (2014:22), niosomes are a class of molecules formed by self-association of non-ionic surfactants in an aqueous phase, which is biodegradable and relatively nontoxic. Since niosomes possess this unique structure, they can load both hydrophilic and lipophilic substances and are an effective way of delivery for the oral, parenteral, ocular, vaginal, inhalation and especially, the transdermal route (Azeem *et al*., 2009:671).

Niosomes were developed and patented by L'Oreal in 1975 and were reported a few years later in 1979 by Handjani-Vila *et al.* (Azeem *et al.,* 2009:671). L'Oreal first used this formulation for cosmetic products to moisturise and enrich the skin.

Niosomes can be divided into three different categories (Figure 2.2), namely small unilamellar vesicles (SUV; 10-100 nm) that consists of one bilayer, multi-lamellar vesicles (MLV) where more than one bilayer is present and large unilamellar vesicles (LUV; 100-300 nm) (Azeem *et al*., 2009:671).

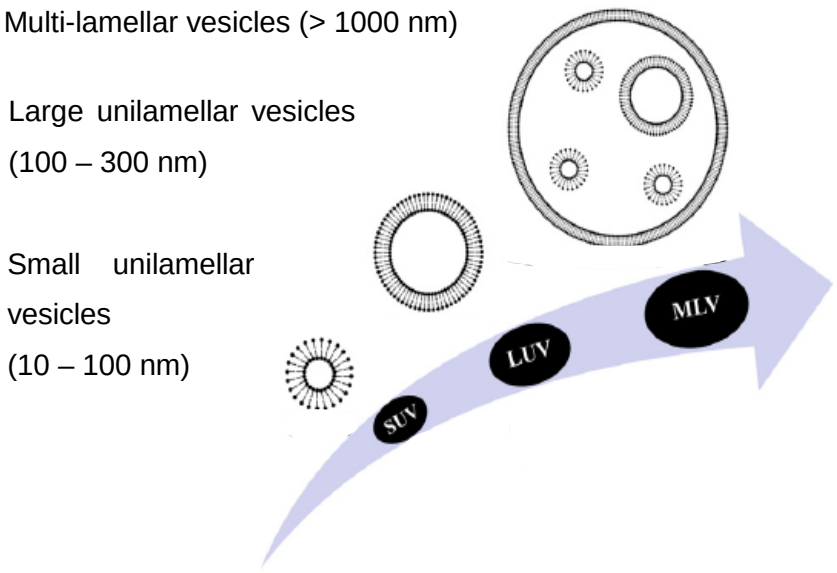


Figure 2.2: Increasing size of niosomes (Azeem *et al*., 2009:671).

Niosomes have advantages when compared to other carriers, including (Azeem *et al*., 2009:671):

- Niosomes are chemically stable and have long storage time compared to liposomes.
- They are osmotically active.
- Due to their hydrophilic heads, their surface modification and surface formulation are very easy.
- One of their properties is that they have a non-ionic nature, which results in high compatibility with biological systems and a low toxicity.
- They are biodegradable and non-immunogenic.
- They can entrap both hydrophilic actives in the vesicular bilayer and lipophilic actives into the aqueous compartment.
- Access to their raw materials is convenient.
- They have a high patient compliance because they have a water-based suspension.
- The handling of the surfactants requires no special precautions compared to phospholipids.
- They increase the oral bioavailability and skin penetration of actives.
- Improving pharmacokinetics and bio-distribution of the active due to higher ratio of surface area to volume.
- Reduces toxicity by accumulation at the target site.
- Enhances intracellular delivery and retention time inside the cell; this leads to an improvement in the therapeutically potential of the active.

The physicochemical characteristics of the niosomes play an important role when determining the quality of the product. These characteristics include the size of the vesicle, morphology, size distribution, charge and zeta-potential, entrapment efficiency, curve of release of the active, stability and viscosity (Abdelkader *et al*., 2010:812).

According to Abdelkader *et al*. (2010:812), the size of a vesicle should be large enough to resist drainage of the active. Considering the size of the vesicles, large multi-lamellar vesicles are more promising than smaller unilamellar ones due to their higher entrapment efficiency and slower drainage. The most important parameter of the niosome is the entrapment efficiency, which is the amount of active concentrated inside the vesicle system (Moghassemi & Hadjizadeh, 2014:31). Zeta-potential is an indication of the charge on the surface of the niosomes and plays

a very important role in the stability of the niosomes. A higher zeta potential leads to increased stability by preventing aggregation and fusion of the vesicles.

Niosomes can be made by using the thin-film hydration method where by an organic solvent, such as chloroform, are mixed with Span® 60 and cholesterol in a round bottomed flask to form the lipid phase. A thin-film of the lipid phase will occur when all the organic solvent has evaporated through rotor evaporation (Yeom *et al.,* 2014:84). This film will then be hydrated with phosphate buffer solution (PBS) at a pH of 7.4 and sonicated three times at 50 Hz for 15 min (Ruckmani & Sankar, 2010:1120). Due to niacinamide being highly water soluble, this means the active will be dissolved in the PBS and then added to the thin-film.

Although niosomes have many advantages, there are a few disadvantages too. This may include hydrolysis and degradation during long storage of niosomes; sedimentation, aggregation or fusion, which directly affects the stability of the product, can also take place (Yeom *et al.,* 2014:83). The proniosomes can be used as an alternative in order to increase stability and shelf life.

2.11 Proniosomes

Proniosomes are a dry powder formulation that transforms into niosomes when hydrated (Rhodes & Hu, 2005:97). According to Rhodes and Hu (2005:97), when formulating the proniosomes the same surfactant mixture that is used when making the niosomes will be used; the only difference in preparation is that the proniosomes include the use of sorbitol. The sorbitol is placed into a roundbottomed flask and attached to a rotary evaporator. The surfactant mixture (lipid phase) will then be sprayed over the sorbitol for the organic solvent to evaporate. After the solvent evaporates, the dry formulation is placed into a desiccator, under a vacuum, overnight in order to dry completely (Rhodes & Hu, 2005:97). When the proniosomes are ready for use, it can be hydrated with PBS (pH 7.4) and sonicated at 50 Hz for 15 min.

According to Marianecci *et al.* (2013:71), sorbitol is used because it is a sugar that acts as a water-soluble carrier. This produces a dry formulation, where the water-soluble particles are covered with a thin-film of surfactants and cholesterol to result in a proniosome. When hydrated, the water phase dissolves the sorbitol and forms a niosome.

If niosomes and hydrated proniosomes have a very low viscosity, this will indirectly lead to an unstable vesicle system. According to Romanowski and Schuller (2006:662), the stability can be influenced by aggregation or leakage of the particles in the vesicle system. Aggregation or leakage can be described by the strong mechanical forces between the particles in the system causing the fragile lipid layer to merge. For this reason, it may be useful formulating the niosomes

into semi-solid dosage forms, which will increase the viscosity, leading to less fusion of the particles. Not only will this lead to a more stable formulation, the product will be more acceptable for the patient and enhance the topical delivery of the active.

2.12 Semi-solid dosage forms

Semi-solid formulations are preparations designed for topical or transdermal use. The topical formulation is designed to deliver the active into the skin and to treat local skin conditions. For transdermal use, they are formulated to deliver the active into the systemic circulation. These formulations are used due to them being less irritating to the skin than solid solutions (Van Abbe *et al*., 1969:110). The topical products include liquid preparations, gels, ointments, creams, pastes and aerosols.

Pharmaceutical gels are semi-solid, two-compartment systems, which are rich in liquid and especially used for topical application (Barry, 2002:529). Depending on the nature of the solvent, gels can be divided into two groups, hydrogels or organogels. The hydrogels are single-phased systems, which contain water, whilst the organogels are two-phased systems and consist of mineral oil and water (Ramchandani & Toddywala, 1997:547). Gels are formulated by using gelling agents (carbomer 934, cellulose derivate and natural gums), water, an active, solvents (propylene glycol), antimicrobial preservatives (methyl- and propylparaben) and stabilisers. The consistency of the gel is affected by the concentration of the gelling agent added. The gelling agent may affect the viscosity of the system, resulting in Newtonian, plastic or pseudo-plastic behaviour (Ramchandani & Toddywala, 1997:547).

Pharmaceutical creams are semi-solid emulsions, which may be used for external application and can either be an o/w or a w/o emulsion. When a cream is applied to the skin, the continuous phase evaporates causing the water-soluble active's concentration to increase in the remaining film, leading to a better percutaneous penetration (Mahato, 2002:187). Creams are prepared by first mixing the oil phase and the water phase separately. The oil phase may consist of ingredients such as mineral oil, cetyl alcohol, stearic acid and lanolin, and the water phase: triethanolamine, propylene glycol, water and preservatives (Hunting, 1993:103).

2.13 Conclusion

In this study, niacinamide will be used for topical application. The physicochemical characteristics of niacinamide are not ideal for topical delivery and for this reason, the active will be encapsulated into a vesicle system, such as niosomes and proniosomes, to try to improve the topical delivery. Furthermore, the niosomes will be formulated into a semi-solid dosage form in order to achieve improved stability, a longer shelflife and easier application of the product.

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Chapter 3

Article for publication in Die Pharmazie

Chapter 3 is written in article format for publication in *Die Pharmazie*. The article is written in US English and the complete authors guide can be found in Appendix H.

Topical delivery of niacinamide encapsulated in niosomes and semi-solid formulations

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Abstract

The aim of this study was to deliver niacinamide topically in order to treat skin conditions such as hyperpigmentation, rosacea, anti-aging and melasma. Niosomes and proniosomes were formulated containing 3% niacinamide, and had a vesicle size of 668.55 ± 101.75 nm and 593.33 ± 70.55 nm, respectively. Furthermore, the zeta-potential, entrapment efficiency, pH and the viscosity of the vesicle systems were determined. There after niosomes were incorporated into a niosome gel and niosome cream on which accelerated stability tests were performed. During this study, the solubility and octanol-buffer distribution coefficient (log D) of niacinamide was determined as 112.70 mg/ml and -0.43, respectively. Membrane diffusion studies were performed to indicate if niacinamide had released from the preparations and the proniosomes presented with the highest median flux, followed by the niosomes, niosomes gel and lastly, the niosome cream. The preparation with the highest median flux during the skin diffusion studies were the niosomes, followed by the niosome gel, the proniosomes and the niosome cream. All the preparations delivered niacinamide into the stratum corneum-epidermis (SCE) and dermis-epidermis (ED). The preparation with the highest median concentration of niacinamide in the SCE was the niosomes, followed by the niosome gel, niosome cream, and lastly, the proniosomes. The niosomes also presented with the highest median concentration of niacinamide into the ED, followed by the niosome cream, the proniosomes and the niosome gel.

Keywords: Niacinamide, Niosomes, Transdermal diffusion studies, Tape stripping, Accelerated stability tests, Semi-solid dosage forms.

1 Introduction

Niacinamide (vitamin B_3) is a water-soluble vitamin, which shows some benefits when applied to the skin (Lupo 2001). The active may act as an antioxidant and possesses anti-inflammatory action, treating conditions such as hyperpigmentation, rosacea, anti-aging and melasma (Mohammed *et al*. 2013; Gehring 2004). Considering the active is water soluble, it may cause a problem penetrating through the lipid domains of the skin. Another problem of the active is the partition coefficient and the ionization. These two physicochemical properties of the active will also lead to little or no skin penetration. During this study, the active was incorporated into a vesicle system, niosomes and proniosomes in order to overcome these physicochemical properties. Niosomes and proniosomes are used since they enhance the topical delivery of the encapsulated active. Niosomes are structures, which are formed by self-association of non-ionic surfactants in an aqueous phase (Moghassemi and Hadjizadeh 2014). These vesicle systems consist of a polar hydrophilic head and a hydrophobic tail, making it possible to encapsulate the hydrophilic active. Vesicle systems are used since they enhance topical delivery (Azeem *et al*. 2009). The proniosomes differ from the niosomes in their formulation (Seleci *et al*. 2016), and have a longer shelf life than the niosomes. The proniosomes are formulated to form a dry powder, which is hydrated to form niosomes, just before use (Rhodes and Hu 2005).

Although these vesicle systems are only pre-formulations, they were incorporated into semi-solid dosage forms, where after accelerated stability tests were performed to determine the stability of the semi-solid dosage forms. Membrane studies were performed to demonstrate the release of niacinamide from the formulation, followed by skin diffusion studies and tape stripping.

2 Investigations, results and discussion

2.1 High performance liquid chromatography

The high performance liquid chromatography (HPLC) method for the determination of niacinamide (for the purpose of diffusion studies), as well as the validation of niacinamide, methylparaben and propylparaben (for the purpose of the concentration assay), was validated in terms of linearity range, accuracy, precision, recovery, specificity and ruggedness. The lower limit of detection (LOD) was 0.003 µg/ml and lower limit of quantification (LOQ) was 0.017 µg/ml for niacinamide. During the study, all the validation parameters for both validations met the requirements.

2.2 The characteristics of the vesicle system

Both the niosomes and the proniosomes were characterized in accordance to the vesicle size, morphology, viscosity, zeta-potential, entrapment efficiency and pH. The results obtained are indicated in Table 1.

2.2.1 Vesicle size

The vesicle size was determined during this study, because it indirectly influences the bio-distribution, clearance and the stability of nanoparticles (Gaumet *et al*. 2008). The average size of the niosomes and proniosomes were approximately 706.388 nm and 559.366 nm, respectively.

2.2.2 Morphology

The morphology of the vesicle systems was investigated to characterize the vesicles.

In Fig. 2, the morphology of the niosomes and the proniosomes are presented and in Fig. 2.a and Fig. 2.b, the niosomes present a round shape containing a lipid layer.

In Fig. 2.c the undissolved sorbitol particles can still be noticed inside the proniosome and in Fig 2.d, it is observed that the proniosomes have a lipid layer on the outside. It is also evident that the proniosomes are not completely spherical.

2.2.3 Viscosity

For the purpose of this study, the determination of the viscosity was an important parameter. Since the product will be used topically, the preparation must be removed easily from the container and applied onto the skin. The viscosity of the niosomes and the proniosomes was

determined as 4.036 ± 0.04 cP and 3.616 ± 0.06 cP, respectively. Thus, these values indicate the viscosities of both preparations were very low for topical application.

2.2.4 Zeta-potential

The zeta-potential can be defined as the difference in the potential on the surface of the particle and the electro region of the solution (Roland *et al*. 2003). The zeta-potential of both the vesicle systems were above - 25 mV, implying that both vesicle systems were stable and flocculation and fusion were unlikely to take place. The zeta-potential of the niosomes and the proniosomes were approximately - 45.11 ± 2.80 mV and - 61.32 ± 2.87 mV, respectively; resulting in the two pre-formulations being a deflocculated system.

2.2.5 Entrapment efficiency

The entrapment efficiency was determined to evaluate the entrapped amount of the active. According to Bayindir *et al*. (2009), niosomes can encapsulate between 19.90% and 86.40%, while proniosomes may encapsulate between 20.23% and 91.95% of the active. During the study, the niosomes and proniosomes entrapped approximately 86.70 ± 5.34% and 32.94 ± 1.17%, respectively. These results correlate well with the literature and are in the acceptable ranges.

2.2.6 pH

The pH plays an important role in this study, because the formulations are applied topically. The pH of both vesicle systems was between 5 and 9, which is ideal for topical products since no harm will be done to the stratum corneum (Naik *et al*. 2000). The pH of the niosomes and the proniosomes were approximately 7.28 ± 0.009 and 6.99 ± 0.012, respectively.

2.3 Stability tests of semi-solid dosage forms

The stability tests were performed on the niosome gel and niosome cream over a period of three months. The International Conference on Harmonisation (ICH) (2006) states that a product may be defined to have significant change if 1) an assay deviates by more than 5% from the initial value, 2) failing to meet the acceptance criteria of appearance and pH, as well as 3) the presence of degradation, which exceeds the acceptance criteria.

The data of the stability tests are presented in Tables 2 to 5. As seen in Table 2 and 3, the formulations were prepared containing an excess of the ingredients, to extend the shelf life of the products (Barnes 2007). This way of increasing the storage time may only be done in products where the dose is not critical and the degradation of the products is nontoxic, such as vitamins. Since the active used in this study is vitamin B_3, the products in this study were formulated containing an excess of the labeled amount in order extend the storage time.

In Table 2 and 3, the concentrations of niacinamide, methylparaben and propylparaben in the niosome gel and niosome cream during the three-month test period are presented. The concentrations of all the ingredients in both the products decreased over the stability test period, except for the concentration of niacinamide in the niosome cream, which increased. During the stability test period, niacinamide, methylparaben and propylparaben also presented with inconsistent concentrations, possibly because of inconsistent mixing during the bulk formulation, or appearance of hydrolysis and condensation reactions (Soni *et al*. 2002). Although the concentration of niacinamide fluctuated over the three months in both products, a USP monograph indicated that the final product could contain between 95 and 120% of the active (USP 2016). The fact that the concentration of niacinamide was still in that range, indicated that the preservatives (methyl- and propylparaben) used were both effective (Haley 2009).

Since the pH of a system indicates the ratio and the concentration of hydrogen ions [H^+] to hydroxide ions [OH^+], it may indicate the stability of a product (Hach Company 2010).

The pH values of both the formulations fluctuated over the stability test period, although no significant change could be noticed. In Tables 4 and 5, the pH values of the niosome gel and niosome cream are presented. The pH of the niosome gel increased during the three-month test period, resulting in a formulation that was more basic and contained more [OH^+] ions than [H^+] ions. The pH of the niosome cream decreased during the stability test period, resulting in a more acidic formulation at the end of the test period and containing more [H^+] ions than [OH^+] ions. This change in hydrogen and hydroxide ion concentration may be due to water production in the system.

According to Hach Company (2014), conductivity is the conduction of energy in a solution and is a result of ion movement. The conductivity of the niosome gel increased during the stability test period, which may be caused by an increase in the amount of electrolytes. This appearance may be the result of breakage of the polymer hydroxyl group's in the gel, which leads to release of hydrogen and hydroxyl ions (Van der Walt 2016). The niosome cream showed a decrease in conductivity for the duration of the study, meaning that less or no ion movements occurred.

The viscosity of the niosome gel and niosome cream fluctuated over the stability test period, which may be due to water production in the system, as presented by the conductivity. Although the viscosities from each product fluctuated during the test period, no physical change in viscosity could be noticed.

Light microscopy was used to determine if any physical changes occurred during the test period. The freshly prepared niosome gel presented with a significant amount of air-bubbles, which decreased during the test period. At the end of month 3 crystals were observed, probably niacinamide. The niosome cream presented with small oil droplets during the initial month, as well as throughout the study. During the study, no flocculation of oil droplets could be noticed. Although changes occurred in both formulations, no significant changes could be observed for the duration of the test period.

The visual appearances assessment indicated a huge transparent lump of Carbopol® in the niosome gel, together with a decrease in volume. The volume decrease may be attributed to air-bubbles, which decreased during the test period. The niosome cream stored at 40 ± 2 °C/75 ± 5% RH (relative humidity), presented with a more oily texture. Furthermore, no color or fragrance changes could be noticed in either product.

In Tables 4 and 5, the mass of the niosome gel and niosome cream is presented. The mass of the niosome gel stayed constant for the duration of the test period, indicating the packaging was acceptable, as no significant amount were lost. As for the niosome cream, the mass deviated during the test period, which may be an indication of evaporation.

Since zeta-potential is a surface property, which measures the electric potential on the surface of a particle, it can indicate whether a formulation is stable. When the zeta-potential of a formulation

is greater than - 25 mV, the formulation could be considered stable resulting in no fusion or flocculation of the particles in the dispersion (Lamba *et al.* 2015). In general, both the formulations presented with stable zeta-potential values for the duration of the stability tests. Both formulations had values exceeding - 25 mV, resulting in no flocculation or aggregation in the niosome gel or niosome cream.

Thus, both products were able to remain stable regarding the concentration assay of niacinamide, pH, mass loss (only the niosome gel), light microscopy, visual appearance and zeta-potential.

2.4 The physicochemical properties of niacinamide

2.4.1 Solubility

The ideal aqueous solubility for a substance to penetrate through the skin is more than 1 mg/ml (Naik *et al*. 2000). During this study, the solubility of niacinamide was determined as 112.70 mg/ml in PBS (phosphate buffer solution; pH 7.4) at 25 °C. This excellent solubility of the active indicates it should penetrate the skin.

2.4.2 The octanol-buffer distribution coefficient

The octanol-buffer distribution coefficient (log D) indicates the balance of solubility of the active in water and oil domains. For a particle to penetrate the skin an optimal log D value between 2 and 3 is required; during this study, the log D value was determined to be - 0.43. This indicates that skin penetration will be challenging with this active (Donnelly *et al*. 2015).

2.5 Diffusion studies

For the diffusion studies, the median values were used rather than the average values. This was done because the median value takes all the data into consideration and represents the skewed data more accurately, as it is not affected by the distribution in data spreads (Dawson and Trapp, 2001; Gerber *et al*. 2008).

2.5.1 Membrane diffusion studies

The aim of the membrane diffusion study was to determine if niacinamide was released from the preparations. The box-plots illustrating the diffusion during the membrane study are presented in Fig. 3. In the box-plots, the median and average flux is presented, together with the minimum and maximum diffused. When comparing the preparations to one another, the proniosomes had

the highest median flux with a value of 1 163.58 µg/cm^2.h, followed by the niosomes (1 162.58 µg/cm^2.h), the niosomes gel (1 042.08 µg/cm^2.h) and lastly, the niosome cream (469.72 µg/cm^2.h).

Therefore, both the pre-formulations and the formulations had released niacinamide, although when comparing the pre-formulations to the formulations, the release of the active was less after the niosomes were mixed into a gel and cream. This influence may be the result of the difference in viscosity between the pre-formulations and the formulations; both formulations presented with a higher viscosity, which could have an indirect effect on the delivery (Ueda *et al*. 2009).

2.5.2 Skin diffusion studies

The purpose of the skin diffusion studies was to indicate if the active was delivered topically and/or transdermally. The aim of this study was to deliver niacinamide topically to treat skin conditions such as ageing, hyperpigmentation, melasma and rosacea (Gehring 2004).

During this study, concentrations of niacinamide were also noticed in the placebo samples and since they were above the LOD and LOQ, it had to be brought into account. After investigating why niacinamide was present in the placebos, it was found it could be obtained through the intake of chicken, beef, pork and fish (Chen and Damian 2014); another way of obtaining the active may be through vitamin supplements or injections. A complete placebo study was performed containing only PBS (pH 7.4) in both the donor and receptor compartment to determine the concentration of niacinamide located in the skin. An average was calculated and subtracted from the results of the diffusion study, to determine the real amount diffused.

Both the pre-formulations and formulations presented with concentrations of niacinamide in the receptor phase during the 12 hr skin diffusion study. Comparing the preparations, the niosomes presented with the highest median flux (0.844 µg/cm^2.h), followed by the niosome gel (0.692 µg/cm^2.h), the proniosomes and lastly, the niosome cream (0.172 µg/cm^2.h).

Comparing the niosomes and proniosomes in accordance to their characteristics, the niosomes had a higher entrapment and presented with more densely packed vesicles over the proniosomes. This appearance may have caused the better penetration presented by the niosomes.

When comparing the two formulations to one another, the niosome gel presented with a higher median flux. Since the niosome gel contains higher water content over the niosome cream, it may have resulted in the stratum corneum being hydrated and in this manner, led to an increase in diffusion of the water soluble active (Bouwastra *et al*. 2003:22).

2.5.3 Tape stripping

The data obtained from the tape stripping were also calculated by subtracting the placebo study. Both the pre-formulation and the formulations delivered niacinamide in the stratum corneum-epidermis (SCE) and epidermis-dermis (ED). Comparing the preparations, the niosomes (2.13 µg/ml) had the highest median concentration in the SCE, followed by the niosome gel (1.94 µg/ml), niosome cream (1.05 µg/ml) and lastly, the proniosomes (0.71 µg/ml).

Considering the stratum corneum is the main barrier of the body, its main purpose is preventing penetration of molecules from the outside environment (Bouwastra *et al*. 2003). This barrier function of the stratum corneum may be reduced by hydrating the skin and may be the reason why the delivery of the hydrophilic active occurred. During the skin diffusion study, the skin was extremely hydrated, as the experiment was performed in a water bath of 37° C for 12 hrs. This enormous hydration of the skin causes the lipid organisation in the stratum corneum to change, resulting in the topical delivery of the hydrophilic active in the SCE (Bouwastra *et al*. 2003).

The results obtained from the tape stripping indicated the niosomes delivered the highest concentration of niacinamide into the SCE. The possible reason why this was so, compared to the niosome gel and niosome cream, could be the result of the difference in viscosities. Both the niosome gel and niosome cream presented with a higher viscosity than the niosomes, which could have indirectly influenced the delivery (Ueda *et al*. 2009).

The niosomes had a higher concentration of niacinamide in the SCE than the proniosomes, which could be due to the characteristics of the vesicle systems, as the niosomes formed more-densely packed lipid vesicles than the proniosomes resulting in the niosomes being in closer contact with the stratum corneum therefore increasing penetration (Liu *et al*. 2008).

2.5.4 Epidermis-dermis

The niosomes (5.02 µg/ml) delivered the highest median concentration of niacinamide into the ED, followed by the niosome cream (2.86 µg/ml), the proniosomes (2.04 µg/ml) and lastly, the niosome gel (1.94 µg/ml).

The concentration of niacinamide located in the ED, also indicated that the penetrating effect of the niosomes was influenced by incorporating them into the semi-solid dosage formulation. This being said, the delivery could have been influenced since the niosome gel and niosome cream were more viscous resulting in less topical delivery (Ueda *et al*. 2009). The niosomes also presented with better delivery in comparison to the proniosomes. As explained previously under tape stripping, the niosomes were more-densely packed than the proniosomes, which formed individually, resulting in the niosomes covering a greater surface area and possibly to better penetration. The possible reason why the niosome cream delivered more of the active into the ED compared to the niosome gel might be attributable to the fact that oil in water (o/w) creams consist of a continuous phase which evaporates after application, causing the water soluble active to concentrate in the adhering film. This then increases the concentration gradient and resulted in a higher percutaneous deposit (Barry 2007).

The study was performed using niosomes, proniosomes, a niosome gel and a niosome cream to determine if the active could be delivered topically. All the preparations were successful in delivering the water-soluble active to the target site.

The box-plots indicating the concentration of niacinamide in the different domains of the skin delivered through each preparation are presented in Fig. 4. Presented in the box-plots are the median and average flux, together with the minimum and maximum.

2.5.5 Statistical analysis

The Tukey multiple comparison test was used to compare the mean concentration values of the SCE, ED and receptor phase with each other. It indicated that significant differences in the niosomes occurred between the SCE and ED ($p = 0.03$) and between the SCE and the receptor phase ($p = 0.003$), but no significant difference between the ED and the receptor phase ($p = 0.596$) was noticed. The proniosomes had significant difference between the SCE and ED ($p = 0.005$), as well as the SCE and the receptor phase ($p = 0.008$). For the proniosomes, no

significant difference between the ED and receptor phase appeared (p = 0.982). The niosomes gel presented with a significant difference between the SCE and the receptor phase (p = 0.025), as well as between the ED and the receptor phase (p = 0.013), but no significant difference could be seen between the SCE and ED (p = 0.955). No significant differences in the niosome cream between the SCE and ED (P = 0.560), SCE and the receptor phase (p = 0.728), ED and the receptor phase (p = 0.195) were noticed.

3 Experimental

3.1 HPLC validation of niacinamide for the purpose of determining the concentration throughout the study

An Agilent 1100 series analytical system, equipped with an Agilent 1100 isocratic pump, autosampler injection mechanism and diode array detector, and a Venusil XBP C_8, 5 µm, 250 x 4.6 mm (Agela Technologies, Newark, DE, USA) column, were used. The mobile phase was used in a ratio of 40:60 methanol and 0.075 M octane sulphonic acid at a flow rate of 1 ml/min. The UV detection was 212 nm with a retention time of 4.5 min.

3.2 HPLC validation of niacinamide, methylparaben and propylparaben for the purpose of the concentration assay

The concentration assay was performed on an Agilent 1100 series analytical system, equipped with an Agilent 1100 isocratic pump, autosampler injection mechanism and diode array detector. A Venusil XBP C_8, 5 µm, 250 x 4.6 mm column (Agela Technologies, Newark, DE, USA) and UV detection at 212 nm was utilised. Elution was achieved by means of a gradient consisting of methanol (A) and 0.075 M octane sulphonic acid (B), buffered to a pH of 3.5 with a few drops of 2 M phosphoric acid. The gradient started with 45 % A and increased linearly to 70% A after 2 minutes, where it was held until 7 minutes. The flow rate was 1 ml/min and the detection times were approximately 3.4 min for niacinamide, 6.9 min for methylparaben and 9.5 min for propylparaben.

3.3 Formulation of niosomes and proniosomes

In this study, niosomes and proniosomes were made by using the thin film hydration method. Niosomes were prepared using cholesterol, a non-ionic surfactant (Span® 60) and an organic

solvent (chloroform), placed together in a glass beaker on a heating plate while stirring with a magnet, to form the lipid phase. The active was then dissolved in purified water to form the aqueous phase, which was mixed into the lipid phase after the organic solvent completely evaporated to form niosomes. The formulation contained 3% niacinamide.

When formulating the proniosomes, a water-soluble carrier, such as sorbitol, is coated with the lipid phase, resulting in a dry formulation. When needed, the proniosomes were hydrated with the aqueous phase containing 3% niacinamide (Seleci *et al*. 2016).

3.3.1 The characteristics of the vesicle system

The characteristics of the vesicle systems were done on both the niosomes and the proniosomes. The characteristics included; vesicle size, morphology, viscosity, zeta-potential, entrapment efficiency and pH.

3.3.1.1 Vesicle size

The vesicle size was determined using the Malvern Zetasizer Nano (Nano SZ) (Malvern instruments, Worcester, UK) at room temperature. Freshly prepared samples were used and diluted to reduce the particle interactions. The test was done in triplicate, and each test was run three times to ensure accuracy.

3.3.1.2 Morphology

The morphology of the vesicle systems was done in conjunction with Dr A Jordaan (Laboratory for Electron Microscopy, North-West University, Potchefstroom Campus) and was determined using the transmission electron microscope (TEM) FEI Tecnai G2 (FEI, Holland) at 120 Kv.

3.3.1.3 Viscosity

The viscosity was determined using the Brookfield® Viscometer model DV-III Ultra (Middleboro, Massachusetts, USA). A SC4-18 Spindle was used and multi-point readings were taken every 10 sec to gather eight readings in total.

3.3.1.3 Zeta-potential

The zeta-potential of the vesicles was done using the Malvern Zetasizer Nano (Nano SZ) (Malvern instruments, Worcester, UK) at room temperature with disposable folded capillary cells.

3.3.1.4 Entrapment efficiency

The entrapment efficiency was determined using the HPLC. First, the pellets and the supernatant was separated with the use of an ultra-centrifuge, at 25 000 rpm at room temperature for 30 min. The supernatant was extracted and diluted before placing it into a HPLC vial for analysis. The entrapment was determined by the equation:

EE% = (Concentration (t) – Concentration (f)/Concentration (t)) x 100 **(1)**

Where concentration (t) is the amount of drug weighed and concentration (f) is the concentration analyzed on HPLC.

3.3.1.5 pH

The pH of the vesicle systems was determined with a Mettler-Toledo pH meter FE20/FG2 at room temperature. The pH of both the niosomes and proniosomes were measured in triplicate.

3.4 Formulation of semi-solid dosage forms

Two semi-solid dosage forms were prepared by using the 3% niosome preparation as a base. A niosome gel and niosome cream were formulated. The niosome gel was prepared by making use of Carbopol® as a rheology modifier in a concentration of 0.5%. After mixing together with a homogenizer, the pH was set at 7.4 with triethanolamine and the air bubbles were removed in a sonicating bath. For the niosome cream, an o/w emulsion was prepared, where the niosomes were incorporated into the water phase.

3.5 Stability tests of semi-solid dosage forms

The stability tests were performed on the niosome gel and niosome cream. Both the formulations were placed in different conditions over a period of three months. The tests were done on the initial month, as well as on months 1, 2 and 3. The formulations were stored at 25 ± 2° C/60 ± 5% RH, 30 ± 2° C/60 ± 5% RH and 40 ± 2° C/75 ± 5% RH. The tests that were performed included a concentration assay, pH, conductivity, viscosity, light microscopy, visual appearances, mass loss and zeta-potential.

3.5.1 Concentration assay

The concentration of niacinamide, methyl- and propyl paraben in the niosome gel and cream was determined using the HPLC. This was done for each formulation at the different storage conditions. The samples were prepared by weighing 1 g of each sample in triplicate before making it up to volume with Milli-Q® HPLC grade water and methanol (HPLC grade), in a ratio of 20:80. A standard sample was prepared containing 350 µg/ml niacinamide, 20 µg/ml methylparaben and 5 µg/ml propylparaben.

3.5.2 pH and conductivity

The pH was determined using the Mettler-Toledo pH meter FE20/FG2 (instrument no P16) at room temperature. The pH of each formulation was determined in triplicate. The conductivity was determined by using the GLASS Mettler Toledo In Lab® 731 electrode.

3.5.3 Viscosity

The viscosity was measured with the Brookfield® Viscometer model DV-III Ultra (Middleboro, Massachusetts, USA). A SC4-25 spindle was used at a rate of 5 rpm. Before testing, the formulations were placed into a water bath to reach a temperature of 25° C. In total, 32 readings were gathered from multi-point readings taken every 10 sec for 5 min.

3.5.4 Light microscopy

Light microscope photos were taken using the Motic microscope (Motic, Hong Kong), equipped with a Moticam 3 camera (Motic, Hong Kong). A portion of each sample was placed onto a glass microscope slide and photographed.

3.5.5 Visual appearance

The samples were visualized on each month and photos were taken with an iPhone 5 equipped with an 8-megapixel camera (Apple Inc. California, USA).

3.5.6 Mass loss

The mass loss during the stability test period was determined by the Shimadzu (Japan) scale. Each sample was weighed in triplicate and subtracted from the mass of the container to determine whether loss of mass occurred.

3.5.7 Zeta-potential

The zeta-potential was measured in triplicate at 25° C by making use of the Malvern Zetasizer Nano (Nano SZ) (Malvern instruments, Worcester, UK). Each sample was diluted before placing it into a disposable folded capillary cell.

3.6 The preparation of PBS

PBS (pH 7.4) was prepared by accurately weighing 1.5 g sodium hydroxide and 6.5 g potassium dihydrogen orthophosphate and dissolving them separately in 400 ml and 250 ml of Milli-Q® HPLC grade water (Merck Millipore, Halfway House, South Africa). The two solvents were added together and buffered with 10% orthophosphoric acid (2 M) to a pH of 7.4. When a pH of 7.4 was reached, it was made up to volume (British Pharmacopoeia, 2014).

3.7 The physicochemical properties of niacinamide

3.7.1 Solubility

The aqueous solubility of niacinamide was determined by adding an excess amount of the active into a test tube containing 3 ml PBS (pH 7.4). The test tube was then placed into a pre-heated shaking water bath at a temperature of 25° C and left overnight. The solution was filtered and diluted the next day before being transferred to a HPLC vial for analysis. This test was done in triplicate.

3.7.2 Log D

A pre-saturated solution was used and prepared by magnetically stirring together *n*-octanol and PBS (pH 7.4) for a period of 24 h. The two phases were then left to separate, where-after 20 ml of the pre-saturated PBS was extracted and placed into a tube. Niacinamide was accurately weighed and dissolved in the 20 ml pre-saturated PBS. After the active had completely dissolved, 3 ml of the mixture was placed into three different test tubes. After this, 3 ml of the pre-saturated *n*-octanol was extracted and placed into each test tube containing the niacinamide and pre-

saturated PBS mixture. The test tubes were placed into a shaking water bath of 32° C and left overnight. The test tubes were then placed into an Eppendorf Centrifuge 5804 R at 3000 rpm for 5 min at 32° C to separate the phases. Approximately 1 ml of each phase was extracted, then the saturated PBS was diluted (1:20) with PBS (pH 7.4) and the saturated *n*-octanol was diluted (1:20) with methanol before being transferred to an HPLC vial for analysis.

3.8. Diffusion studies

3.8.1 Membrane diffusion studies

The membrane release studies were performed using vertical Franz diffusion cells. The cells were placed in a pre-heated water bath, maintained at a constant temperature of 37° C. Polyvinylidene fluoride (PVDF) membrane filters with a pore size of 0.45 µm were used during this study. The receptor phase (PBS at pH 7.4) was removed at hourly intervals six consecutive times and then replaced with a fresh receptor phase. The samples were transferred to HPLC vials for analysis.

3.8.2 Skin preparation

Female Caucasian skin, obtained after abdominal cosmetic surgery, was used during this study. Once collected, the skin was placed in a freezer to be frozen at - 20° C for no longer than 24 hrs. The skin was dermatomed to a thickness of 400 µm, cut into circles of 15 mm in diameter, then placed on filter paper with the stratum corneum facing up. The skin was then left to air dry, where after it was enclosed with aluminum foil and stored at - 20° C until needed. Ethical approval (NWU-00114-11-A5) was granted by the North-West University Ethics Committee for "Application for the use of biological material obtained from human subjects in experiments."

3.8.3 Skin diffusion studies

The dermatomed skin was cut into suitably sized pieces and placed in the Franz diffusion cells with the stratum corneum facing the donor compartment. The average diffusion surface of the Franz cells is 1.25 cm^2. After the donor compartment was placed on top of the receptor phase, the receptor compartment was filled with 2 ml of PBS (pH 7.4) and the donor compartment with the different preparations,. The total amount in the receptor phase was extracted at predetermined time-intervals, there after it was transferred into HPLC vials for analysis. For the

placebo study, PBS (pH 7.4) was placed in both the donor and receptor phase, then the same method was performed.

3.8.4 Tape stripping

After the Franz cells were dismantled, the skin was removed and pinned to a piece of Parafilm® and placed on a solid surface. The skin was dabbed dry with a clean paper towel to remove the remaining formulation. 3M Scotch® Magic Tape™ was cut into pieces and placed on the diffusion surface of the skin and removed, until the stratum corneum was removed completely. The first strip was discarded to prevent possible contamination. The remaining 15 strips, containing the active and SCE, were then placed into a polytop filled with 5 ml of PBS (pH 7.4). The remaining diffusion area (ED) was then cut into small pieces and placed into another polytop also containing 5 ml of PBS (pH 7.4). The polytops were left overnight at 4° C in order for the active ingredient to dissolve into the extraction solution. Both these samples were analyzed the next day on the HPLC (Pellett *et al.* 1997:91).

3.8.5 Statistical analysis

The data obtained from the membrane release studies were used to calculate the percentage released (%), as well as the cumulative amount per area against time (µg/cm^2.h) of the active that diffused after 6 hrs. The data obtained from the skin diffusion studies were used to calculate the percentage diffused (%), as well as the cumulative amount per area against time (µg/cm^2.h) of the active that diffused through the skin into the receptor phase after 12 hrs. The data obtained from the tape stripping and pieces of cut, diffused skin were used to determine the concentration (µg/ml) of the active delivered into the SCE and the ED, respectively.

The data obtained from the membrane and skin diffusion studies, as well the data from the SCE and ED, were used during the statistical analysis. First, a two-way analysis of variance (ANOVA) was performed to test for an interaction effect between formulation and skin layer. This was followed by a one-way ANOVA to determine if any statistical differences in the mean concentration between formulations occurred in the data. The same was done to compare the skin layers. Then a Tukey multiple comparison test was performed to determine between which

pairs of formulations or skin layers the significant differences occurred. The tests indicated that significant difference occurred when a p-value of smaller than 0.05 was presented.

There was a statistically significant interaction effect ($p < 0.001$) between the means for each skin layer per formulation therefore, it was decided to do a one-way ANOVA to compare different formulations within each skin layer separately; skin layers were also compared within each formulation.

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Tables

Table 1: The characteristic of the niosomes and the proniosomes

Characteristics	Niosomes	Proniosomes
Vesicle size	706.388 nm	559.366 nm
Viscosity	4.036 cP	3.616 cP
Zeta-potential	- 44.78 mV	- 61.32 mV
Entrapment efficiency	86.85%	66.34%
pH	7.28	6.99

Table 2: The concentration of niacinamide, methylparaben and propylparaben in the niosome gel during the stability test period

	Initial	Month 1	Month 2	Month 3
25 ± 2 °C/60 ± 5% RH				
Niacinamide	105.153	102.128	103.089	100.950
Methylparaben	111.100	81.657	94.569	83.288
Propylparaben	108.554	80.771	79.759	78.067
30 ± 2 °C/60 ± 5% RH				
Niacinamide	105.153	105.747	104.089	99.566
Methylparaben	111.100	81.531	94.569	76.691
Propylparaben	108.554	86.254	81.244	107.091
40 ± 2 °C/75 ± 5% RH				
Niacinamide	105.153	107.378	103.275	101.280
Methylparaben	111.100	80.251	90.686	51.683
Propylparaben	108.554	75.453	78.484	71.526

Table 3: The concentration of niacinamide, methylparaben and propylparaben in the niosome cream during the stability test period

	Initial	Month 1	Month 2	Month 3
25 ± 2 °C/60 ± 5% RH				
Niacinamide	103.501	108.047	103.624	104.740
Methylparaben	123.716	89.548	99.403	99.924
Propylparaben	115.350	81.966	81.718	101.004
30 ± 2 °C/60 ± 5% RH				
Niacinamide	103.501	111.489	107.271	105.280
Methylparaben	123.716	92.165	102.713	97.895
Propylparaben	115.350	84.241	86.156	116.286
40 ± 2 °C/75 ± 5% RH				
Niacinamide	103.501	111.363	105.713	103.623
Methylparaben	123.716	92.763	99.403	95.650
Propylparaben	115.350	85.55	83.030	116.701

Table 4: The results obtained from the niosome gel during the stability test period

	Initial	Month 1	Month 2	Month 3
25 ± 2 °C/60 ± 5% RH				
pH	6.46	8.30	8.20	7.82
Conductivity (µs/cm)	151.83	489.00	522.66	515.66
Viscosity (cP)	48820.67	46958.75	38871.09	55652.81
Mass (g)	20.308	20.607	20.578	20.543
Zeta-potential (mv)	-87.030	-67.244	-51.266	-71.800
30 ± 2 °C/60 ± 5% RH				
pH	6.46	7.79	7.67	7.53
Conductivity (µs/cm)	151.83	404.66	456.66	503.66
Viscosity (cP)	48820.67	53121.56	42194.06	45585.31
Mass (g)	20.308	20.574	20.528	20.552
Zeta-potential (mv)	-87.03	-69.05	-56.13	-79.73
40 ± 2 °C/75 ± 5% RH				
pH	6.46	8.13	7.91	7.35
Conductivity (µs/cm)	151.83	498.33	605.33	410.0
Viscosity (cP)	48820.67	36075.34	30102.19	28841.87
Mass (g)	20.308	19.576	19.527	19.513
Zeta-potential (mv)	-87.030	-67.160	-53.333	-81.130

Table 5: The results obtained from the niosome cream during the stability test period

	Initial	Month 1	Month 2	Month 3
25 ± 2 °C/60 ± 5% RH				
pH	7.31	6.88	7.00	6.85
Conductivity (µs/cm)	222.66	245.33	108.73	109.40
Viscosity (cP)	149944.800	234446.900	97256.250	85858.120
Mass (g)	20.678	20.977	18.793	18.583
Zeta-potential (mv)	-43.83	-54.04	-51.26	-60.50
30 ± 2 °C/60 ± 5% RH				
pH	7.31	6.88	6.89	6.79
Conductivity (µs/cm)	222.66	187.03	208.33	96.56
Viscosity (cP)	149944.800	249403.100	32578.750	81899.620
Mass (g)	20.678	20.944	25.321	25.211
Zeta-potential (mv)	-43.83	-58.32	-56.13	-53.76
40 ± 2 °C/75 ± 5% RH				
pH	7.31	6.80	6.73	6.70
Conductivity (µs/cm)	222.66	59.96	198.86	58.43
Viscosity (cP)	149944.800	126165.600	296390.600	105606.250
Mass (g)	20.678	19.944	20.051	19.996
Zeta-potential (mv)	-43.83	-58.46	-53.33	-52.534

Table 6: The final formulas for the vesicle systems

Ingredients	**Quantities**
Niosomes	
Cholesterol	600 mg
Span® 60	300 mg
Niacinamide	300 mg
Milli-Q® water	10 ml
Chloroform	10 ml
Proniosomes	
Cholesterol	600 mg
Span® 60	300 mg
Niacinamide	300 mg
Milli-Q® water	10 ml
Chloroform	10 ml
Sorbitol	2000 mg

Table 7: The final formulas for the semi-solid formulations

Ingredients		Quantities (%m/m)
Niosome gel		
Niosomes (3% niacinamide)		95 ml
Carbopol®		0.50%
Methylparaben		0.20%
Propylparaben		0.05%
Propylene glycol		5 ml
Triethanolamine		± 1 ml
Niosome cream		
Lipid phase (A)	Liquid paraffin	10.00%
	Tween® 20	1.00%
	Cetyl alcohol	10.00%
	Methylparaben	0.20%
Aqueous phase (B)	Niosomes (3% niacinamide)	72.25%
	Propylene glycol	3.00%
	Span® 20	1.00%
	Propylparaben	0.05%
	Veegum®	2.50%

Figures

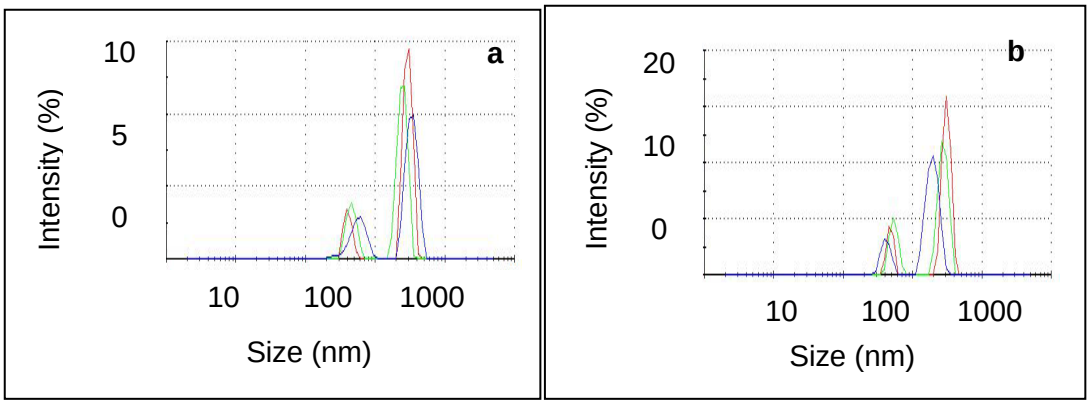

Figure 1: Size distribution of a) niosomes and b) proniosomes

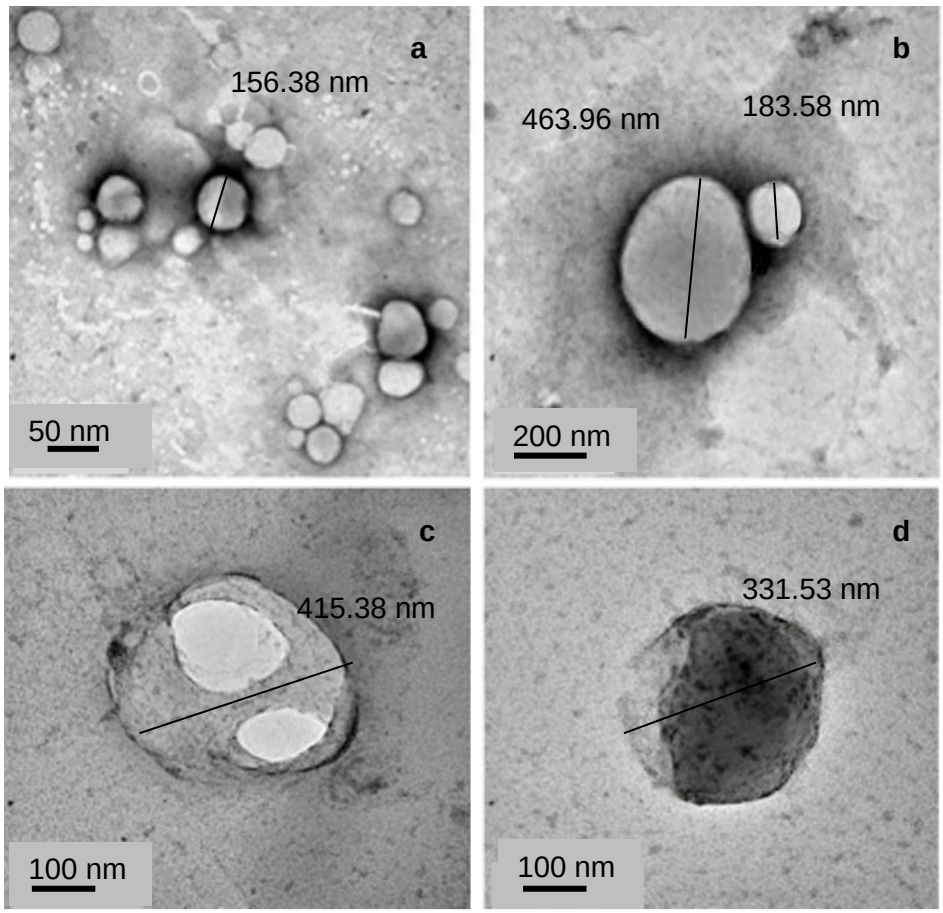

Figure 2: Transmission electron microscope micrographs of round shaped niosomes (a and b) and the proniosomes (c and d)

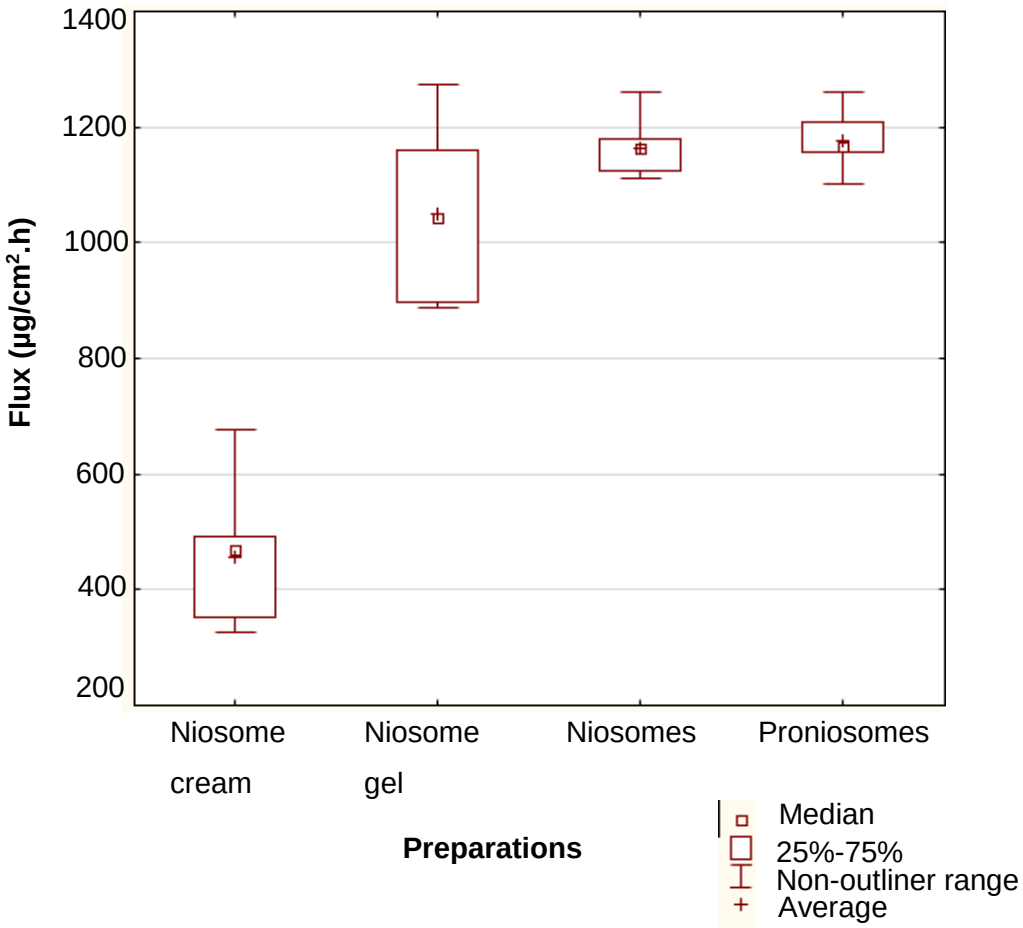

Figure 3: The box-plots of the membrane diffusion study of all four preparations, illustrating the median (small square) and average (plus sign) flux of niacinamide

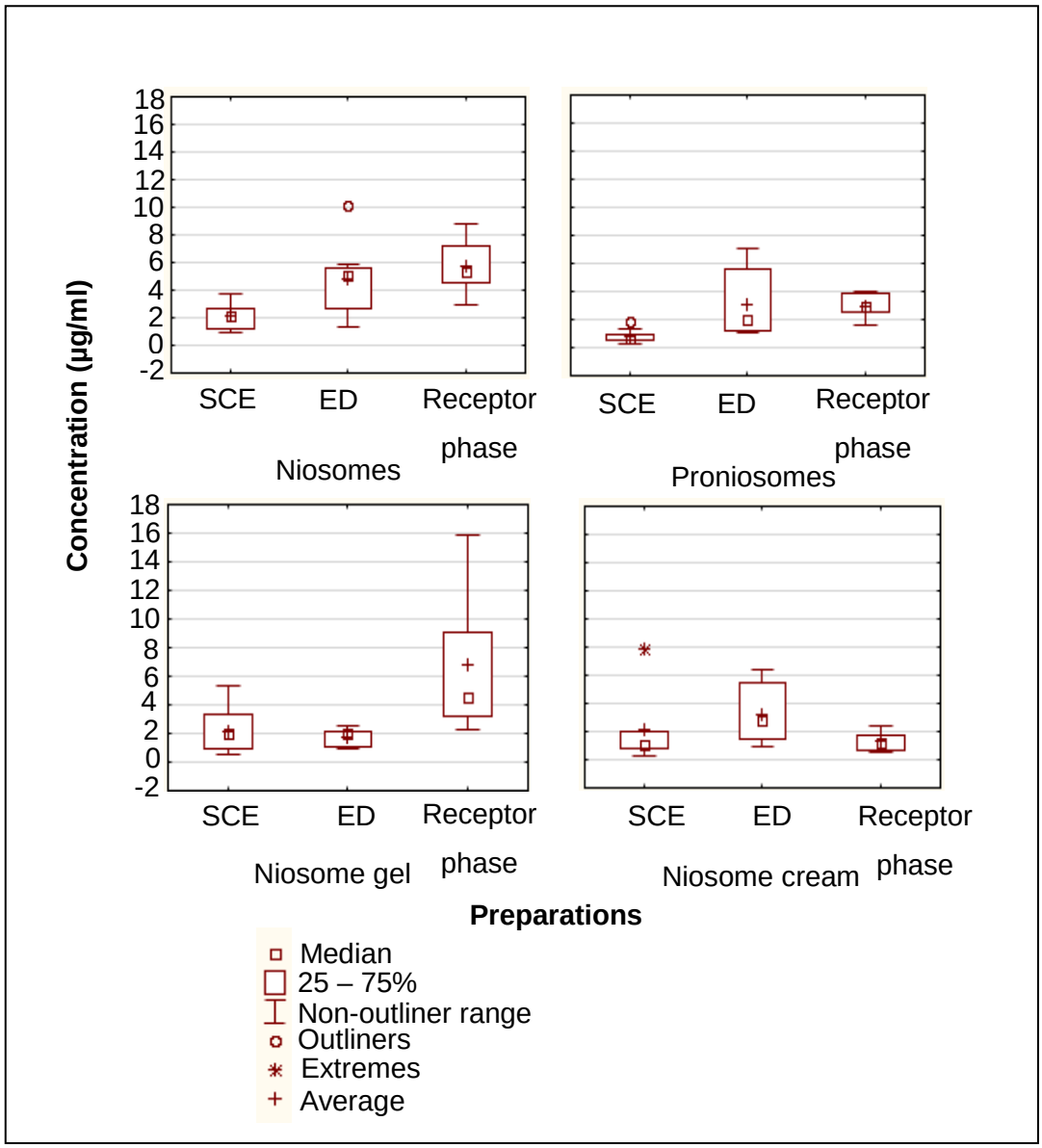

Figure 4: The box-plots of the skin diffusion and tape stripping studies of all four preparations, illustrating the median (squared box) and average (plus sign) concentration of niacinamide

Chapter 4

Final conclusion and future prospects

The aim of this study was to design a topical preparation containing the active ingredient, niacinamide. This preparation must be designed to deliver the active topically in order to treat skin conditions such as hyperpigmentation, rosacea, anti-aging and melasma. Since the active belongs to the vitamin B group and is highly water soluble (Lupo, 2001:468); skin penetration might be easily reached (Naik *et al*., 2000:319), however, niacinamide has a distribution coefficient (log D) of -0.32 (Jacobs, 2009:ii), which is not ideal for skin penetration.

Consequently, the objectives for this study were to:

- Validate an HPLC method to determine the concentration of niacinamide.
- Determine the aqueous solubility and the distribution coefficient of niacinamide at pH 7.4.
- Formulate a vesicle system such as niosomes and proniosomes, containing niacinamide.
- Determine the physical characteristics of the vesicle system (pH sensitivity, entrapment efficiency, morphology, zeta-potential, surface properties, viscosity and vesicle size).
- Formulate a gel and cream which contains the niosomes.
- Perform membrane studies to determine the release of niacinamide from the niosomes, proniosomes, niosome cream and niosome gel.
- Perform diffusion studies with vertical Franz cells, by using dermatomed, female Caucasian skin, to determine the concentration of niacinamide being delivered through the skin and tape stripping of the skin after completion of the diffusion study in order to determine if the active could be delivered into the skin.
- Validate an (HPLC) method for the purpose of the stability test analysis on the niosome gel and niosome cream.
- Perform accelerated stability testing on the niosome cream and niosome gel at 0, 1, 2 and 3 months. The conditions will consist of three different temperatures at a specific RH: ± 25° C/60% RH; ± 30° C/60% RH and ± 40° C/75% RH.

First, an HPLC method was validated in a controlled laboratory environment at the Analytical Technology Laboratory, North-West University, Potchefstroom, South Africa. This was done to

determine the concentration of niacinamide throughout the study. During the study all the validation parameters met the requirements.

The physiochemical characteristics of niacinamide were determined to indicate if skin penetration of the active was possible. The aqueous solubility of niacinamide, which was determined as 112.70 mg/ml, predicted good skin penetration. However, the log D value, of - 0.43, indicated that the active would not be the ideal substance to be applied topically. No single physico-chemical characteristic of an active can be used as indication of the possibility to topically deliver an active. A combination of these characteristics plays a role in delivery.

In order to overcome the possible barrier for topical delivery, the active was formulated into niosomes and proniosomes.. The niosomes and proniosomes were formulated to contain 3% of the active and physically characterised (pH, entrapment efficiency, morphology, zeta-potential, size and viscosity). The size of the niosomes and proniosomes was approximately 668.55 nm and 593.33 nm, respectively, which correlated well with values found in literature (Bolzinger *et al*., 2012:163). Both the vesicle systems were stable with a zeta-potential of below - 25 mV. The niosomes and proniosomes had zeta-potential values of - 45.11 mV and - 61.32 mV, respectively. The viscosities of both these systems were too low for easy topical application and for this reason the niosomes were incorporated into a niosome gel and a niosome cream.

Membrane release studies were performed on both the pre-formulations (niosomes and proniosomes) and also on the formulations (niosome gel and niosome cream). The membrane studies were performed to determine whether release of the active from the dosage forms occurred. During the release studies, the proniosomes had the highest median flux of 1 163.58 µg/cm^2.h, followed by the niosomes (1 162.58 µg/cm^2.h), the niosome gel (1 042.08 µg/cm^2.h), with the niosome cream having the lowest median flux of 469.72 µg/cm^2.h. Skin diffusion studies were performed to determine if the active was delivered topically or transdermally. After the skin diffusion studies, tape stripping was performed to determine whether the active was delivered into the SCE or ED. Although the aim of the study was to deliver the active topically, both the pre-formulations and formulations delivered the active into the receptor phase, SCE and the ED. During the skin diffusion studies, the niosomes had the highest median flux of 0.844 µg/cm^2.h, followed by the niosome gel (0.692 µg/cm^2.h), the proniosomes (0.462 µg/cm^2.h), with the niosome cream having the lowest median flux of 0.172 µg/cm^2.h.

The preparation with the highest median concentration in the SCE was the niosome gel (2.15 µg/ml), followed by the niosome cream (2.09 µg/ml), the niosomes (2.08 µg/ml) and the proniosomes (0.83 µg/ml). The preparation with the highest median concentration in the ED was

the niosomes (5.02 µg/ml), followed by the niosome cream (2.86 µg/ml), the proniosomes (2.04 µg/ml) and the niosome gel (1.94 µg/ml).

Accelerated stability test were done on the niosome gel and niosome cream. According to the ICH (2006:3), the formulations were stored, at ± 25° C/60% RH; ± 30° C/60% RH and ± 40° C/75% RH for a period of three months. Physico-chemical characterisation included:

- Concentration assay
- pH and conductivity
- Viscosity
- Light microscopy
- Visual appearance
- Mass loss
- Zeta-potential

The ICH (2006:3) states that a product may be defined to have a significant change if an assay deviate more than 5% from the initial value; The formulations were unstable regarding the viscosity, conductivity, mass loss (only the niosome cream), as well as the concentration assay of the parabens. Both products were stable regarding the concentration of the active ingredient, pH, mass loss (niosome gel), light microscopy, visual appearance and zeta-potential. Since the concentration of niacinamide stayed between 95 and 110%, the products may still be effective when applied topically.

The future prospect for further investigation includes the following:

- Formulate more ideal vesicle systems in order to prevent the transdermal penetration.
- Determine the clinical effectiveness and clinical sensitivity of niacinamide in the vesicle systems incorporated in the gel and cream, by making use of the Corneometer® CM 825, Visioscan® VC 98, Cutometer® dual MPA 580 and Mexameter® MX 18.
- Investigate the dermal toxicity of niacinamide, by performing methylthiazol tetrazolium (MTT)- and lactate dehydrogenase (LDH)-assays.
- Examine the dermal metabolism of niacinamide in the vesicle system to determine whether the following enzyme is present, i.e. Cytochrome P450 and causes metabolism of the active.

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Appendix A

Validation of the HPLC method for niacinamide

A.1 Purpose of the validation

An HPLC validation was used for the quantification of niacinamide and was done in conjunction with Prof Jan Du Preez at the Analytical Technology Laboratory, North-West University, Potchefstroom Campus, South Africa. The purpose of this validation was to ensure the analytical method was accurate and precise in order to analyse niacinamide. After the data was collected and processed, the percentage standard deviation (SD) and relative standard deviation (RSD) of each test were calculated.

A.2 Chromatographic conditions

The chromatographic conditions for the validation of niacinamide are given in the table below.

Table A.1: Chromatographic conditions

Analytical instrument	Agilent 1100 series analytical system, equipped with an Agilent 1100 isocratic pump, autosampler injection mechanism and diode array detector was used. It was installed with ChemStation Rev. A06.02 data acquisition and software analysis (Agilent Technologies, Palo Alto, CA, USA). The analysis was performed at room temperature (25 °C).
Column	Venusil XBP C8, 5 µm, 250 x 4.6 mm (Agela Technologies, Newark, DE, USA)
Mobile phase	Methanol/0.075 M octane sulphonic acid in a ratio of 40:60, buffered to a pH of 3.5 with a few drops of 2.000 M phosphoric acid
Flow rate	1 ml/min
Injection volume	20 µl
Detection	UV at 212 nm
Retention times	4.5 min
Stop time	6.0 min

Solvent	Phosphate buffer solution (PBS) buffered with a few drops of 2 M phosphoric acid to adjust the pH to 7.4

A.3 Standard preparation

For the standard preparation, 5 mg of niacinamide was accurately weighed and placed into a 100 ml volumetric flask. The sample was made up to volume with Milli-Q® HPLC grade water (Merck Millipore, Halfway House, South Africa).

A.4 Sample preparation

For the preparation of the sample, 20 mg of niacinamide was accurately weighed and placed into a 100 ml volumetric flask, then made up to volume with PBS (pH 7.4). Approximately 5 ml of the solution was then diluted to 20 ml. A further 5 ml of the solution was diluted to 50 ml to get a final concentration. The last step was performed in duplicate.

A.5 Validation parameters

Validation is where the performance characteristics of the procedure meet the requirements of the analytical applications. The performance characteristics used in the validation method were linearity, range, lower limit of quantification (LOQ) and upper limit of detection (LOD), accuracy, precision, ruggedness and robustness (Bansal & DeStefano, 2007:109).

A.5.1 Linearity and range

Linearity of an analytical procedure is the ability to obtain test results that are proportional to the amount of the analyte in the sample. The range of an analytical procedure can be described as the interval between the upper and the lower concentration of the analyte in the sample, which shows the analyte has a suitable level of precision, accuracy and linearity. The data can be described by the linear equation where *y* is the peak area, *m* is the slope, *x* is the concentration and *c* is the *y*-intercept (Johnson & Van Buskirk, 1998:101).

$$y = mx + c \qquad \textbf{Equation A.1}$$

A.5.1.1 Procedure for linearity testing

Three different samples were prepared by weighing 22.49 mg of niacinamide and diluting it to different concentrations ranging from 0.1 – 112.4 µg/ml. Volumes of 1.0, 2.5, 5.0, 10.0, 15.0 and 20.0 µl were injected into the HPLC for analysis.

A.5.1.2 Acceptance criteria

For the linear regression analysis to be acceptable, a regression coefficient of ≥ 0.99 is needed for a range of 80 – 120% of the target concentration (Shabir, 2005:9).

A.5.1.3 Results and discussion

For the HPLC system to be stable, the correlation coefficient (r^2) value must be ≥ 0.999. Since niacinamide had a value of 0.999, it illustrates the method was stable over a concentration range of 0.1 – 112.4 µg/ml.

Table A.2: Linearity results of niacinamide

Standard (µg/ml)	Peak area
0.112	12.4
0.281	29.8
0.562	58.5
1.125	115.3
1.687	173.8
2.249	242.3
5.623	545.6
11.245	1109.7
16.868	1651.3
22.490	2211.7
56.225	5305.4
112.450	10040.1
r^2	**0.999**
y-intercept	**61.07**
slope	**89.91**

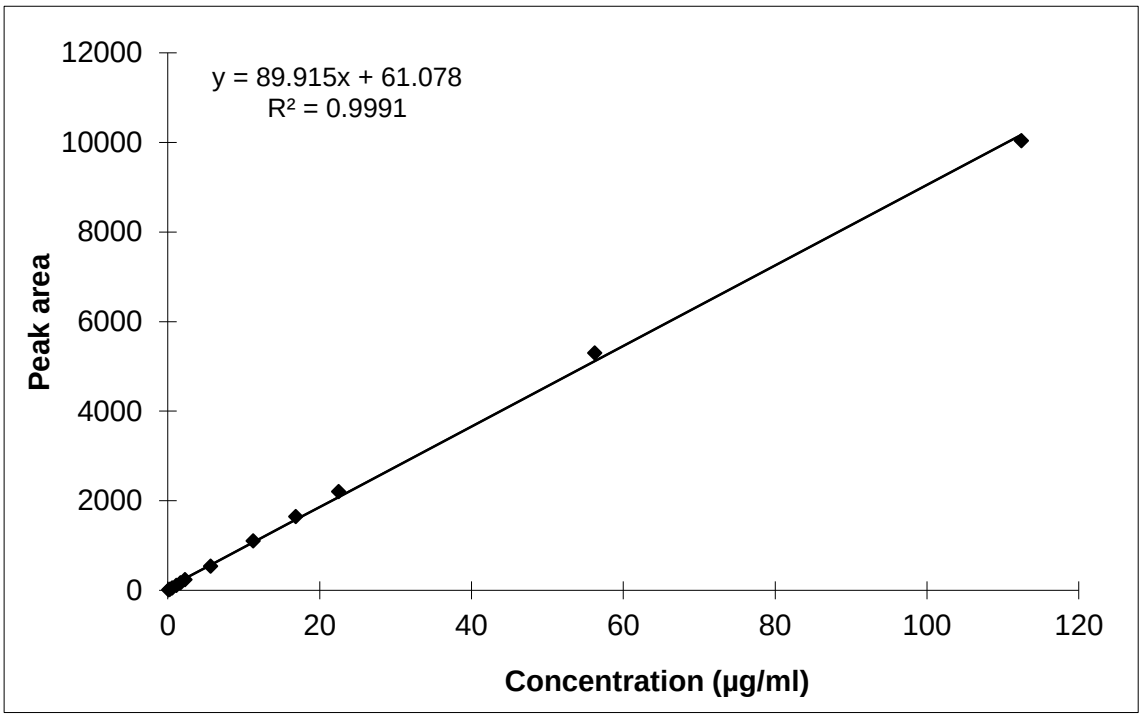

Figure A.1: Line regression graph of niacinamide

A.5.2 Limit of quantification and Limit of detection

LOQ refers to the lowest concentration of an analyte in a sample that can be accurately measured, where the LOD is used to detect the presence or the absence of an analyte in a sample. For the purpose of the LOD, a standard sample will be diluted into six concentrations and injected into the HPLC. For the LOQ, the lowest concentration will be determined by diluting the standard solution in order to obtain six samples (Shabir, 2005:10).

A.5.2.1 Procedure for LOQ and LOD

A standard solution was prepared, as described in Section A.3, containing 0.0686 µg/ml niacinamide. Different volumes (0.5, 1 and 2.5 µl) of the standard were injected into the HPLC. Each of the samples was injected six times.

A.5.2.2 Acceptance criteria

According to the Centre for Drug Evaluation and Research (CDER) (2001:6), the acceptable criteria for the accurate quantification of low concentrations are when the %RSD is less than 20% for at least six samples.

A.5.2.3 Results and discussion

Table A.3: The results of LOQ and LOD of niacinamide

Injection volume (µl)	**0.5**	**1.0**	**2.5**
Concentration (µg/ml)	0.003	0.006	0.017
Peak area	0.29 0.23 0.23 0.21 0.18 0.20	0.60 0.63 0.70 0.60 0.40 0.50	0.80 0.73 0.83 0.81 0.88 0.80
Mean peak area	0.22	0.57	0.81
SD	0.03	0.10	0.04
%RSD	15.44	16.91	5.49

As illustrated above, the LOD of niacinamide is 0.003 µg/ml and the LOQ is 0.017µg/ml. Since the %RSD of niacinamide is less than 20%, it is acceptable.

A.5.3 Accuracy

The accuracy of an analytical procedure is the degree of closeness of the observed or tested concentration to the normal concentration and also termed as trueness.

A.5.3.1 Procedure for accuracy

A.5.3.1.1 Preparation of the standard solution

Approximately 5 mg of niacinamide was weighed and placed into a 100 ml volumetric flask and made up to volume with PBS (pH 7.4). The sample was transferred into HPLC vials and injected in five different volumes (5, 10, 15, 20, 25 µl) on the HPLC.

A.5.3.1.2 Preparation of the sample solution

For the samples, niacinamide was weighed in triplicate and prepared as described in Section A.4. Each sample was diluted three times in order to get different concentrations.

A.5.3.2 Acceptance criteria

The acceptable criteria of accuracy states the recovery must be between 98% and 102% and that a relative standard deviation (%RSD) of 2.0% or less is required (Shabir, 2005:9).

A.5.3.3 Results and discussion

Table A.4: Accuracy of niacinamide

Concentration spiked (µg/ml)	**Peak area**			**Recovery**	
	1	**2**	**Mean**	**µg/ml**	**%**
5.0	399.5	407.8	411.0	5.0	99.0
5.0	409.7	408.0	411.0	5.0	99.7
5.2	413.0	409.0	411.0	4.9	96.8
24.0	1916.6	1921.5	1919.1	22.6	94.1
20.1	1687.5	1683.2	1685.4	19.9	98.8
19.9	1683.2	1683.2	1685.4	19.8	99.7
54.0	4580.3	4592.8	4586.6	53.6	91.3
50.9	4275.0	4260.0	4267.5	49.9	98.0
50.3	4171.6	4169.7	4170.7	48.8	97.0
				Mean	**98.0**
				SD	**1.7**
				%RSD	**1.8**

The mean recovery of niacinamide is 98.0% and the %RSD is 1.8%, which are within the requirements.

A.5.4 Precision

The precision of an analytical procedure is a degree of closeness obtained from a series of measurements gathered from a homogeneous sample that is prepared under the same test method (Johnson & Van Buskirk, 1998:103). It is considered at three levels: repeatability, intermediate precision and reproducibility. Repeatability is a precision operating under the same conditions over a short interval of time and can also be termed as intra-assay precision. Intermediate precision refers to the accuracy within laboratory variations, such as different days,

different analysts, different equipment, etc. Reproducibility refers to the precision between different laboratories.

A.5.4.1 Repeatability (intra-day precision)

A.5.4.1.1 Procedure for repeatability

Standard solutions were made containing 12.8; 25.5; 38.3; 51.0; 63.8 µg/ml niacinamide and placed into HPLC vials. The standard solution was injected in duplicate to obtain a calibration curve. Three samples were prepared (19.9; 20.1; 23.5 µg/ml) and injected in different volumes.

A.5.4.1.2 Acceptance criteria

The acceptable criteria for the intra-day precision, according to the CDER (2001:5), are that the mean recovery may not exceed 15% of the true value, where a %RSD of 2.0% or less will be acceptable.

A.5.4.1.3 Results and discussion

Table A.5: Intra-day precision of niacinamide

Concentration spiked (µg/ml)	**Peak area**			**Recovery**	
	1	**2**	**Mean**	**µg/ml**	**%**
20.55	1919.5	1933.8	1926.7	23.3	113.3
20.55	1938.7	1929.9	1934.3	23.4	113.7
20.55	1911.7	1959.2	1935.5	23.4	113.8
				Mean	**113.60**
				SD	**0.23**
				%RSD	**0.20**

Since the mean value of niacinamide is 113.60 and the %RSD less than 2%, it is acceptable according to the CDER.

A.5.4.2 Reproducibility (inter-day precision)

A.5.4.2.1 Procedure for reproducibility

A standard solution of 21.37 µg/ml and three sample solutions (20.0 µg/ml) were prepared. The standard solution was placed into HPLC vials and injected, in duplicate, in different volumes of 5,

10, 15, 20 and 25 µl. The precision was determined by analysing three different samples prepared over three consecutive days.

A.5.4.2.2 Acceptance criteria

As mentioned in Section A.5.4.1.2, the acceptable criteria for reproducibility according to CDER (2001:5) is that the mean recovery may not exceed 15% of the true value, where a %RSD of 2.0% or less is required (Shabir, 2005:9).

A.5.4.2.3 Results and discussion

Table A.6: Inter-day precision of niacinamide

	Day 1	Day 2	Day 3	Between days
Recovery (%)	113.30 113.70 113.80	103.70 104.10 104.20	99.10 97.70 98.10	
Mean	**113.60**	**104.00**	**98.30**	**105.30**
SD	**0.22**	**0.22**	**0.59**	**0.34**
%RSD	**0.19**	**0.21**	**0.60**	**0.32**

According to the CDER (2001:5), the mean recovery may not exceed 15% of the true value. Since days 1, 2 and 3 have a mean value of less than 15% of the true value, the criteria are acceptable. The %RSD of each day is less than 2%, meaning the %RSD is acceptable according to Shabir (2005:9).

A.5.5 Ruggedness

The ruggedness of a system reflects the chemical or physical stability of an analyte in a given matrix under certain conditions for a given time interval. Ruggedness is divided into sample stability and system repeatability. Samples will be prepared and analysed each hour for 24 hrs, under room temperature CDER (2001:7).

A.5.5.1 System repeatability

A.5.5.1.1 Procedure for system repeatability

A standard solution was prepared (206.8 µg/ml) and placed into HPLC vials for analysing. The sample was injected six times on the same day.

A.5.5.1.2 Acceptance criteria

For this criterion, the peak area and the retention times should have a %RSD of 1% (Shabir, 2005:10).

A.5.5.1.3 Results and discussion

Table A.7: The system repeatability of niacinamide

	Peak area	**Retention times (min)**
	2005.6	4.252
	2035.3	4.257
	2019.6	4.260
	2052.1	4.255
	2010.2	4.256
	2033.3	4.263
Mean	**2023.9**	**4.258**
SD	**15.62**	**0.004**
%RSD	**0.77**	**0.091**

Since the %RSD is 0.091% for niacinamide, it shows good system repeatability for the active niacinamide.

A.5.5.2 Sample stability

A.5.5.2.2 Preparation of the sample

A sample (50.2 µg/ml) was prepared as described in Section A.4. This sample was injected over a period of 24 hrs to determine the stability of the sample.

A.5.5.2.3 Acceptance criteria

The degradation of an analyte is indicated with the %RSD. For an acceptable amount of analyte to undergo degradation, a %RSD has to be less than 2% (ICH, 2005:4).

A.5.5.2.4 Results and discussion

Table A.8: Ruggedness of niacinamide

Time (h)	Peak area	%Recovery
0	3640.6	100.0
1	3741.2	102.8
2	3723.5	102.3
3	3631.4	99.7
4	3663.1	100.6
5	3755.7	103.2
6	3679.9	101.1
7	3707.9	101.8
8	3718.3	102.1
9	3722.8	102.3
10	3712.5	102.0
11	3688.9	101.3
12	3734.2	102.6
13	3690.8	101.4
14	3746.8	102.9
15	3699.4	101.6
16	3696.9	101.5
17	3692.4	101.4
18	3725.4	102.3
19	3734.1	102.6
20	3987.9	109.5
21	3705.6	101.8
22	3708.3	101.9
23	6330.2	99.7
24	3408.3	93.6
Mean	**3713.8**	**102.0**

SD	**64.81**	**1.78**
%RSD	**1.75**	**1.75**

Since the %RSD is 1.75%, it is acceptable and indicates that niacinamide is stable over a time-period of 24 hrs.

A.5.7 Specificity

The aim of this test is to detect the analyte with the presence of the matrix, impurities, degrades and metabolites (Johnson & Van Buskirk, 1998:99).

A.5.7.1 Procedure for specificity

To test the specificity of the active, the following reagents was used: distilled water, hydrochloric acid (0.1 M), sodium hydroxide (0.1 M) and 10% peroxide. A standard solution was made and 1 ml of the reagents was added to 1 ml of standard solution in different test tubes. The samples were left overnight and tested the next day.

A.5.7.2 Acceptance criteria

For the criteria to be accepted, the degraded samples should not interfere with the determination of niacinamide (ICH, 2005:4).

A.5.7.3 Results and discussion

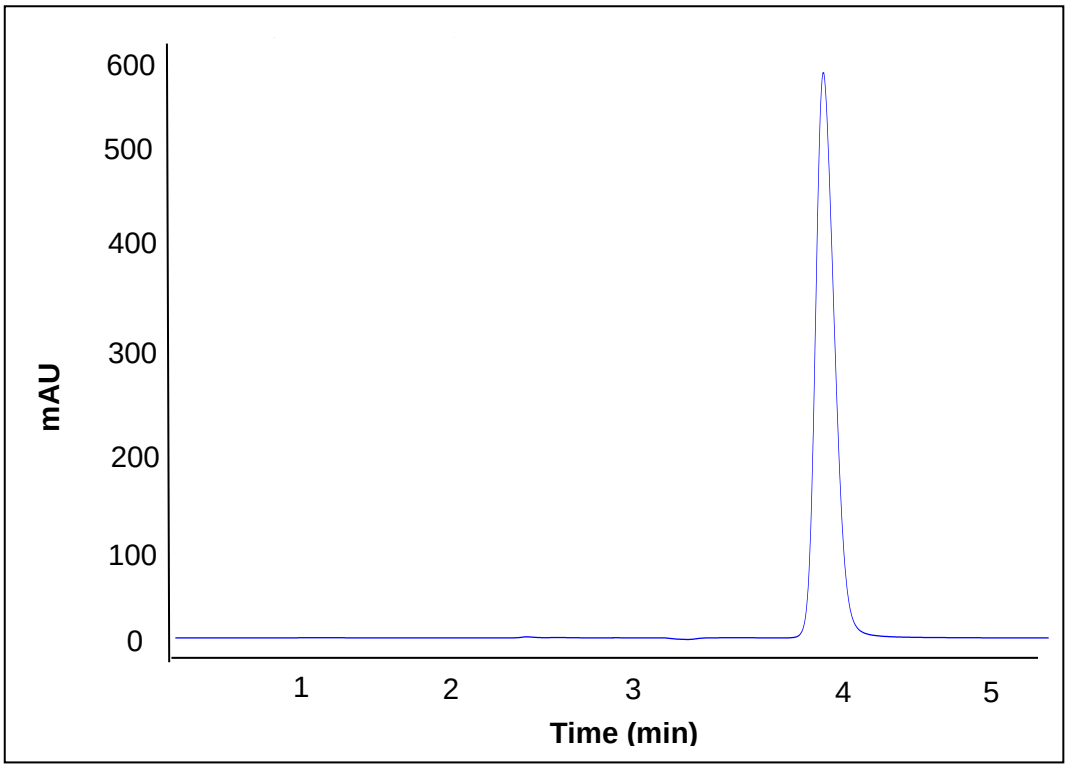

Figure A.2: A chromatogram of a standard solution of niacinamide

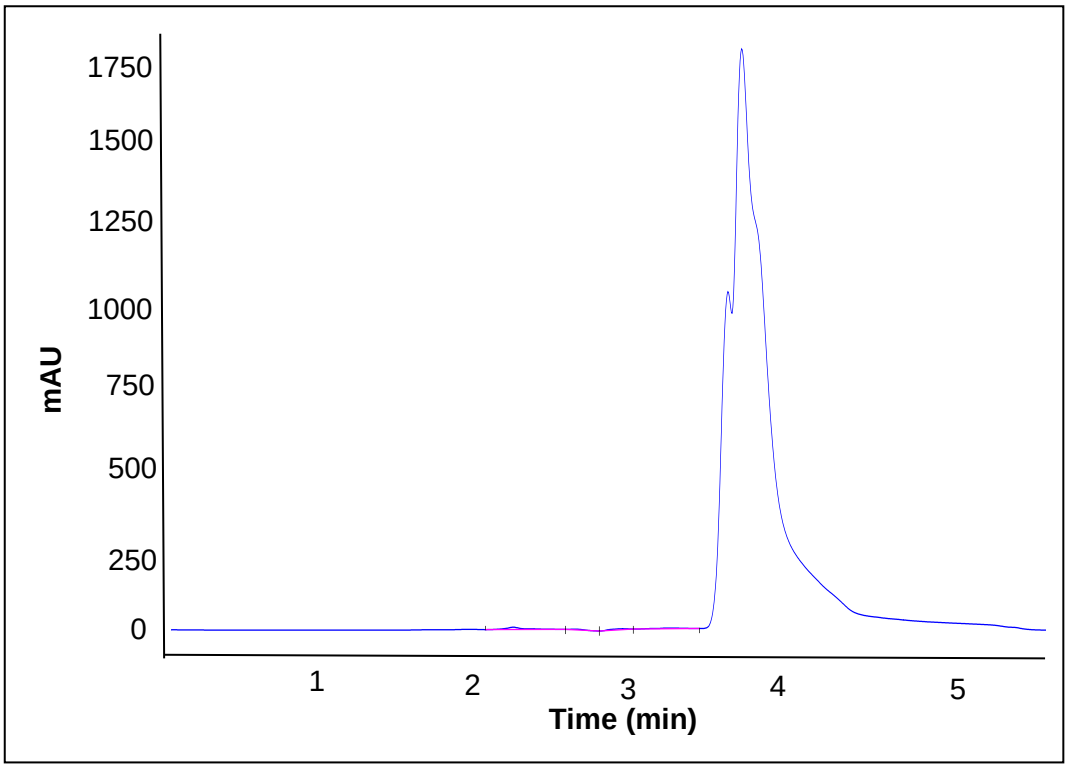

Figure A.3: Chromatogram of niacinamide mixed with sodium hydroxide (0.1 M)

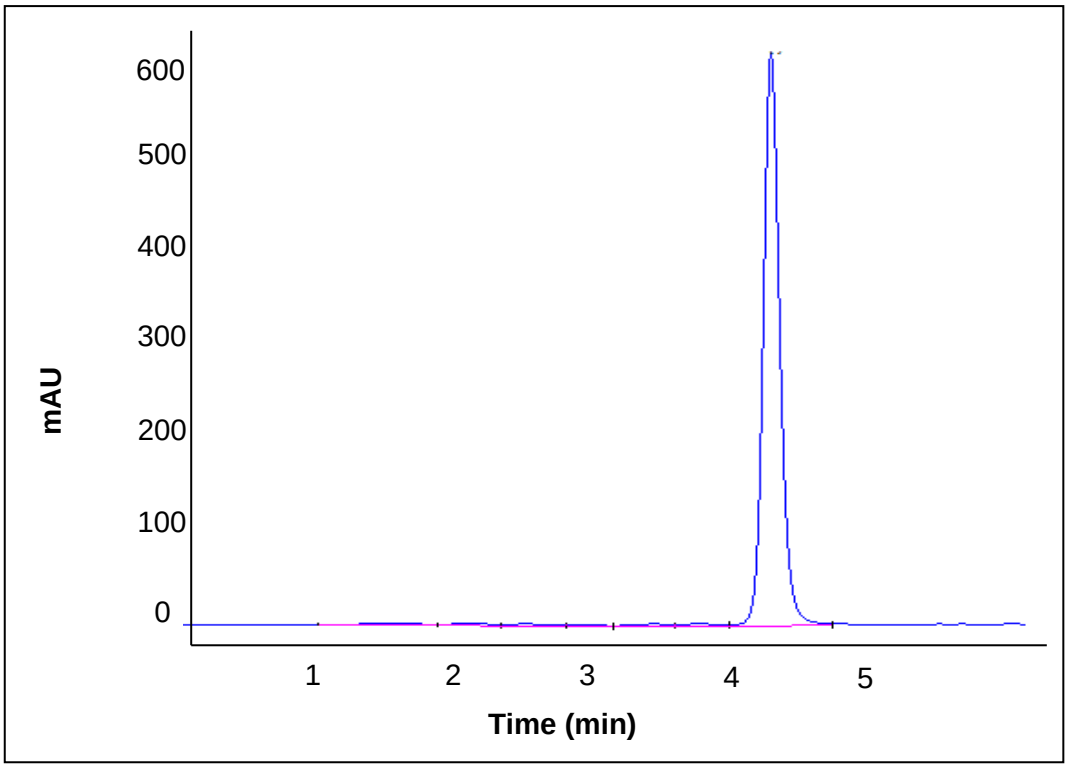

Figure A.4: Chromatogram of niacinamide mixed with hydrochloric acid (0.1 M)

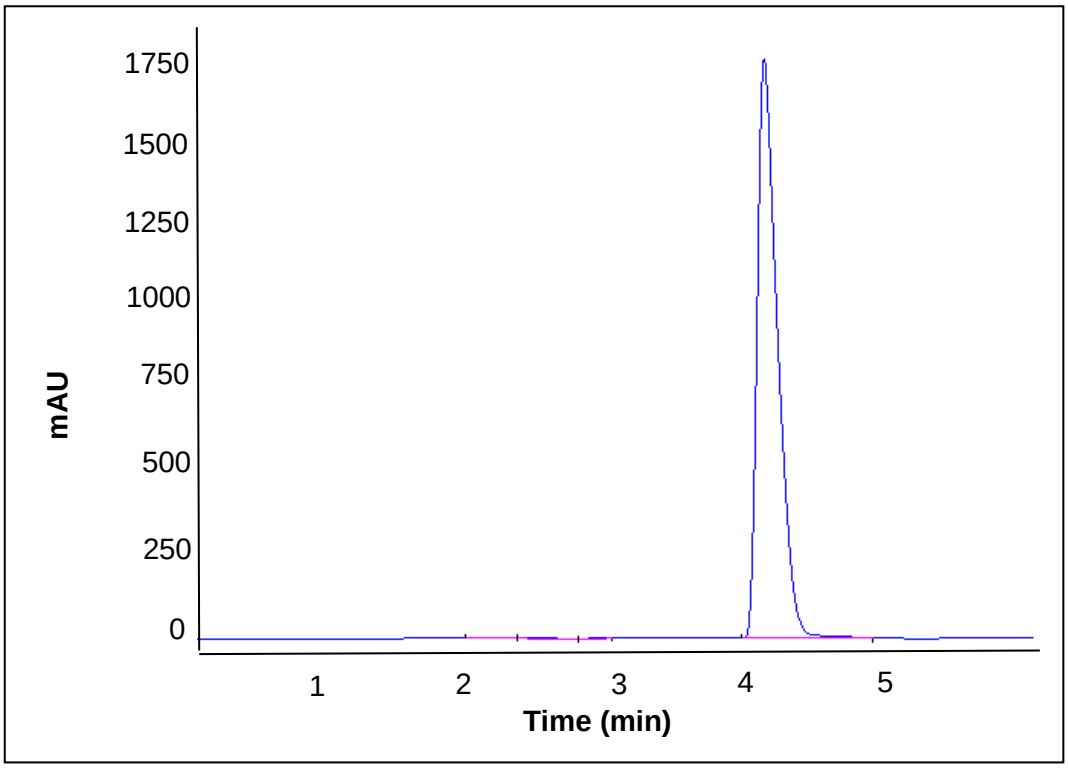

Figure A.5: Chromatogram of niacinamide mixed with distilled water

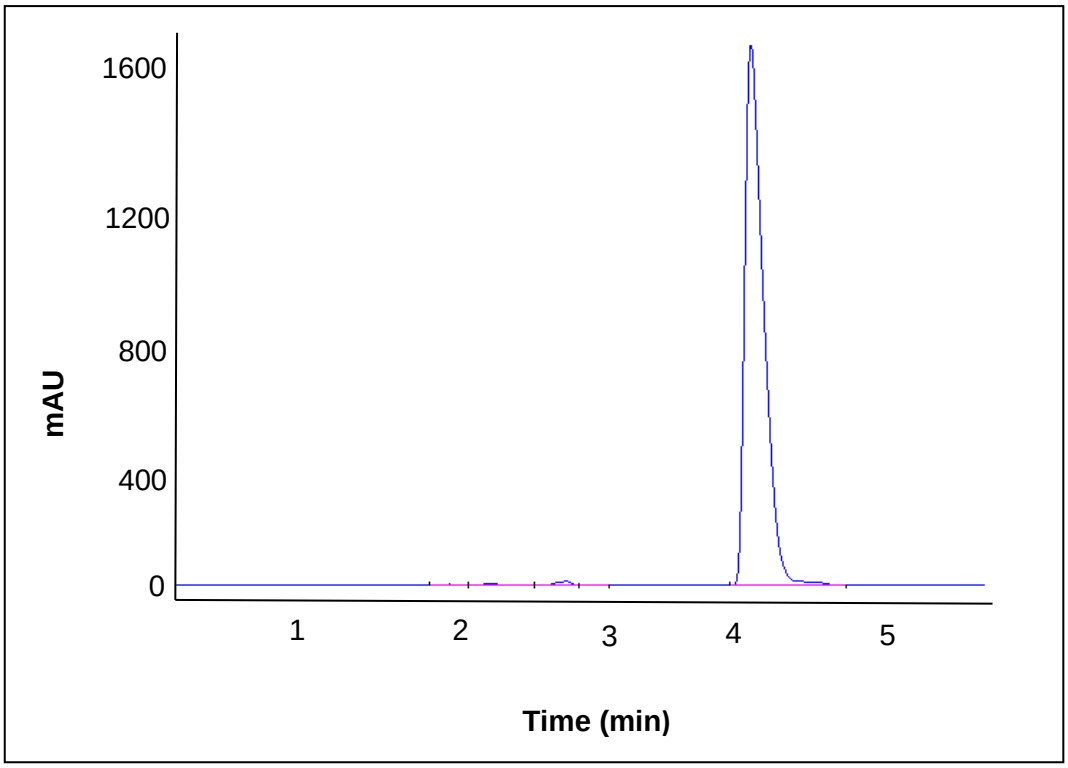

Figure A.6: Chromatogram of niacinamide mixed with 10% peroxide

When comparing the standard solution (Figure A.2) to Figures A.3 to A.6, the active could still be detected, thus, adding reagents would not influence the determination of the active.

A.5.8 Robustness

Robustness measures the analytical procedure capacity to remain unaffected by small variations in the method parameters and indicates the repeatability during normal usages (Shabir, 2005:11).

A.5.8.1 Procedure for robustness

For the robustness, a standard sample was prepared containing 20 mg niacinamide, placed into a 100 ml volumetric flask and made up to volume with PBS (pH 7.4).

A.5.8.2 Acceptance criteria

For the criteria to be acceptable, the analyte must remain unaffected by small variations in the method parameters (ICH, 2005:4).

A.5.8.2 Results and discussion

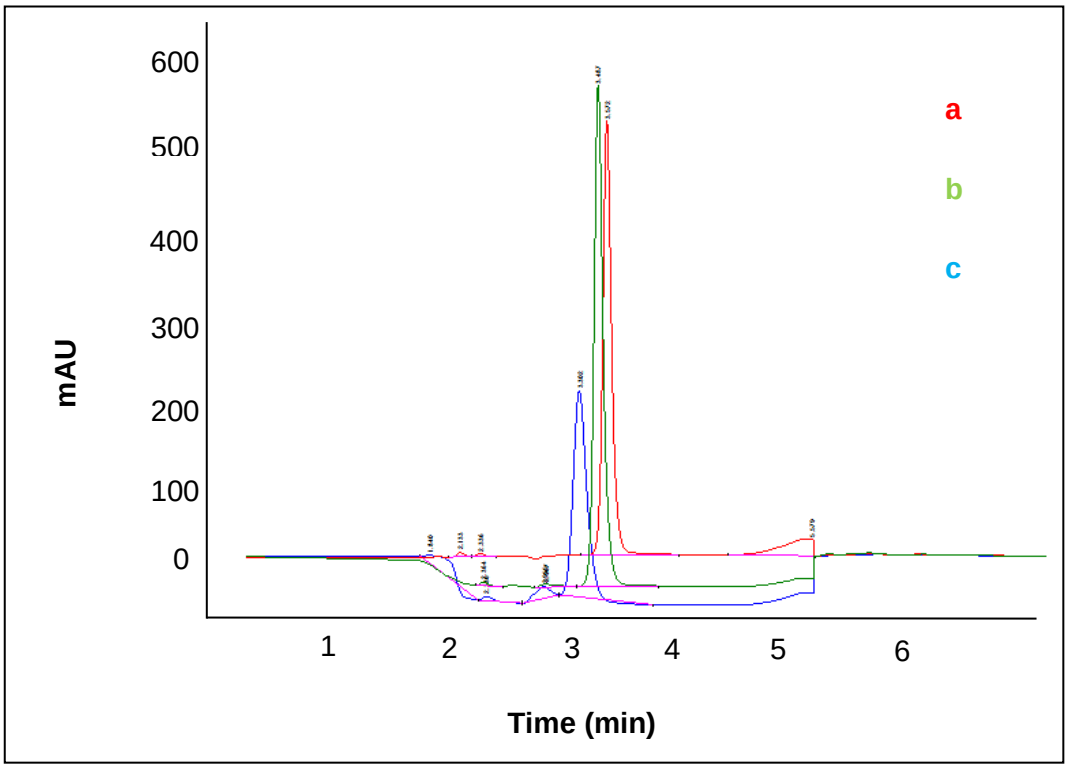

Figure A.7: Chromatogram of niacinamide with small variations in the method parameters: a) standard solution of niacinamide, b) results when increasing the wavelength, injection volume and increase flow tempo and c) the result of decreasing wavelength and injection volume and decreasing the flow tempo.

As seen in Figure A.7, niacinamide could still be detected with various changes in the method parameters. Although a difference in the peak area can be noted, it may be explained by the increasing and decreasing of the injection volume.

A.6 Conclusion

The HPLC method of niacinamide was validated and found to be acceptable in all the above-described criteria. This means the method may be used for the determination of the active throughout the study.

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Appendix B

Formulation of niosomes and proniosomes

B.1 Introduction

Niacinamide which is part of the vitamin B group; is water soluble and has been shown to have a few benefits when applied to the skin (Lupo, 2001:467). If topical delivery is needed, niacinamide does not have the ideal physical and chemical properties to reach the target sight. Therefore, the aim of this study was to formulate niacinamide into a vesicle system to try to enhance topical delivery.

Niosomes and proniosomes are vesicle systems which are formed by self-assembly of non-ionic surfactants (Ruckmani & Sankar, 2010:1119). Niosomes were initially produced in the cosmetic industry and later studied for their potential use in drug delivery (Moghassemi & Hadjizadeh, 2014:23). According to Ruckmani and Sankar (2010:1119), a vesicle system may provide a better bio-distribution and a better degree of drug targeting, especially when sustained release is required.

Niosomes are amphiphiles and consist of a hydrophilic head group and a hydrophobic tail (Marianecci *et al*., 2013:67). Consequently, they can encapsulate both hydrophilic and lipophilic actives (Moghassemi & Hadjizadeh, 2014:34). According to Marianecci *et al*. (2013:67), niosomes can encapsulate various types of actives because of the different components used to manufacture them. Niosomes are produced by dissolving surfactants and cholesterol in an organic substance to form the lipid phase, where after an aqueous phase is added and the mixture with continuous stirring. Surfactants are the main reason why vesicles form in the dispersion, while cholesterol is used to provide the dispersion with rigidity and permeability over the cell membranes. The organic phase is only used to dissolve the surfactants and the cholesterol. By using the aforementioned ingredients, niosomes can be formulated by utilising different types of methods, such as:

- the thin-film hydration method;
- the hand shaking method;
- the bubble method;
- ether injection method;
- sonication method;

- heating method.

During this study, the thin-film hydration method was used. This method entails making use of surfactants such as Span®, which is dissolved in an organic solvent and placed in a round bottomed flask. By using a rotary vacuum, the organic solvent evaporates forming a thin film inside the flask. An aqueous solution containing the active ingredient is added to the thin film in the flask causing niosomes to form (Moghassemi & Hadjizadeh, 2014:22).

According to Marianecci *et al*. (2013:67), proniosomes are more or less formulated by using the same technique as formulating niosomes; the only difference is that proniosomes involve the coating of a water-soluble carrier with the lipid phase. This water-soluble carrier may include sorbitol, glucose or lactose. Coating the sugar with the lipid phase, results in a dry formulation of a thin film covering each particle. When hydrated, the sugar dissolves into the aqueous phase and entraps the drug to form niosomes. Proniosomes are formulated because it is more stable compared to niosomes.

B.2 Ingredients used to formulate niosomes and proniosomes

The raw materials that were used during the manufacturing of niosomes and proniosomes were cholesterol, Span® 60, chloroform, sorbitol and purified water.

B.2.1 Cholesterol

Cholesterol is used in the formulation of niosomes and proniosomes because of its interaction with the non-ionic surfactant. This interaction can influence the physical properties, as well as the structure of the niosomes formed. The physical properties include the aggregation, ion permeability, the fusion process, elasticity, enzymatic activity, as well as the shape and size (Mahale *et al.,* 2012:48-49).

B.2.2 Span® 60

Span® 60 is a non-ionic surfactant which contains both a polar and a non-polar compartment that is responsible for a high interfacial activity. According to Kumar and Rajeshwarrao (2011:209), non-ionic surfactants are used to compose vesicle systems since they improve the stability and are less toxic than the amphoteric and cationic forms. They tend to be less toxic, since they are less irritating and maintain a pH range as near as possible to physiological pH. The functions of these surfactants may include acting as a wetting agent, a solubilising agent, an emulsifier and a permeability enhancer (Kumar & Rajeshwarrao, 2011:209).

B.2.3 Chloroform

Chloroform is an organic solvent used to dissolve the cholesterol and the surfactant. After mixing the chloroform with cholesterol and the surfactant, the organic solvent evaporates leaving a lipid phase.

B.2.4 Purified water

As the solubility of the active ingredient depends on the pH of the hydration medium , Milli-Q® HPLC grade water will be used, since niacinamide has good solubility in purified water.

B.2.5 Sorbitol

Sorbitol is a water-soluble carrier used during the manufacturing of proniosomes. When formulating the proniosomes, the water-soluble carrier is coated with the lipid phase (chloroform, span and cholesterol) to form a thin film covering every particle. When hydrating the sorbitol mixture (also known as a proniosome), the sorbitol dissolves into the hydration medium to entrap the drug and form niosomes (Marianecci *et al*., 2013:67).

B.2.6 Niacinamide

Niacinamide is a water-soluble vitamin used in this study for topical delivery in order to treat skin conditions (Pfuhl *et al.,* 2005:1045). The formulation involves entrapping the active into a vesicle system in order to enhance topical delivery. When formulating niosomes and proniosomes, niacinamide is dissolved into the hydration medium (purified water) to be encapsulated into the vesicle system when hydrated.

B.3 Methods

B.3.1 Formulation of the niosomes and proniosomes

For the formulation of the niosomes, four different dispersions were prepared in order to determine which of the niosomes had the highest level of stability, entrapment efficiency (EE%), as well as the best size and polydispersity index (PdI) to enhance topical delivery. The ratio of cholesterol, Span® 60 and niacinamide was approximately 2:1:1. Three of the four different samples contained 1, 2 and 3% niacinamide respectively, whilst the fourth was a placebo. The proniosomes were made using the niosomes formula that had the best stability, according to their EE%, size and PdI. The proniosomes were formulated containing various amounts of sorbitol (2, 3 and 4 g) to determine which formula had the best characteristics, in accordance to the niosomes, after hydration. In order to ease reading, the following abbreviations were given to the

different formulations: niosomes containing 1% niacinamide (**N1**), niosomes containing 2% niacinamide (**N2**), niosomes containing 3% niacinamide (**N3**) and the niosomes placebo (**NP**). All the proniosomes were formulated to contain 3% niacinamide, thus only the amount of sorbitol differed. They were abbreviated as: proniosomes containing 2 g sorbitol (**P1**), proniosomes containing 3 g sorbitol (**P2**) and proniosomes containing 4 g sorbitol (**P3**). The placebo proniosomes were formulated to contain no active with different amounts of sorbitol and given the following abbreviations: placebo proniosomes containing 2 g sorbitol (**PP1**), placebo proniosomes containing 3 g sorbitol (**PP2)** and placebo proniosomes containing 4 g sorbitol (**PP3**).

B.3.2 Characterisation of the vesicle system

Characterisation was performed on the niosomes ((**N1**), (**N2**), (**N3**) and (**NP**)). This was done to determine which of the pre-formulations had the best characteristics (stability, EE%, size and PdI) to result in the final niosome-dispersion, which was subsequently used to formulate the proniosomes.

The characterisation tests were also performed on the proniosomes ((**P1**), (**P2**), (**P3**) and (**PP1**), (**PP2**), (**PP3**)), to determine which of the proniosomes had the best characteristics compared to the niosomes in order to select the final proniosome-dispersion.

B.3.2.1 Morphology

The morphology of the niosomes and proniosomes was done in conjunction with Dr A Jordaan (Laboratory for Electron Microscopy, North-West University: Potchefstroom Campus) and was determined using the Transmission electron microscope (TEM) FEI Tecnai G2 (FEI, Holland) at 120 Kv. The TEM was only used to determine whether the niosomes and proniosomes had formed in order to perform other tests. The placebo of both the niosomes and the proniosomes were used since no actives may be included when working with the TEM. First the vesicle systems (niosomes and proniosomes) were diluted one drop into 20 ml of Milli-Q® water. After dilution, three drops of each vesicle system was placed onto a separate copper grid (carbon-coated 300 mesh) and allowed to dry. The excess fluid was removed with filter paper and one drop of osmium was placed on each vesicle system. The osmium was left to evaporate, where after uranyl acetate and lead citrate were dropped onto the sample. The osmium, uranyl acetate and lead citrate were used to colour the proteins to improve the visibility of the vesicle systems. The copper grid was then placed into the TEM for analysis.

B.3.2.2 Vesicle size

The vesicle size and PdI of the niosomes and the proniosomes was determined with the Malvern Zetasizer Nano (Nano SZ) (Malvern instruments, Worcestershire, UK) at 25 °C. The vesicle systems used were prepared on the same day the testing was conducted. All the vesicle systems were diluted with Milli-Q® water to reduce particle interactions (1 ml/20 ml). The test was performed in triplicate.

B.3.2.3 Zeta-potential

The zeta-potential was done to determine which of the preformulated vesicle systems had the best stability. This test was performed on the same day the vesicle systems were prepared. The zeta-potential was tested with the Malvern Zetasizer Nano (Nano SZ) (Malvern instruments, Worcestershire, UK), at a temperature of 25 °C, using disposable folded capillary cells. The samples were diluted with Milli-Q® water to reduce particle interaction in (1 ml/20 ml). The tests were done in triplicate.

B.3.2.4 Entrapment efficiency

For the niosomes, the supernatant was separated using the Optima L-100XP ultra-centrifuge (Beckman coulter) at 25 000 rpm at room temperature (25 °C) for 30 min. For the proniosomes, the samples were diluted (1 ml/20 ml) with Milli-Q® water, as the sorbitol causes the vesicle systems to freeze. The supernatants concentration was estimated by using HPLC and each sample of supernatants was diluted before being transferred to an HPLC vial. The entrapment efficiency of each sample was done in triplicate. The encapsulation was determined by the following equation:

$$EE\% = \frac{\text{Concentration (t) - Concentration (f)}}{\text{Concentration (t)}} \times 100 \qquad \textbf{Equation B.1}$$

Where concentration (t) is the amount of drug weighed and concentration (f) is the concentration of free drug analysed on the HPLC.

B.3.3 Formulation of niosomes

As previously mentioned, the thin-film hydration method was used to formulate niosomes. The cholesterol and Span® 60 were weighed and dissolved in chloroform in a beaker to form the lipid phase. Subsequently, niacinamide was weighed and dissolved in water in a separate beaker to produce the water phase.

The lipid phase was placed on a heating pad in a fume hood with a magnetic stirrer and the heat was set at 60 °C (this is the temperature at which chloroform evaporates). The sample was left for approximately one hour until all the chloroform evaporated and a thin layer of lipid phase formed on the bottom of the beaker. The aqueous phase was then added to the mixture and stirred for another 5 min until a homogeneous mixture was produced.

Following this, the niosomes were placed in an ice bath to cool down before sonication. The sample was sonicated for 1 min on ice in order to get all the particle sizes in one range. The ice kept the sample stable as the sonicator became extremely hot while sonicating.

Table B.1: Preformulation of niosomes formula

Ingredients	(**N1**)	(**N2**)	(**N3**)
Cholesterol	100 mg	200 mg	300 mg
Span® 60	200 mg	400 mg	600 mg
Niacinamide	100 mg	200 mg	300 mg
Milli-Q® water	10 ml	10 ml	10 ml
Chloroform	10 ml	10 ml	10 ml

Table B.2: Niosomes placebo formula

Ingredients	(**NP**)
Cholesterol	600 mg
Chloroform	10 ml
Span® 60	300 mg
Milli-Q® water	10 ml

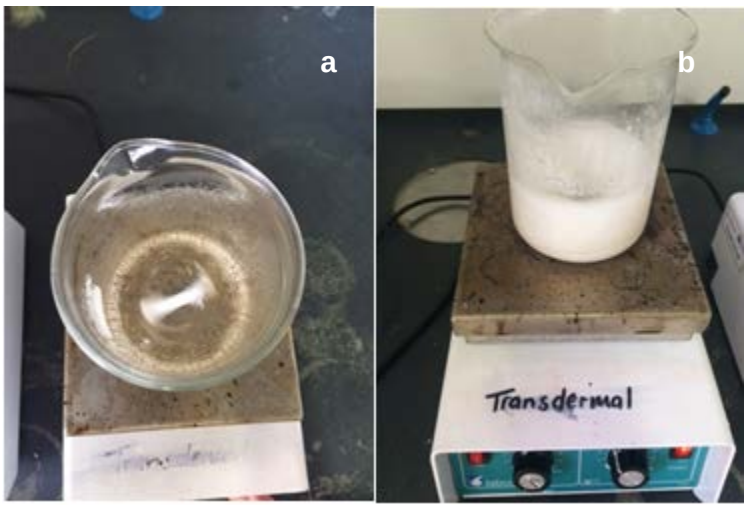

Figure B.1: Formulation process of niosomes: a) the lipid phase of the niosomes and b) niosomes after the water phase was added.

B.3.3.1 Results and discussion of preformulated niosomes

B.3.3.1.1 Morphology

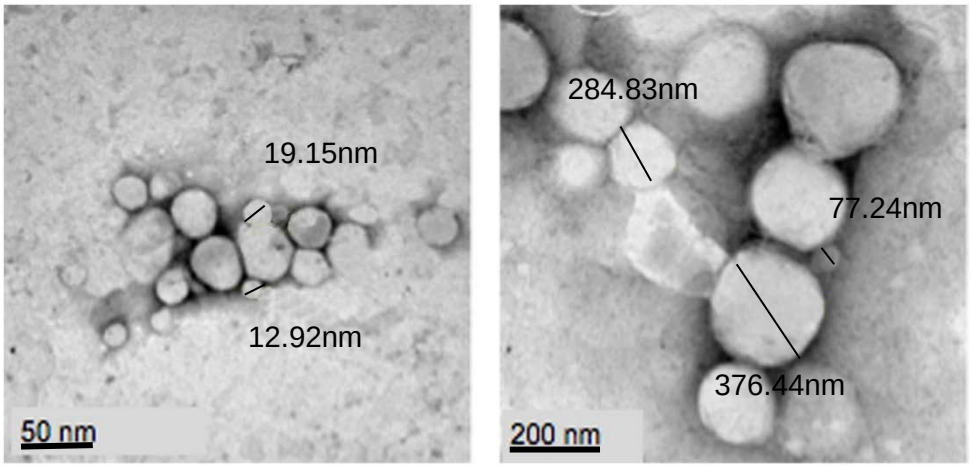

Figure B.2: Transmission electron microscope micrographs of the round shaped niosomes.

The results from the TEM are shown in Figure B.2. This test was only done on the placebo forms of the niosomes (**NP**), since no active ingredients were allowed when working with the TEM. As seen in Figure B.2, round shaped vesicles formed, each containing a lipid layer on the outside.

B.3.3.1.2 Vesicle size

This was done to determine which sample had the most suitable size and PdI. For the (**N1**), (**N2**) and (**N3**), the average sizes were approximately 376.75 ± 1.39 nm, 629.07 ± 3.49 nm and 660.2 ±4.98 nm, respectively. The (**NP**) had an average size of 552.57 ± 0.18 nm. Bolzinger *et*

al. (2012:163) state that particles with a size smaller than 3 µm in diameter will penetrate through the intracellular pathway, while particles containing a size of 3 – 10 µm will easily penetrate through the pilosebaceous follicles. All the different dispersions had particle sizes in the nano-scale range, which may promote topical penetration. All the formulations had a relatively small size, but the (**N1**) had the smallest and therefore be considered as the formulation to be used throughout the study. In Table B.3, it can be seen that the entrapment of the active could influence the size of the vesicle system, since the vesicle systems were bigger when more active was added.

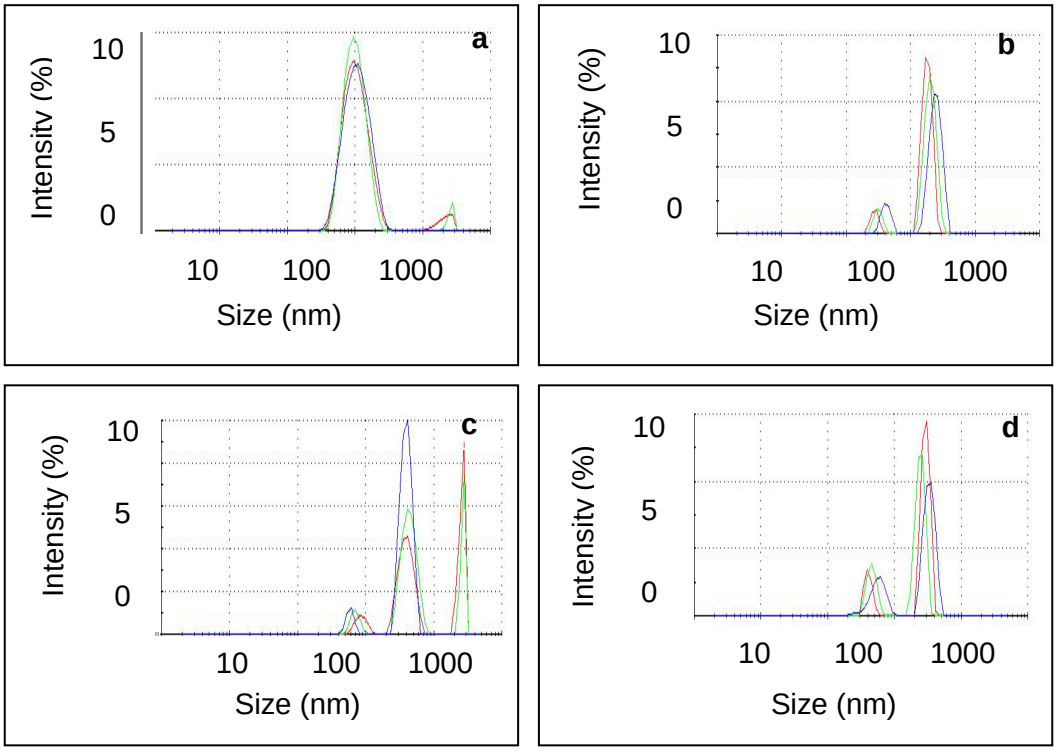

Figure B.3: Vesicle sizes of the preformulated niosomes: a) the vesicle size of the (**NP**), b) the vesicle size of the (**N1**), c) the vesicle size of the (**N2**) and d) the vesicle size of the (**N3**).

Table B.3: The average vesicle size and average PdI of the preformulated niosomes

Vesicle system	Average vesicle size	Average PdI
(**N1**)	376.75 ± 1.39 nm	0.56 ± 1.29
(**N2**)	629.07 ± 3.49 nm	0.71 ± 0.48
(**N3**)	660.20 ± 4.98 nm	0.87 ± 0.12
(**NP**)	552.57 ± 0.18 nm	0.61 ± 0.38

The PdI was also performed in triplicate and the average PdI values for the (**N1**), (**N2**) and (**N3**) were 0.56 ± 1.29, 0.71 ± 0.48 and 0.87 ± 0.12, respectively. The average PdI for the (**NP**) was

0.61 ± 0.38. According to Gaumet *et al*. (2008:2), the PdI value indicates the size distribution in the formula. When the PdI values is near to one or very high, it demonstrates a high size distribution resulting in a more polydispersed medium. Comparing the formulas, the (**N1**) is a more monodispersed medium resulting in a dispersion to have a low size distribution, while the (**N3**) is a more polydispersed medium. As seen in Figure B.3, the sizes of the vesicles range from 100 nm to a 1000 nm. Consequently, the niosomes were more polydispersed than monodispersed, which can indirectly influence the stability of the system.

B.3.3.1.3 Zeta-potential

Table B.4: The average zeta-potential of the preformulated niosomes

Vesicle system	Average zeta-potential
(**N1**)	- 45.24 mV ± 8.16
(**N2**)	- 42.73 mV ± 3.82
(**N3**)	- 48.63 mV ± 4.17
(**NP**)	- 44.78 mV ± 3.27

The zeta-potential was measured to determine which of the preformulations was most stable. The average zeta-potential of the (**N1**), (**N2**) and (**N3**) was - 45.24 mV ± 8.16, - 42.73 mV ± 3.82and - 48.63 mV ± 4.17, respectively. The (**NP**) had an average zeta-potential of - 44.78 mV ± 3.27. The values ranged from - 42.73 mV to - 48.63 mV, therefore all the niosomes were stable and no flocculation occurred. Although all the formulations were stable, the (**N3**) had the best stability with a zeta-potential of - 48.63 mV.

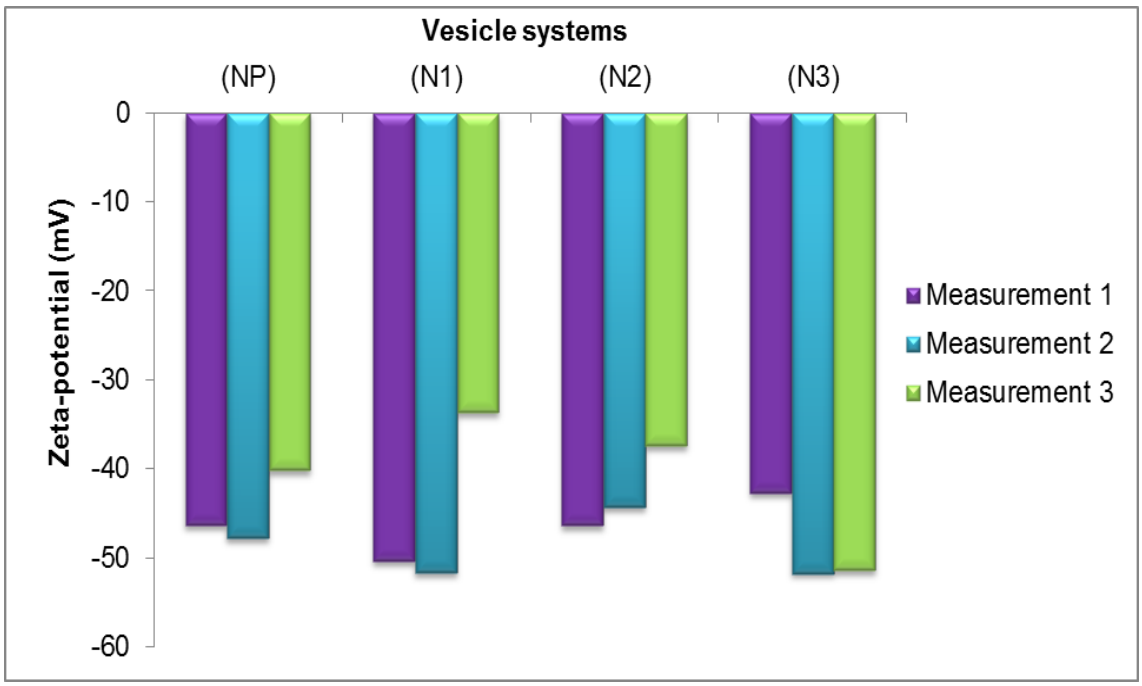

Figure B.4: The zeta-potential of the pre-formulated (**N1**), (**N2**) and (**N3**) as well as the (**NP**).

B.3.3.1.4 Entrapment efficiency

The EE% is an important parameter when considering which of the niosomes is to be used throughout the study. As seen in Table B.5, all the average EE% values were good and consequently the niosomes with the highest average EE% may lead to better penetration and effect. In this case, the (**N3**) presented with the highest average EE% value and could therefore be considered for use throughout the study. Bayindir *et al*. (2009:2052) reported that niosomes can encapsulate between 19.90% and 86.40% of an active. In Table B.5, it can clearly be seen that the values in this study correlate well. It was decided that 3% niacinamide would be used to formulate the proniosomes because of the high entrapment.

Table B.5: Entrapment efficiency (EE%) of (**N1**), (**N2**) and (**N3**)

Vesicle system	Average EE (%)
(**N1**)	68.52 ± 1.81
(**N2**)	79.48 ± 0.34
(**N3**)	86.85 ± 6.52

B.3.4 Method to formulate proniosomes

Proniosomes was made using chloroform, Span® 60, cholesterol and sorbitol. First cholesterol and Span® 60 were weighed and placed into a beaker. Then chloroform was added in a 2:1:1 ratio to the mixture to form the lipid phase. Thereafter, sorbitol was weighed and placed into a beaker on the hotplate with a magnetic stirrer. The temperature was set at 60 °C and the lipid phase was slowly dropped onto the sorbitol until all the sorbitol were wetted and subsequently left to dry before the rest of the mixture was added. This process was repeated until all the lipid phase was poured onto the sorbitol. The dry sorbitol was left overnight in a desiccator to dry completely. The proniosomes were hydrated by using the aqueous phase (niacinamide dissolved in water) when needed. After the aqueous phase was added, the proniosomes were stirred for 5 min and sonicated on ice for approximately 1 min.

Table B.6: Preformulation of the proniosomes containing 3% niacinamide with different amounts of sorbitol

Ingredients	(**P1**)	(**P2**)	(**P3**)
Cholesterol	600 mg	600 mg	600 mg
Span® 60	300 mg	300 mg	300 mg

Niacinamide	300 mg	300 mg	300 mg
Sorbitol	2000 mg	3000 mg	4000 mg
Milli-Q® water	10 ml	10 ml	10 ml
Chloroform	10 ml	10 ml	10 ml

Table B.7: Preformulation proniosomes placebo formula with different amounts of sorbitol

Ingredients	(**PP1**)	(**PP2**)	(**PP3**)
Cholesterol	600 mg	600 mg	600 mg
Span® 60	300 mg	300 mg	300 mg
Sorbitol	2000 mg	3000 mg	4000 mg
Milli-Q® water	10 ml	10 ml	10 ml
Chloroform	10 ml	10 ml	10 ml

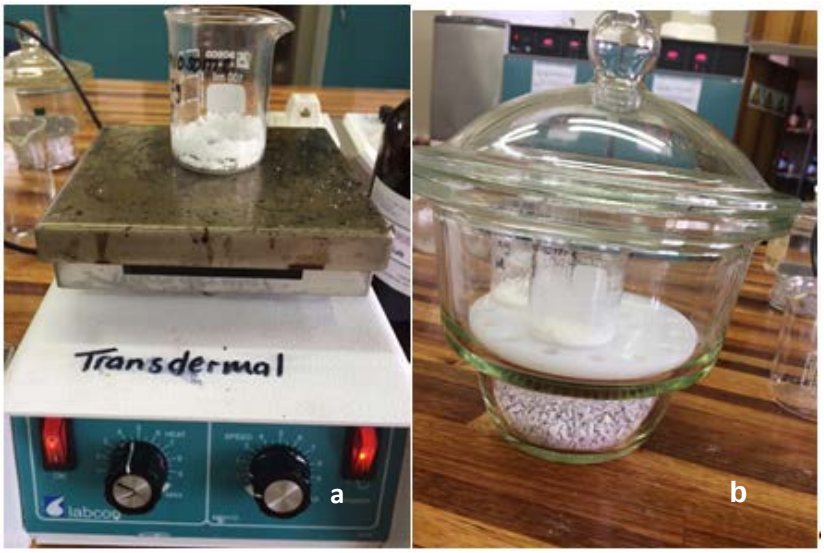

Figure B.5: The preparation of proniosomes: a) sorbitol placed onto a magnetic stirrer in order to let the lipid phase evaporate and b) proniosomes placed into a desiccator and left over night to dry completely.

B.3.4.1 Results and discussion of preformulated proniosomes

B.3.4.1.1 Morphology

TEM micrographs are shown in Figure B.6. This was only performed on the placebo forms of proniosomes as no active ingredients are allowed when working with the TEM. As seen in Figure B.6, the niosomes formed from the proniosomes were much bigger than the original niosomes in Figure B.2 and less roundly shaped, which could be due to the sorbitol forming much bigger vesicles before hydration. In Figure B.6 it is also noticed that the lipid layer on the outside of the vesicles seems to be damaged, which could be a result of the hydration processes, causing a rupture in the lipid membrane when the aqueous phase moved through the membrane to dissolve the carrier.

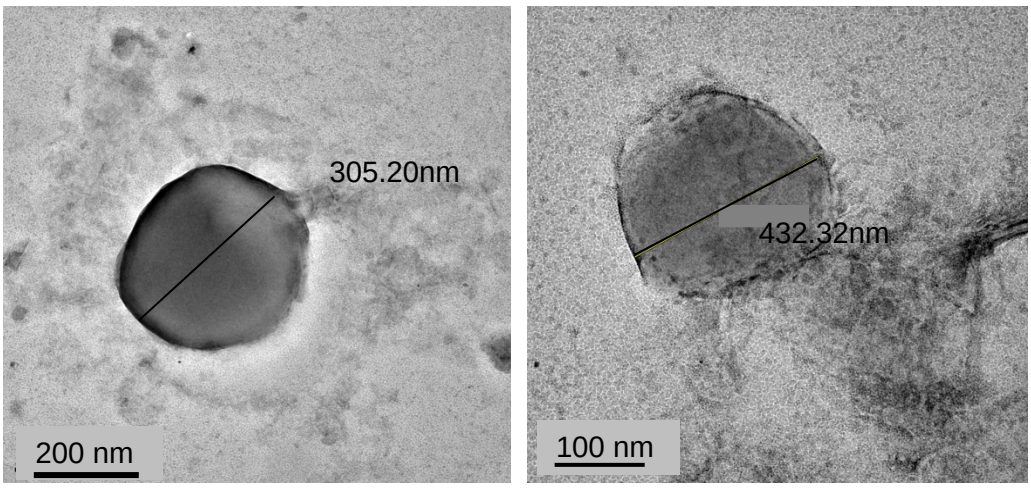

Figure B.6: Transmission electron microscope micrographs of the round shaped placebo proniosomes.

B.3.4.1.2 Vesicle size

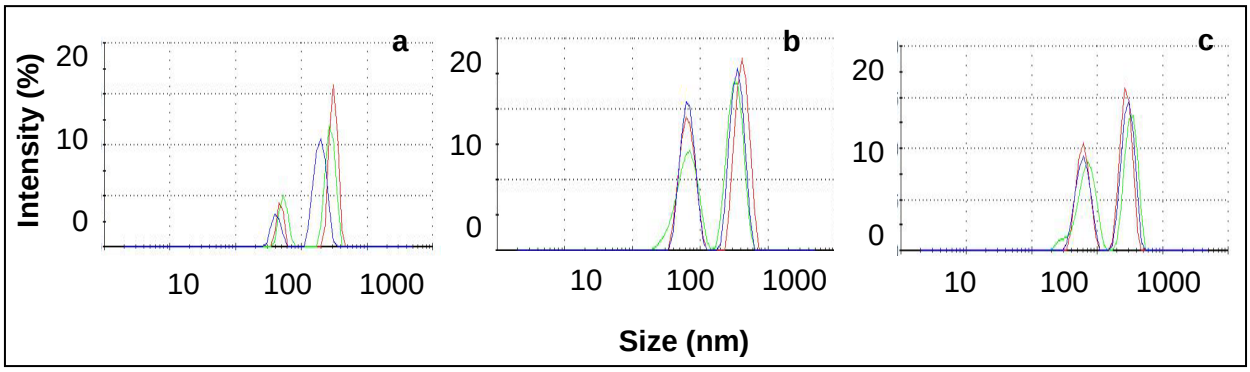

Figure B.7: Vesicle sizes of proniosomes containing 3% niacinamide with different amounts of sorbitol: a) (**P1**), b) (**P2**) and c) (**P3**).

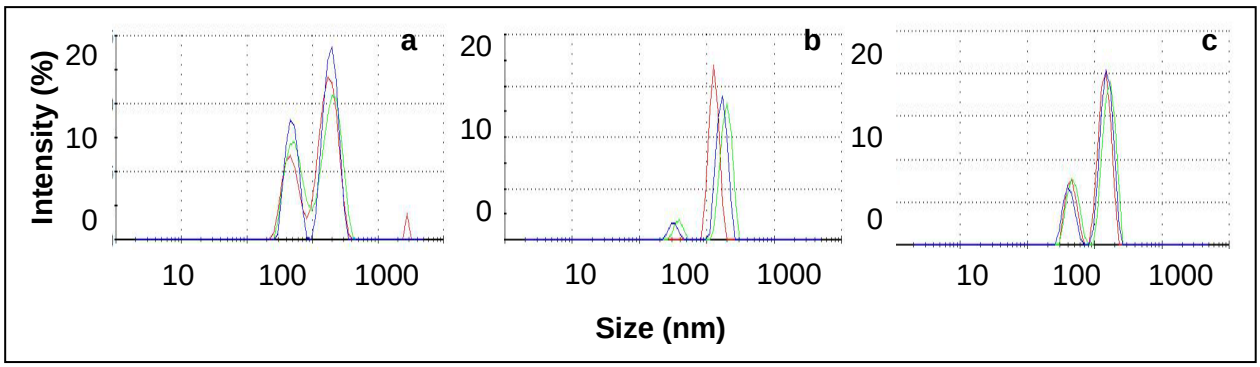

Figure B.8: The vesicle size of placebo proniosomes with different amounts of sorbitol: a) (**PP1**), b) (**PP2**) and c) (**PP3**).

For the proniosomes, the vesicle sizes and the PdI was measured in order to determine which of the preformulations would be the most suitable for the final dispersion. For each of the

proniosome-preformulations containing the active, a placebo proniosome-preformulation was made to determine whether the active or the amount of sorbitol used will influence the size of the vesicle system. For the proniosomes containing the active, (**P1**), (**P2**) and (**P3**) had average sizes of 496.75 ± 80.09 nm, 580.07 ± 19.24 nm and 576.17 ± 35.43 nm, respectively. For the placebo proniosomes, (**PP1**), (**PP2**) and (**PP3**) had average sizes of 451.54 ± 3.21 nm, 738.93 ± 17.98 nm and 474.58 ± 7.38 nm, respectively.

As stated before, according to Bolzinger *et al*. (2012:163), sizes smaller than 3 µm will penetrate through the intracellular pathway and in this manner lead to better topical delivery. When examining the proniosomes containing the active, the size of the vesicles became larger as the sorbitol increased. Hence, the (**P1**) presented with the smallest particle size when compared to (**P2**) and the (**P3**). When comparing the particle size of the placebo proniosomes with that of the proniosomes containing actives, although only minor differences were observed, incorporating the active in the proniosomes had an influence on the size. Thus the (**P1**) would be a good candidate to consider since it presented with the smallest size.

Figures B.7 and B.8 indicates the size distribution of the particles in the dispersion. As seen in the Figures both the proniosomes containing the active and the placebo forms presented with a size distribution between 100 nm and 1000 nm. Since the PdI value indicates the size distribution the approximate average PdI values of the (**P1**), (**P2**) and (**P3**) were as follow: 0.72 ± 0.41, 0.67 ± 0.39 and 0.73 ± 0.17, respectively. For the (**PP1**), (**PP2**) and (**PP3**), the average PdI values were 0.64 ± 1.26, 0.57 ± 0.88 and 0.77 ±0.52, respectively.

The PdI of the proniosomes (containing the active and the placebo) demonstrated they are polydispersed, resulting in the dispersions to have a high size distribution. By adding the active, the PdI increased although the differences were too small to make a direct conclusion. When looking at the size range between the proniosomes and niosomes it is quite the same and any one of the above proniosomes formulations would be ideal.

B.3.4.1.3 Zeta-potential

The zeta-potential of the proniosomes was measured to determine which preformulation would be ideal with properties as close as possible to that of the niosomes. The zeta-potential of the proniosomes was also done in triplicate. The average zeta-potential of the (**P1**), (**P2**), (**P3**) were as follows: - 50.55 ±3.55mV, - 51.54 ± 6.99 mV and - 51.95 ± 5.80 mV, respectively. For the (**PP1**), (**PP2**) and (**PP3**), the average zeta-potential values were - 49.27 ± 1.65 mV, - 38.78 ± 7.92 mV and - 61.32 ± 3.52 mV, respectively. It can be seen in Figures B.9 and B.10 that the zeta-potential was influenced by adding the active. All the values increased, causing the formula to be more stable when the active was encapsulated. According to Roland *et al*.

(2003:87), a dispersion may be classified to be stable when a zeta-potential value of greater than – 25 mV are presented. Since all the formulations' zeta-potential values were greater than - 25 mV, it demonstrates that all the preparations were stable and that any formula could be used throughout the study.

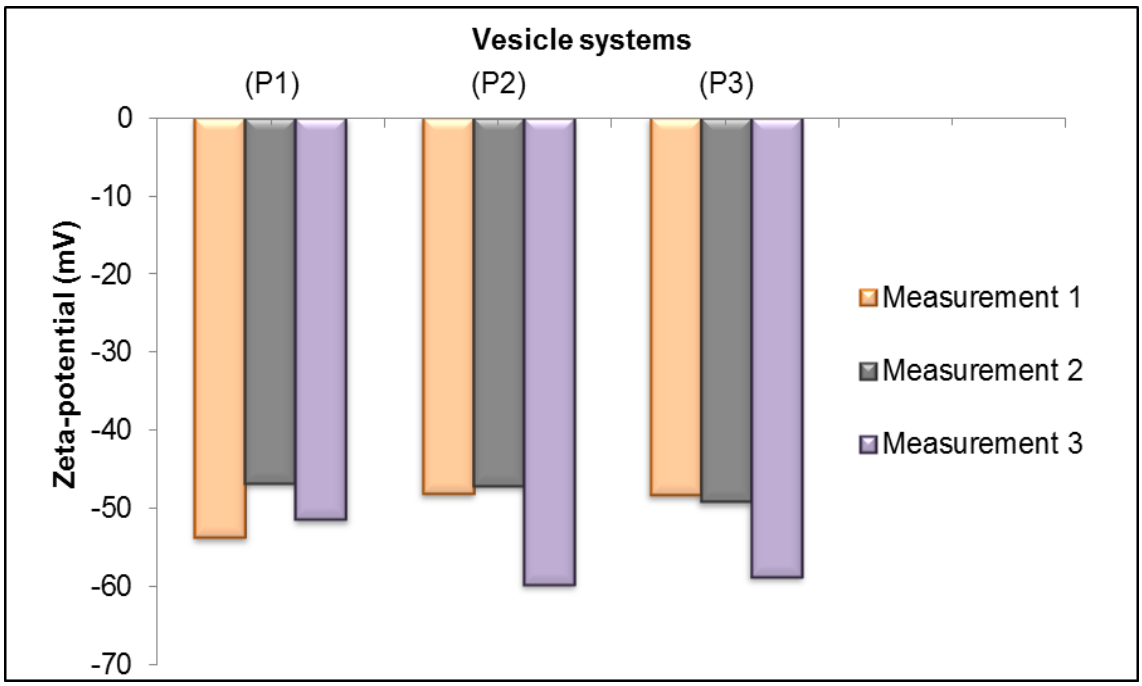

Figure B.9: The zeta-potential of the proniosomes containing 3% niacinamide with different amounts of sorbitol.

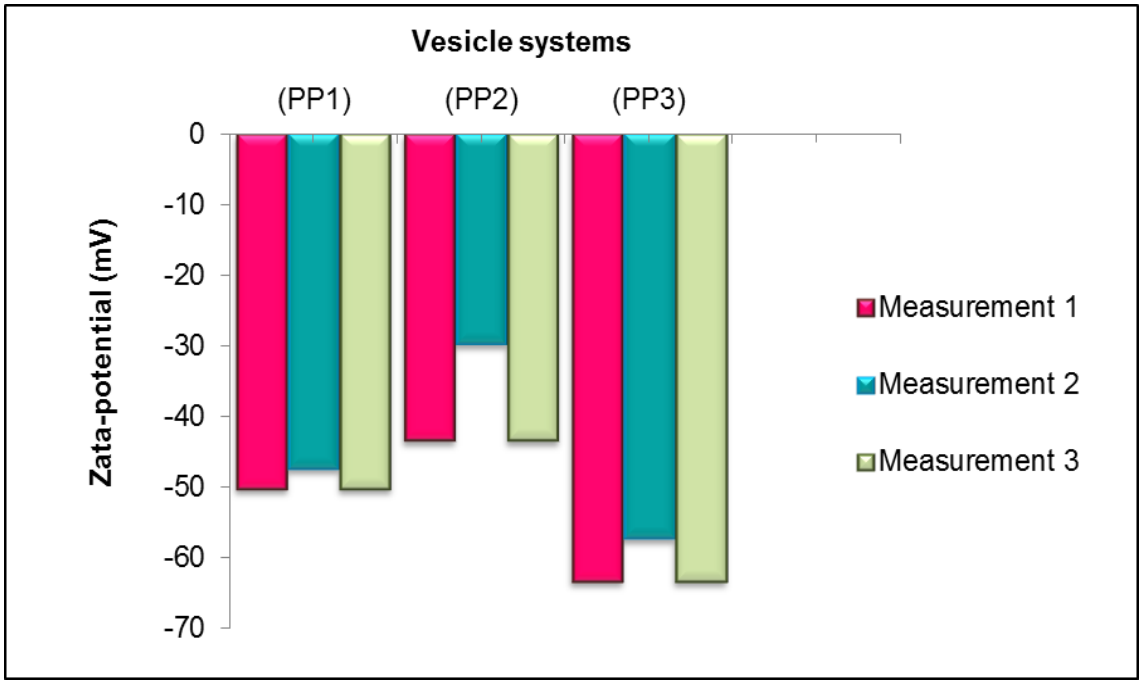

Figure B.10: The zeta-potential of the placebo proniosomes with different amounts of sorbitol.

B.3.4.1.3 Entrapment efficiency (EE%)

The (**P1**), (**P2**) and (**P3**) had an average EE% of 31.29 ± 0.92%, 33.78 ± 0.13% and 33.06 ± 0.16%, respectively. These EE% values are much lower than that of the (**N3**), which had an EE% value of 86.85%. The reason for this poor EE% of the proniosomes may be due to the fact that not all the sorbitol completely dissolved during the hydration process, causing more of the active to be located in the water phase than being entrapped into the vesicle. As all the proniosomes had more or less the same value of entrapment, any of them could be used throughout the study.

Table B.8: The EE% of the preformulated proniosomes containing 3% niacinamide with different amounts of sorbitol

Pro-vesicle system	Average EE% (%)
(**P1**)	31.29 ± 0.92
(**P2**)	33.78 ± 0.13
(**P3**)	33.06 ± 0.16

B.4 Final formula selected for formulating niosomes and proniosomes

B.4.1 The final formula of the niosomes

For the formulation of the niosomes, the thin-film hydration method was used as described in Section B.3.3.

Table B.9: Ingredients used to formulate the final niosomes containing 3% niacinamide (**N3**)

Ingredients	(**N3**)
Cholesterol	600 mg
Chloroform	10 ml
Niacinamide	300 mg
Span® 60	300 mg
Milli-Q® water	10 ml

The niosomes were extremely free-flowing, almost the composition of milk, white in colour and odourless, and could not be placed directly on the skin without formulating it into a semi-solid dosage form.

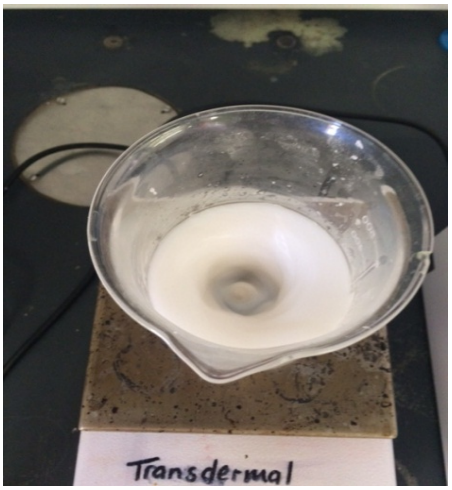

Figure B.11: The final niosomes containing 3% niacinamide (**N3**).

B.4.2 The final formula of the proniosomes

For the formulation of the proniosomes, the same method as discussed in Section B.3.4 was used. This involves coating a water-soluble carrier (like sorbitol) with the lipid phase to form a dry powder also known as proniosomes. When hydrated, the aqueous phase dissolves the carrier and turns the proniosomes into niosomes.

Table B.10: The ingredients used to formulate the final proniosomes containing 3% niacinamide and 2 g sorbitol (**P1**)

Ingredient	(**P1**)
Cholesterol	600 mg
Span® 60	300 mg
Niacinamide	300 mg
Sorbitol	2000 mg
Milli-Q® water	10 ml
Chloroform	10 ml

The proniosomes were a fine, odourless, cloudy white powder, but when hydrated with the aqueous phase, it was a free-flowing liquid with a low viscous that could not be applied to the skin.

Figure B.12: The final formulation of proniosomes containing 3% niacinamide and 2 g sorbitol (**P1**).

B.5 Conclusion

To determine the optimal formulation of the niosomes and the proniosomes a few parameters had to be investigated, including the characteristics of the vesicle system (TEM, vesicle size, zeta-potential and the EE%). The optimal formula of the niosomes contained 3% niacinamide (**N3**) and was selected because of its high value of entrapment. Thus this would be the ideal niosome-dispersion, since its vesicle size, as well as the zeta-potential was within the prescribed values. The optimal formula for the proniosomes contained 3% niacinamide with 2 g sorbitol (**P1**). Although most of the results seem to be the same (even the EE%) for the (**P1**), (**P2**) and (**P3**), the formulation process of the (**P1**) was much faster than the (**P2**) and (**P3**); it also had the smallest particle size which may result in better topical delivery.

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Appendix C

Physical characteristics of the final vesicle system

C.1 Introduction

Since vesicle systems are prepared by combining lipids and surfactants, the composition will influence the physicochemical characteristics of the vesicle such as vesicle size, charge, thermodynamic phase, lamellarity and bilayer elasticity. These characteristics indirectly affect the behaviour of the vesicle system, as well as the effectiveness of drug delivery (Honeywell-Nguyen & Bouwstra, 2005:68). Furthermore, vesicle size influences the biodistribution, clearance and the stability of nanoparticles, therefore, determining it is very important (Gaumet *et al*., 2008:2). According to Gaumet *et al*. (2008:2), the ideal size for a particle to penetrate skin, subcutaneous tissue and mucous membranes is less than 6 nm. Thus the bigger the size, the lower the transdermal penetration will be (Denet *et al*., 2004:665). When referring to the distribution in size, we make use of the polydispersity index (PdI). When calculating the PdI, the mean size of the particle, the refractive index of the solvent, the angle at which the measurement took place, as well as the variance of the distribution are taken into account (Gaumet *et al*., 2008:2). When PdI values are considered from 0 to 1, a PdI value lower than 0.1 is associated with the vesicles in the formulation being distributed homogeneously, while a high PdI value will demonstrate a wide distribution in size of the vesicles in the formulation (Gaumet *et al*., 2008:2). Thus, we can say that the particle size distribution of the vesicle system is directly proportional to the stability of the dispersion.

Additionally, the stability can also be related to the zeta-potential, which can be defined as the difference in the potential between the surface of the particle and the electroneutral area of the solution (Roland *et al*., 2003:87). When the zeta-potential shows a low value (less than - 25 mV), it indicates flocculation in the system, since the attraction forces exceed the repulsive forces. When the zeta-potential is high (higher than - 25 mV), it indicates a deflocculated system in which the particles are dispersed, since the repulsive forces exceed the attractive forces (Roland *et al*., 2003:87).

Since the aim is to deliver these vesicle systems topically, the pH must be brought into account. According to Barry (2007:576), solutions applied to the skin, not having the correct pH may cause harm to the stratum corneum. For this reason the ideal pH for a solution applied to the skin must be between five and nine (Naik *et al*. 2000:319).

The viscosity can be described by Stokes' law and will indicate the rate at which two phases will separate. Where ρ_1 is the separation between liquid 1 and ρ_2 is the separation between liquid 2, g is the gravity, η the viscosity of the medium and r the radius of the particle (Roland *et al*., 2003:88).

$$V=\frac{2r^2(\rho_1-\rho_2)g}{9\eta}$$ **Equation C.1**

The viscosity do not only indicates the stability of a product, but also effects the patient compliances. Customers prefer a product which can be easily removed from the container when applied (Barry, 2007:595).

C.2 Preparation of niosomes and proniosomes

Niosomes are vesicle systems made of non-ionic surfactants, cholesterol, chloroform and a water phase. As described is Section B.3.3, niosomes were made by using the thin-film hydration method. The proniosomes was made by coating a water soluble carrier with a thin lipid film to form a niosome when hydrated. The method for formulating proniosomes is described in Section B.3.4. For the purpose of this chapter, only the vesicle systems used throughout the rest of the study are tested, to indicate if the chosen vesicle systems possess the ideal characteristics for topical application. The niosomes containing 3% niacinamide (**N3**), proniosomes containing 3% niacinamide with 2 g sorbitol (**P1**), as well as the niosome placebo (**NP**) and proniosome placebo with 2 g sorbitol (**PP1**) were identified for inclusion in this phase of the study. The (**NP**) and (**PP1**) were prepared in this chapter to determine if the active had any effect on the characteristics of the vesicle system.

C.3 Physical characteristics of the vesicle system

C.3.1 Morphology

The morphology of the (**NP**) and the (**PP1**) was studied in conjunction with Dr A Jordaan (Laboratory for Electron Microscopy, North-West University: Potchefstroom Campus), using the transmission electron microscope (TEM) FEI Tecnai G 2 (FEI, Holland) at 120 Kv. After the niosomes and proniosomes were diluted with water, three drops of each vesicle system were placed on a separate copper grid and allowed to dry (Sezign-Bayindir & Yuksel, 2012:830). The excess was removed with filter paper and a drop of osmium was placed on each sample. After the osmium evaporated, uranyl acetate and lead citrate were placed onto the samples causing the proteins to colour. The copper grid, which contained the coloured niosomes and proniosomes was placed into the TEM to be analysed.

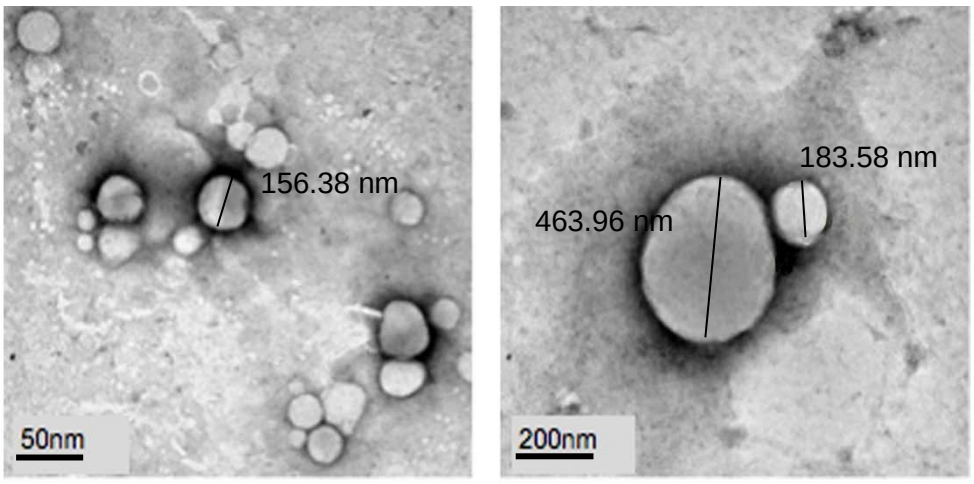

Figure C.1: Transmission electron microscope micrographs of round shaped niosomes

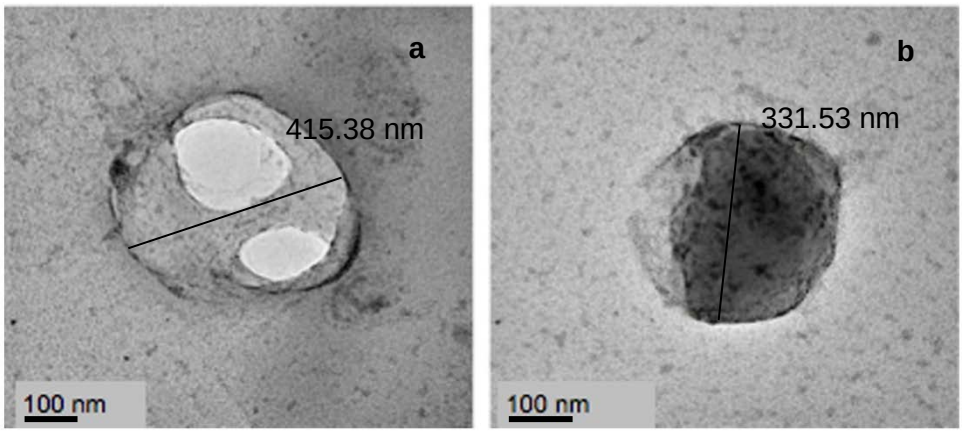

Figure C.2: Transmission electron microscope micrographs of round shaped proniosomes; a) proniosomes where the sorbitol has not yet fully dissolved and b) proniosomes were the sorbitol fully dissolved with a radius of 331.53 nm.

As seen in Figures C.1 and C.2, both vesicle systems contained a lipid layer on the outside and the proniosomes were larger than the niosomes. Another appearance observed in the micrographs, is that the niosomes formed more vesicles compared the proniosomes. Since no active may be added when working with the TEM, the placebos could not be compared to the products containing the active. We were only able to establish that vesicles did indeed form.

C.3.2 Vesicle size

Both the average vesicle size and PdI of formulas (**N3**), (**P1**), (**NP**) and (**PP1**) were determined using the Malvern Zetasizer Nano (Nano SZ) (Malvern instruments, Worcestershire, UK) at a temperature of 25 °C in triplicate. The vesicle systems were freshly prepared before the testing was performed. Both the vesicles systems were diluted in a ratio of 1:20 with Milli-Q® HPLC grade water (Merck Millipore, Halfway House, South Africa) to reduce particle interactions.

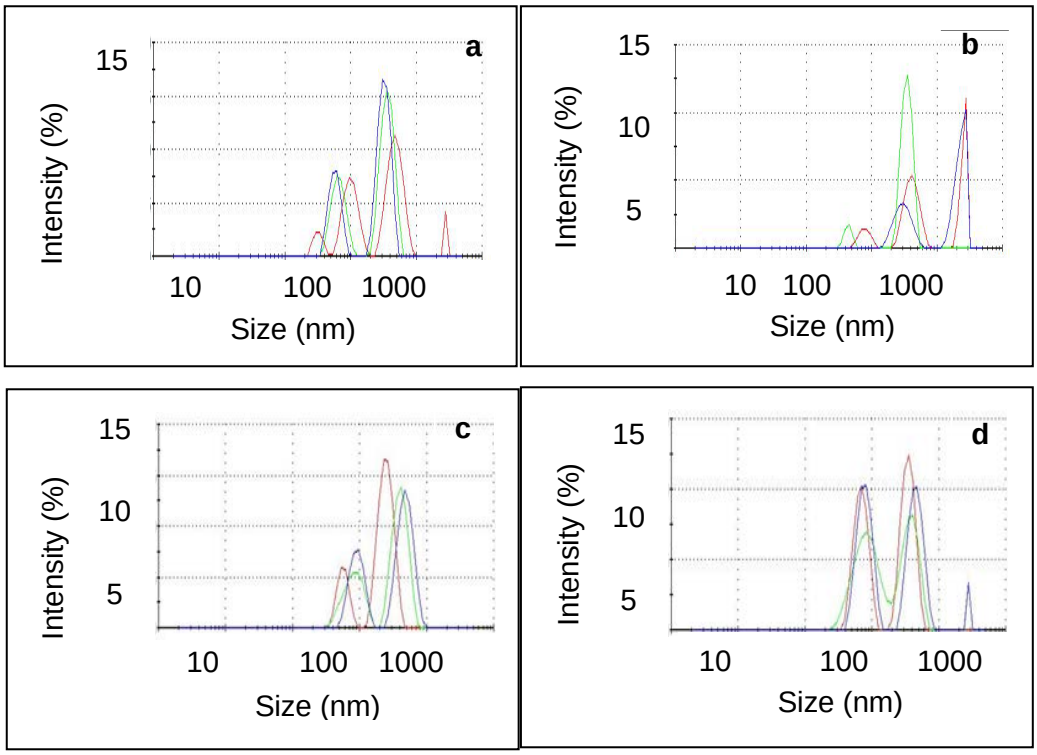

Figure C.3 The vesicle size distribution of: a) the (**NP**), b) the (**N3**), c) the (**PP1**) and d) the (**P1**)

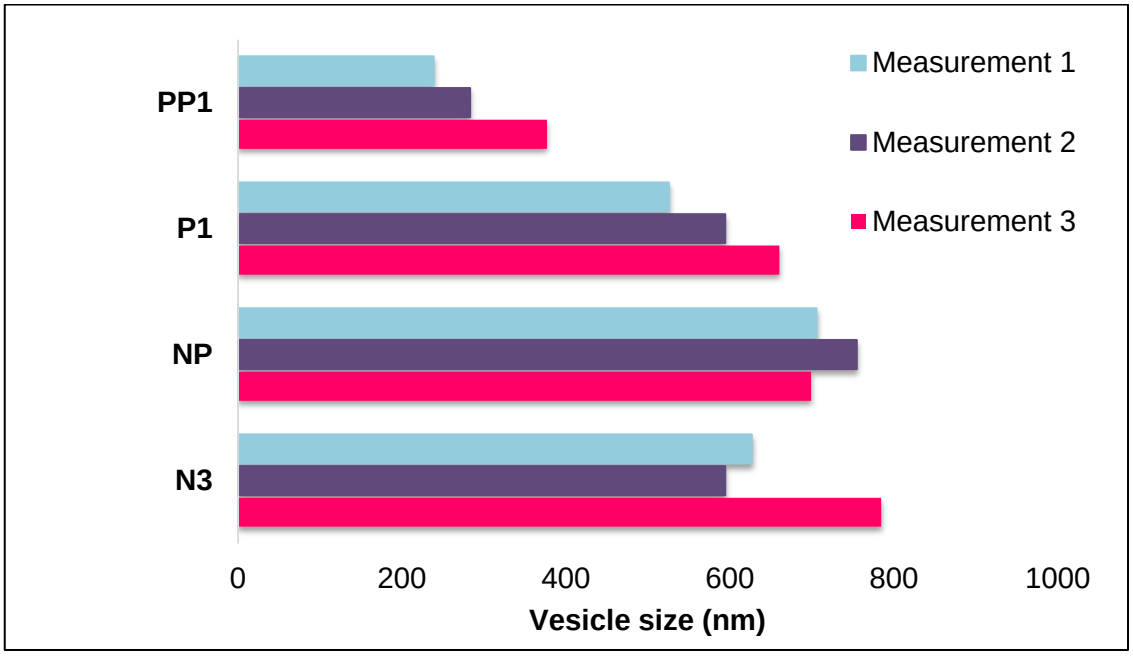

Figure C.4 The average vesicle size of the of the niosomes (**N3**) and proniosomes (**P1**) compared to their placebos (**NP**) and (**PP1**)

Figure C.3 presents the vesicle size distribution of the (**N3**) compared to the (**NP**), (**P1**) and (**PP1**). In Figure C.3, it can be noticed the sizes range between 100 nm and 1000 nm, which is an indication of a large size distribution for all four vesicle systems. The (**NP**) presented with an

average size of 719.73 ± 30.44 nm, while the (**N3**) had an average size of 668.55 ± 101.75 nm. Thus, the incorporation of the active had no significant influence on the size of the niosomes. When comparing the proniosomes to one another, the (**PP1**) had an average size of 299.66 ± 70.55 nm and the (**P1**) presented with an average size of 593.33 ± 70.55 nm. It is noticeable that by incorporating the active into the proniosomes it had an influence on the size, since the vesicles containing the active were much larger. When comparing the (**N3**) with (**P1**), the sizes of the niosomes were larger than those of the proniosomes. Although these sizes seem large for topical delivery, literature states that particle size smaller than 3 µm in diameter penetrates through the intercellular pathway, while sizes between 3 and 10 µm can easily penetrate the pilosebaceous follicles (Bolzinger *et al*., 2012:163). Since the vesicle sizes were determined to be in the nano size scale, both the niosomes and proniosomes would penetrate the skin easily, since the nanometre is three orders smaller than the micrometre.

Table C.1: The polydispersity index of the (**N3**) and the (**P1**), as well as the placebos ((**NP**) and (**PP1**))

Vesicle system	Average PdI
(**N3**)	0.884 ± 0.08
(**NP**)	0.862 ± 0.10
(**P1**)	0.876 ± 0.07
(**PP1**)	0.802 ± 0.19

As stated in Section C.1, the PdI of a system is associated with the homogeneity of the dispersion. When looking at the values in Table C.1, the average PdI of the (**NP**) and the (**N3**) were 0.862 ± 0.10 and 0.884 ± 0.08, respectively. This indicates the niosomes (with and without the active) are not homogeneous, since the values are closer to one. The (**PP1**) and the (**P1**) had a value of 0.802 ± 0.19 and 0.876 ± 0.07, respectively, meaning the size distributions in the proniosomes (with and without the active) were very wide and therefore the proniosomes are also not homogeneous.

C.3.3 Zeta-potential

The zeta-potential was determined on the same day the vesicle systems were prepared. The zeta-potential was determined with the Malvern Zetasizer Nano (Nano SZ) (Malvern instruments, Worcestershire, UK) at a temperature of 25 °C using disposable folded capillary cells. The samples were diluted with Milli-Q® water to reduce particle interaction and the experiment was done in triplicate.

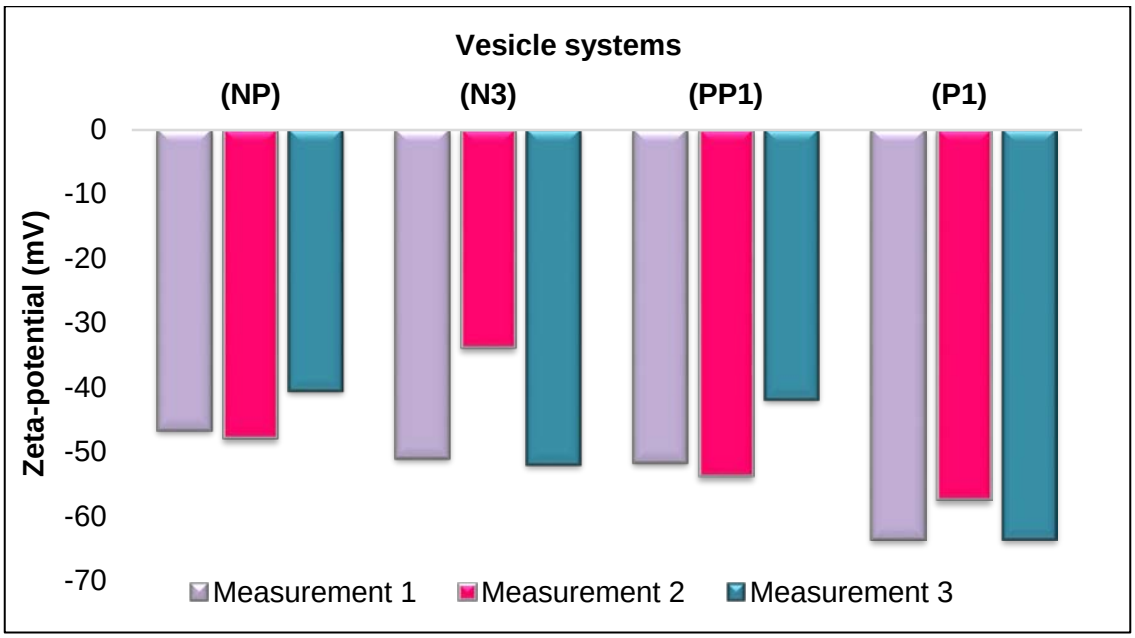

Figure C 5 The zeta-potential of the final niosomes and proniosomes with and without the active

As mentioned before, the zeta-potential is an indication of the stability of the vesicle system. Since all the zeta-potential values exceeded - 25 mV, it could be concluded that both the formulas were stable. These high zeta-potential values indicate that both the systems are deflocculated (Roland *et al*., 2003:87) and flocculation and fusion is unlikely to take place. When looking at the data after adding the active to the vesicle system, it appears the vesicle system became even more stable with average zeta-potential values of the (**N3**) and the (**P1**), - 45.11 ± 2.80 mV and -61.32 ± 2.87 mV, respectively.

C.3.4 Entrapment efficiency

Table C.2: The entrapment efficiency of (**N3**) and (**P1**) vesicle system

	Niosomes	**Proniosomes**
Entrapment (%)	82.66	31.15
	94.31	34.00
	83.13	32.89
Average	86.70 ± 5.34	32.94 ± 1.17

To determine the EE% of the niosomes (**N3**) and proniosomes (**P1**), the supernatant was separated from the vesicles using an ultra-centrifuge at 25 000 rpm at room temperature (25 °C) for 30 min. After being centrifuged, the supernatant was separated to leave the pellets behind.

The concentration of the supernatant was estimated by using HPLC. For both vesicle systems, the samples were diluted (1:20) with Milli-Q® water before transferring them into HPLC vials for analysis. The EE% of the niosomes and proniosomes were done in triplicate. The encapsulation was determined using the same equation as used in Section B.3.2.4

Tables C.2, presents the EE% of both the vesicle systems. According to Bayindir *et al*. (2009:2052), the entrapment of the niosomes was found to be between 19.90% and 86.40%. According to Ammar *et al*. (2011:149), proniosomes can encapsulate between 20.23 % and 91.95 % of the active. This data shows that EE% of vesicle systems can vary between studies. During this study, the niosomes and proniosomes encapsulated 86.70 ± 5.34% and 32.94 ± 1.17% respectively. These results correlate well with the literature and the EE% was within the acceptable ranges. From the data above, it was noticed that the niosomes had the highest EE%, while the proniosomes had the lowest.

C.3.5 pH

The pH of the niosomes and proniosomes was determined with a Mettler-Toledo pH meter FE20/FG2 (instrument no P 16) at room temperature. The experiment was done in triplicate.

Table C.3: The average pH values of the (**N3**), the (**NP**), the (**P1**) and the (**PP1**)

Vesicle system	Average pH
(**N3**)	7.28 ± 0.009
(**NP**)	6.90 ± 0.410
(**P1**)	6.99 ± 0.012
(**PP1**)	6.90 ± 0.041

Since the niosomes and proniosomes were formulated to be applied topically, the pH of the system is of importance. According to Barry (2007:576), the stratum corneum is quite resistant to alterations in pH, ranging between three and nine. Thus, both the vesicle systems' presented with pH values in this range, no harm could be done to the stratum corneum and according to Naik *et al*. (2000:319), it's the ideal pH for topical preparations. As seen in Table C.3, the (**N3**) had an average pH of 7.28 ± 0.009 and the (**P1**) had an average pH value of 6.99 ± 0.012. These excellent pH values may be explained by the buffering effect of the niosomes, since niacinamide has a pH range of 3.0 – 3.5 (Jacobs, 2009:11).

C.3.6 Viscosity

The viscosities of the vesicle systems were determined using the Brookfield® Viscometer model DV - III Ultra (Middleboro, Massachusetts, USA). A volume of 6.7 ml of each vesicle system was placed into a water bath to reach room temperature (25 °C). A SC4 - 18 spindle at a stirring rate of 200 rpm was used for all the samples. Multi-point readings were taken every 10 sec for a total time of 1 min and 20 sec in order to gather eight readings.

Table C.4: The viscosity of the vesicle systems

Vesicle system	Average viscosity (cP)
(**N3**)	4.036 ± 0.04
(**NP**)	5.532 ± 0.31
(**P1**)	3.616 ± 0.06
(**PP1**)	4.937 ± 0.07

Since the vesicle systems were formulated for topical application, the viscosity was determined in order to resolve whether the preparations could be applied easily. According to Barry (2007:595), customers normally prefer products, which are easily transferred from the container and spread smoothly on the skin. Table C.4 presents the viscosity values of the vesicle systems. Thus, all four preparations had very low viscosities, which indicated they were free flowing and would not be preferred by customers. These low viscosities also indicate that phase separation may occur, resulting in poor stability. For this reason the vesicle systems will be incorporated into a semi-solid dosage form in order to enhance the stability and customer compliance.

C.4 Conclusion

The characteristics of the vesicle system indirectly influence their behaviour, stability as well as the effectiveness of drug delivery, and is very important parameter to determine. As seen in the results, the vesicle sizes had a wide distribution, but the vesicles themselves were still very small which may promote delivery of the active. The vesicle sizes of the proniosomes were much smaller than the niosomes. The TEM results showed that the niosomes and proniosomes formed and that each one contained a lipid layer. The zeta-potential values of both the vesicle systems exceeded - 25 mV, indicating that both formulas were stable. The high negative value indicates a deflocculated system that leads to no flocculation and fusion of the particles in the dispersion. The pH values of the niosomes and proniosomes showed good results with values between four and seven, which is ideal for skin products. Another reason for buffering the pH between these

values is to have no interactions with the mobile phase during HPLC analysis and with the receptor phase during diffusion studies. The EE% showed that the amount of the active encapsulated into the niosomes was higher than the amount encapsulated in the proniosomes, but still correlated well with the literature. The viscosity of the system was measured to determine if the product would be acceptable for skin application. The viscosity of both the niosomes and the proniosomes were very low and would not be an acceptable product. As viscosity leads to a product not being acceptable for topical application as well as poor stability, the vesicle systems will be incorporated into a gel and cream, in order to enhance the patient compliances and stability. This will be discussed in Appendix D.

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Appendix D

The formulation of semi-solids containing niacinamide encapsulated in niosomes for topical delivery

D.1 Introduction

Several approaches have been used to develop new cosmetic systems in contrast to the classic old forms, such as creams and gels, and consequently, this has resulted in the discovery of vesicular systems which have different shapes, forms and sizes. The objectives were to design delivery systems with controlled release, as well as a system which can encapsulate actives in order to protect it against degradation (Pay *et al*., 2006:175).

Niosomes are non-ionic surfactant vesicles that may be prepared from various classes of non-ionic surfactants to encapsulate an aqueous medium (Ruckmani & Sankar, 2010:1119). According to Barel *et al*. (2001:181), when these particles are incorporated into a gel form and applied to the skin, they enhances the skin's humidity, leading to an improvement of the penetration of the active and better cosmetic efficiency. Conversely, when incorporating these particles into a cream it enhances the occlusivity of the cream, causing a better bioavailability and efficacy (Barel *et al*., 2001:181).

In this study, semi-solid formulations were prepared from niosomes, since the niosome dispersion had a very low viscosity and could not be applied on to the skin. As stated above, incorporating these systems into semi-solid dosage forms also leads to a few advantages. By incorporating the vesicular system into a semi-solid dosage form it will increase viscosity, which may lead to better topical delivery as the contact time with the skin is longer.

D.2 Ingredients used to formulate semi solid dosage forms

D.2.1 Niosomes

For the preparation of niosomes, which are produced by mixing surfactants with cholesterol and an organic substance to form the lipid phase together with water to form the aqueous phase, the Fin Film hydration method, as described in Section B.4.1, was used. This method included mixing a lipid phase (consisting of surfactants, cholesterol and an organic substance) with an aqueous phase. The formulas of the niosomes were modified to create a semi-solid dosage containing 3% niacinamide.

D.2.2 Semi-solid formulations

D.2.2.1 Carbopol®

According to Draganoiu *et al.* (2009:110), Carbopol® consists of high molecular weight polymers of acrylic acid cross-linked with either allyl sucrose or allyl ethers of pentaerythritol. These agents can be used in liquid or semi-solids as rheology modifiers and may also be called carbomers, Acrypol® and Pemulen™. Carbopol® can be used in a wide variety of pharmaceutical preparations because of its wide range of functionalities and in this study, it will be used for multiple reasons, namely as a bio-adhesive material, a controlled-release agent, an emulsion stabiliser and a suspending agent.

D.2.2.2 Propylene glycol

Propylene glycol also known as 1,2-propanediol, is used today as a solvent, extractant and a preservative in parenteral and non-parenteral pharmaceutical formulations. According to Weller (2009:592), propylene glycol can also act as an antimicrobial preservative, disinfectant, humectant, plasticiser, solvent and stabilising agent. In this study, propylene glycol will be used as a solvent for the poorly soluble parabens.

D.2.2.3 Triethanolamine

According to Goskonda (2009:754), triethanolamine is used as an alkalising agent in topical pharmaceutical formulations. In this study, the triethanolamine will be used to adjust the pH of the semi-solid, in order to be more acceptable for skin application.

D.2.2.4 Methyl- and propylparaben

According to Haley (2009:441), methylparaben and propylparaben are mostly used as an antimicrobial agent in food, cosmetics and different pharmaceutical products. Parabens show antimicrobial activity in a pH range of 4 to 8 and are most effective against yeasts and molds. The activity of the parabens may also be increased by combining different types of parabens (methyl-, ethyl-, propyl- and butylparaben) or other excipients, such as propylene glycol; these combinations will cause a synergistic effect in antimicrobial activity. For topical preparations, methylparaben should be used in concentrations of 0.02 to 0.30% and propylparaben in concentrations of 0.01 to 0.60%; in this formulation the parabens will be used for its antimicrobial action. Since the pH of the semi-solid formulations will be buffered at 7, this will be an ideal medium to activate the parabens.

D.2.2.5 Tween® 20

Tween® 20, also known as polysorbate 20, is a hydrophilic non-ionic surfactant that can act as an emulsifying agent, solubilising agent and a wetting agent. Due to the fact the surfactant can act in all these ways, it can be used in pharmaceutical preparations such as oil-in-water (o/w) emulsions, essential oils and even oral and parenteral suspensions (Zhang, 2009:551). Tween® 20 is relatively safe as it is non-toxic and non-irritating however, there have been reports of hypersensitivity when applied topically or intramuscular. For the purpose of this study, Tween® 20 will be used as an emulsifying agent.

D.2.2.6 Span® 20

Sorbitan esters are a mixture of partial esters of sorbitol and its mono-anhydride fatty acids. They are lipophilic non-ionic surfactants which may act as a dispersing agent, emulsifying agent, solubilising agent, as well as a wetting agent (Zhang, 2009:675); it will be used in this study as a dispersing agent.

D.2.2.7 Cetyl alcohol

According to Unvala (2009:155), cetyl alcohol is used in cosmetics and pharmaceutical formulations such as lotions, creams and ointments. In this study it is used because it has water-absorptive, emollient and emulsifying properties and enhances the stability, improves the texture and increases the consistency of topical formulations. According to Unvala (2009:155), the emollient properties can be described by the absorption and retention of cetyl alcohol in the dermis. This can cause a velvety texture, since the cetyl alcohol smooths and lubricates the skin.

D.2.2.8 Liquid paraffin

According to Sheng (2009:445), liquid paraffin is a mineral oil used in topical pharmaceutical formulations. Its use in this study is because of its wide range of properties which include acting as an emollient, lubricant, oleaginous vehicle, as well as a solvent.

D.2.2.9 Veegum®

Veegum®, which may also be called magnesium aluminium silicate, is used for the formulation of tablets, ointments and creams. In topical formulations this product is used as a viscosity enhancer, suspending and stabilising agent in concentrations between 1 – 10% (Palmieri, 2009:395). In the niosome cream, Veegum® will be used as a viscosity enhancer.

D.3 Methods used for formulation

D.3.1 Niosomes

As mentioned in Section B.3.3, niosomes were made using the thin-film hydration method. During the preparation of the niosomes for the semi-solid dosage forms a few adaptations were made, which included adding a higher concentration of the active to the niosome formulation, in order to get a final formulation containing 3% niacinamide.

D.3.2 Semi-solid dosage forms

D.3.2.1 Niosome gel formulation

According to Barry *et al*. (2002:529), pharmaceutical gels are a two-compartment semi-solid system that is rich in liquid formed by using a natural or synthetic polymer, which builds a three-dimensional matrix throughout a hydrophilic liquid. Polymers used for the formulation of gels can be divided into natural and synthetic polymers. Natural polymers may include natural gums such as pectin, agar and alginic acid and semi-synthetic materials include hydroxypropyl methylcellulose, carboxymethylcellulose, as well as a synthetic polymer such as Carbopol®.

According to Mitsui (1997:353), in order to manufacture these products the correct equipment is needed. For example, the mixer should be able to uniformly mix the substances together and equipment should be able to remove the air bubbles since this tends to be a problem. According to Harry (1973:374), two types of gels can be manufactured, one that is like a simple thickened lotion and another that is very thick and non-flowing (thixotropic gel). The thickened lotion type is quite simple to prepare and includes adding a solid gellant to the liquid phase while stirring at a high speed with a propeller. The non-flowing gels include a much more advanced method as the final product will contain a great amount of air bubbles. Consequently, the non-flowing gel should be prepared under a vacuum in order to completely remove the air (Harry, 1973:374).

D.3.2.1.1 Pre-formulation of niosome gels

During the pre-formulation of the niosome gel, a trial-and-error approach was used. Several formulations were made using different ingredients to determine which would be the best candidate for the study. The formulations were prepared, left overnight then investigated visually. The choice of the best formulation was made based on the viscosity, colour, homogeneity, consistency and phase separation aspects.

D.3.2.1.2 Final niosome gel formulation

The final formulation was chosen according to the viscosity, colour, homogeneity, consistency and phase separation aspects and then used to prepare the final formulation.

According to Gupta *et al*. (2010:250), gels for topical delivery may be formulated through the use of hydroxypropyl methylcellulose, Carbopol® and sodium alginate. These agents are used because the hydrophilic polymers are non-greasy and can easily be washed off the skin They also have a high molecular weight which leads to minimal skin penetration, causing the polymers to be non-toxic.

For the niosome gel, Carbopol® was used as a rheology modifier in a concentration of 0.5%. The gel was made by preparing the niosomes as stated in Section D.3.1, then Carbopol® was added and the mixture was left to soak for about 15 min for the Carbopol® to hydrate. The rest of the ingredients (as shown in Table D.1) were added before mixing. The formulation was homogenised at 1000 rpm for about 1 hrs in order to get a uniform formulation. The pH of the gel was set at 7.4 with triethanolamine, where after the air bubbles were removed by placing the formulation into a sonication bath for 10 min.

Table D.1: Formula of the niosome gel

Ingredients	Quantities
Niosomes (3% niacinamide)	95 ml
Carbopol®	0.50%
Methylparaben	0.20%
Propylparaben	0.05%
Propylene glycol	5 ml
Triethanolamine	± 1 ml

After the manufacturing of the final formulation, the gel was prepared in bulk for the purpose of stability testing at different storage conditions.

D.3.2.1.3 Results

The niosome gel had a white colour, due to the niosomes used being a white milky solution, and was odourless. The niosomes gel applied easily to the skin, but was sticky because of the cholesterol used when preparing the niosomes.

D.3.2.2 Niosome cream formulation

Creams are semi-solid emulsions that have been formulated for external use to treat several skin conditions (Barry *et al.,* 2002:530). According to Mitsui (1997:341), it is called an emulsion since two liquids, such as oil and water, are mixed together to form a stable dispersion. The main ingredients used to manufacture a cream are oily ingredients, aqueous ingredients, surfactants, preservatives and active(s) as well as excipients (Mitsui 1997:342). The oily ingredients include hydrocarbons, waxes (beeswax and lanolin), fatty acids, higher alcohols (stearyl alcohols, cholesterol) and synthetic esters. The aqueous phase includes humectants (propylene glycol, sorbitol), alcohols (ethanol) and purified water (Mitsui 1997:344).

In general, creams are manufactured by adding all the aqueous phase ingredients together and heating them to 70 °C. Compounds of the lipid phase are then mixed in a separate beaker and stirred while heating at 70 °C. After reaching temperature and all the solid ingredients in the oil phase melted, the oil phase is mixed into the water phase while stirring to form an o/w emulsion (Mitsui, 1997:344).

D.3.2.2.1 Pre-formulation of creams

The pre-formulation of the niosome cream was based on the same principle as for the niosome gel. A trial-and-error approach was used and the best formulation was chosen. Three different samples were made containing different ingredients. The samples were left overnight and the best formulation chosen on grounds of viscosity, colour, homogeneity and absence of phase separation.

D.3.2.2.2 Final formulation of creams

The lipid phase and the aqueous phase, as seen in Table D.2, was mixed separately (each in its own beaker) in a water bath at 80° C. After both phases reached temperature, and all the solid particles in the lipid phase had melted, the lipid phases were gradually stirred into the water phase to form a cream. The final formulation of the cream was made in bulk to perform stability tests.

Table D.2: Formula of niosome cream

Ingredients	Quantities (%m/m)
Lipid phase (A)	
Liquid paraffin	10.00%
Tween® 20	1.00%
Cetyl alcohol	10.00%
Methylparaben	0.20%
Aqueous phase (B)	
Niosomes (3% niacinamide)	72.25%
Propylene glycol	3.00%
Span® 20	1.00%
Propylparaben	0.05%
Veegum®	2.50%

D.3.2.2.3 Results

The cream had a good viscosity and could be applied easily to the skin. The niosomes cream had a brownish colour, which could be explained since Veegum ®, which has a brown colour, was used in the formulation.

D.4 Conclusion

Niacinamide was encapsulated into a vesicle system to enhance topical delivery. These vesicles were incorporated into a 3% niosome gel and 3% niosome cream. Each formulation was stored at ± 25 °C/60% relative humidity (RH), ± 30 °C/60% RH and ± 40 °C/70% RH for three months, according to the ICH (2003:3) guidelines (Mitsui 1997:191). The stability of the formulations will be discussed in Appendix G.

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Appendix E

Diffusion studies of niosomes, proniosomes, niosome gel and niosome cream

E.1 Introduction

According to Barry (2007:571), diffusion is the random motion of molecules from one region to another. This motion of molecules may be controlled by the physiological and physicochemical properties of the skin and the properties of the penetrant.

Considering the skin, the stratum corneum is the main barrier, as it consists of protein-enriched and lipid-enriched intracellular domains to provide resistance to diffusion (Baroni *et al*., 2012:257). Conversely, the epidermis also acts as a control element; with mostly small water-soluble molecules and non-electrolytes diffusing even more in the absence of this skin layer (Venter *et al*., 2001:169).

Since the quantification of drug concentrations in the skin is very complex; the use of excised human skin mounted on a suitable *in vitro* test is a good alternative (Leveque, *et al*., 2004:323). Consequently, the diffusion of a substance for topical or transdermal delivery may be tested using vertical Franz diffusion cells (Chattaraj *et al*., 1995:120).

During this study, vertical Franz diffusion cells were used for the membrane- and skin diffusion studies to determine the release and the transdermal delivery of the active from the preparation, respectively.

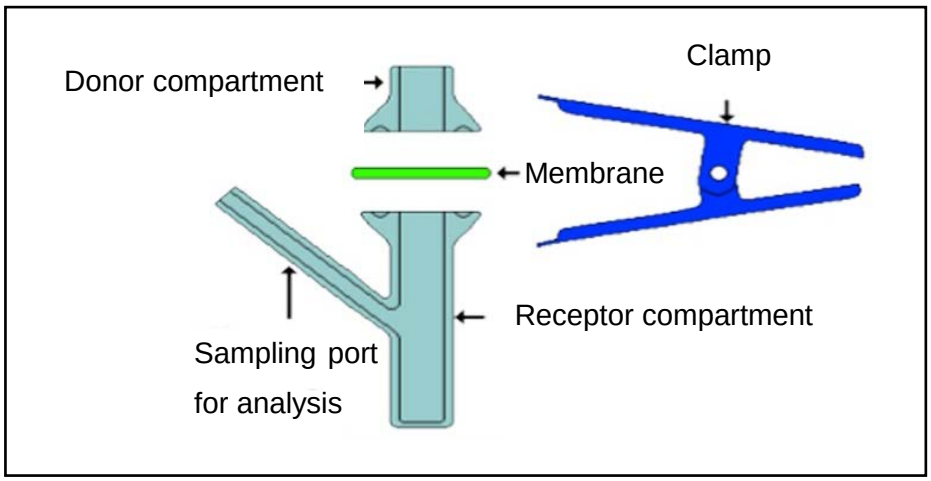

Figure E.1: Graphic illustration of a vertical Franz diffusion cell

E.2 Methods

E.2.1 HPLC analysis

The HPLC was used to determine the concentration of niacinamide during diffusion studies. This was done in conjunction with Prof Jan Du Preez at the analytical Technology Laboratory, North-west University, Potchefstroom campus, South Africa. The chromatographic conditions used are described in Section A.2.

E.2.2 Preparation of PBS (pH 7.4)

During this study the PBS (pH 7.4) was prepared by weighing 1.5 g sodium hydroxide (NaOH) and dissolving it in 400 ml of Milli-Q® water (Merck Millipore, Halfway House, South Africa), followed by 6.5 g of potassium dihydrogen orthophosphate (KH_2PO_4) being accurately weighed and dissolved in 250 ml of Milli-Q® water. The two solvents were added together and buffered with 10% orthophosphoric acid (H_3PO_4; 2 M) to a pH of 7.4 (British Pharmacopoeia, 2014). After the pH was set, the solution was made up to a volume of 1000 ml with Milli-Q® water.

E.2.3 The aqueous solubility of niacinamide

For the determination of the aqueous solubility; a saturated solution was made. This was done by adding an excess amount of the active into a test tube containing 3 ml of PBS (pH 7.4). The mixture was placed into a water bath (25° C) on a magnetic stirrer overnight. After 24 hrs the mixture was filtered and diluted and each sample was placed into an HPLC vial to be analysed. The experiment was done in triplicate.

E.2.4 The octanol-buffer partition coefficient (log D) of niacinamide

A saturated *n*-octanol and PBS (pH 7.4) mixture was prepared by magnetically mixing the two components together for 24 hrs, after which the solution was left to allow for the phases to separate. After separation 20 ml of the pre-saturated PBS was extracted and mixed with 30 mg niacinamide. After the niacinamide was dissolved, 3 ml of the mixture was extracted and placed into a test tube; where after 3 ml of pre-saturated *n*-octanol was added (this was done in triplicate). The mixture was then placed into a pre-heated water bath (32 °C) and left overnight. The following day the test tubes were removed from the water bath and placed into an Eppendorf Centrifuge 5804R for 5 min at 3 000 rpm, which caused the phases to separate. Each phase was withdrawn, where after the saturated PBS was diluted with PBS (1:20) and the saturated *n*-octanol phase was diluted with methanol (1:20). The samples were then placed into HPLC vials for analysis, where after the log D was determined by using Equation E.1.

Log D = Concentration in *n*-octanol/Concentration in PBS **Equation E.1**

E.2.5 The preparation of the donor phase

The preparations used in the donor phase were the pre-formulations (niosomes and proniosomes), as well as the formulations (niosome gel and niosome cream). The preparation of the niosomes and proniosomes were described in Sections B.4.1 and B.4.2, respectively. The formulation of the niosome gel and niosome cream were fully described in Sections D.3.2.1.3 and D.3.2.2.1, respectively.

E.2.6 The preparation of the receptor phase

The receptor phase was filled with PBS (pH 7.4), which is described in Section E.2.2.

E.2.7 Diffusion studies

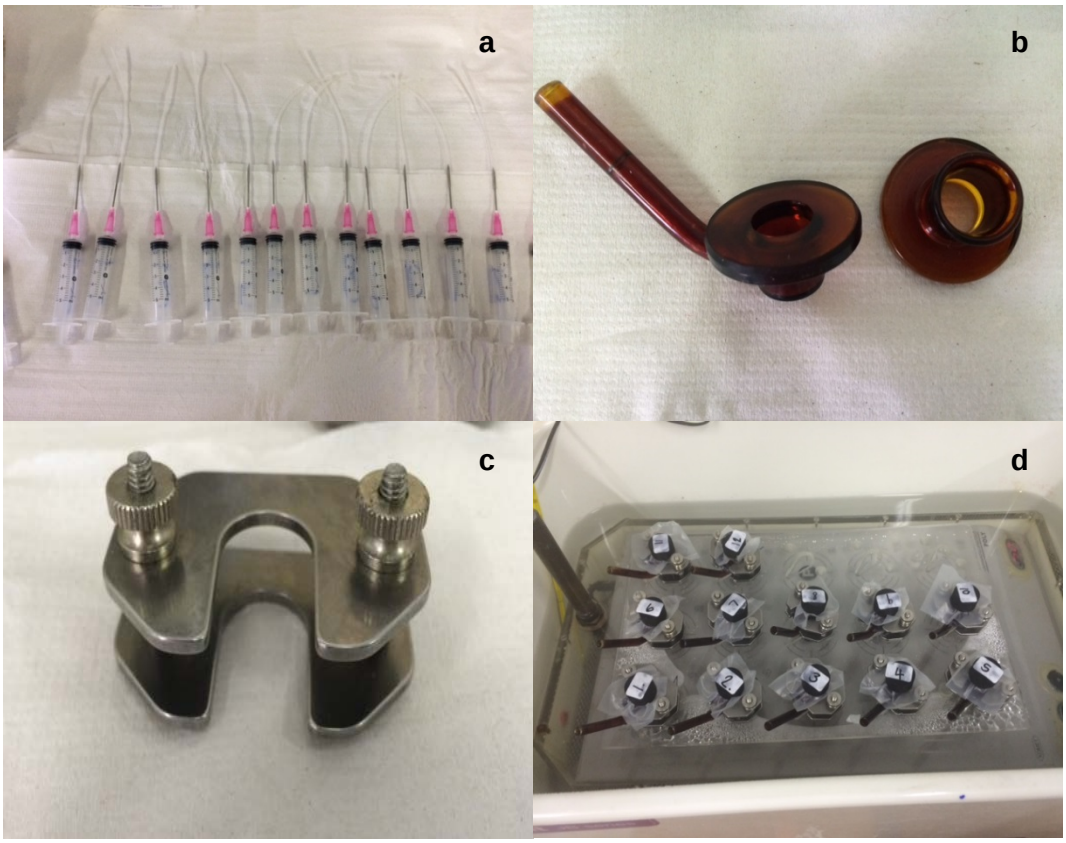

Figure E.2 Photographs of a) the syringes (5 ml) used to extract the sample from the receptor phase at certain time intervals, b) amber vertical Franz diffusion cells, c) horse shoe clamp and d) loaded Franz diffusion cells in a water bath of 37 °C

For the membrane- and skin diffusion studies, vertical Franz cells were used. Polyvinylidene fluoride (PVDF) membrane filters, with a pore size of 0.45 µm or dermatomed skin, were placed over the opening of the receptor phase. The donor phase was placed onto the receptor phase

and sealed with Dow Corning® vacuum grease to prevent leakage. The two compartments were then secured with a horse shoe clamp. The donor compartments were filled with 1 ml of the preparation and covered with Parafilm® to prevent evaporation. A magnetic stirring bar was placed; into each receptor compartment, where after it was filled with PBS (pH 7.4). The Franz cells were then placed onto a magnetic stirrer into a pre-heated water bath at a temperature of 37 °C, the temperature of the water bath was maintained throughout the experiment. The entire receptor volume was extracted at certain time intervals. After every extraction, the receptor phase was filled with 2 ml fresh PBS (pH 7.4) held at a temperature of 37 °C. The samples were placed into HPLC vials and analysed by the HPLC.

E.2.8 Membrane diffusion

The aim of the membrane studies was to determine whether niacinamide was released from the preparations. The same method as discussed in Section E.2.7 was used for the membrane diffusion studies. PVDF membrane filters were placed between the donor- and the receptor phase. Extraction samples were taken hourly for 6 hrs.

E.2.9 Skin diffusion studies

E.2.9.1 Preparation of skin

In this study, female Caucasian skin obtained after cosmetic abdominoplasty surgery was used. Ethical approval (NWU-00114-11-A5) was granted by the North-West University Ethics Committee for “Application for the use of biological material obtained from human subjects in experiments,” for the period 25 August 2011 to 24 August 2016. After collection, the skin was frozen at - 20 °C for no longer than 24 hrs. The full thickness skin was dermatomed to ensure a specific thickness of 400 µm for the diffusion studies. After being dermatomed, the skin was punched into circles with a diameter of 15 mm, placed with the stratum corneum facing up on filter paper. The skin was left to air dry, closed with aluminium foil and stored in a freezer at - 20 °C until used.

E.2.9.2 Skin diffusion

For the skin diffusion studies the same method discussed in Section E.2.7 was used. Dermatomed human skin was mounted between the receptor and donor compartments. The entire volume of the receptor compartment was extracted after every 20 min for 2 h and there after every 2 hrs up to 12 hrs. After the skin diffusion studies; tape stripping was performed.

E.2.9.3 Tape stripping

Tape stripping was used to determine the amount of the active present in the stratum corneum-epidermis (SCE) and the epidermis-dermis (ED), after skin diffusion studies. After the Franz cells were dismantled, the skin was removed and pinned to a piece of Parafilm® placed on a solid surface. The skin was dabbed dry with a clean paper towel to ensure all the remaining formulation was removed. After removing the remaining formulations, 3 M Scotch® Magic Tape™ was cut into pieces equal in size to the diffusion area. The pieces of tape were placed on the diffusion surface and removed, this was done until the stratum corneum was removed completely. The first strip was discarded in order to prevent possible contamination and the remaining 15 strips, which contained the active and stratum corneum-epidermis, were each placed into their own polytop containing 5 ml PBS (pH 7.4). The remaining diffusion area was then cut into small pieces and placed into their own polytop also containing 5 ml of PBS (pH 7.4). The polytops were left overnight at 4 °C in order to dissolve the entire amount of active ingredient into the PBS (pH 7.4). The following day all the samples were filtered and placed into HPLC vials for analysis (Pellett *et al.,* 1997:91).

E.2.10 Statistical data analysis

The data obtained from the membrane release studies were used to calculate the percentage released (%), as well as the cumulative amount per area against time (µg/cm^2.h) of the active that diffused after 6 hrs. The data obtained from the skin diffusion studies was used to calculate the percentage diffused (%), as well as the cumulative amount per area against time (µg/cm^2.h) of the active that diffused through the skin into the receptor phase after 12 hrs. The data obtained from the tape stripping and pieces of cut diffused skin was used to determine the concentration (µg/ml) of the active delivered into the SCE and the ED, respectively.

The average and mean flux was determined differently during this study; although the words are synonyms to each other, the mean was calculated by Statistical Services (North-West University, Potchefstroom Campus) and the average was calculated by means of a graph (method used internationally). The average flux (in the membrane- and skin diffusion studies) was determined by plotting the average cumulative amount per area (of all the Franz cells used together) against time, in order to obtain the slope of the straight line, also known as the average flux. The mean flux (in the membrane- and skin diffusion studies) was determined by plotting the cumulative amount per area (of each individual Franz cell) against time. The slope of each Franz cell was then added together and divided by the total amount of Franz cells used to obtain the mean flux value.

The data used for statistical analysis was the results obtained from the membrane- and skin diffusion studies, as well the data from the SCE and ED. A two-way analysis of variance (ANOVA) was performed to test for an interaction effect between formulation and skin layer. That was followed by one-way ANOVA's to determine if any statistical differences in mean concentration between formulations occurred in the data. The same was done to compare the skin layers. A Tukey multiple comparison test was then performed to determine between which pairs of formulations or skin layers the significant differences occurred. The tests indicated that significant difference occurred when a p-value smaller than 0.05 were presented.

There was a statistically significant interaction effect ($p < 0.001$) between the means for each skin layer per formulation, therefore it was decided to do one-way ANOVA's to compare different formulations within each skin layer separately, skin layers were also compared within each formulation.

E.3 Results and discussion

E.3.1 The aqueous solubility

Due to significant solubility in water, the aqueous solubility in this study was determined at 25 °C. When the samples were kept at 32 °C crystallisation took place the instant that the saturated solution was removed from the water bath. According to Naik *et al*. (2000:319), the ideal aqueous solubility for a substance to be passively delivered is > 1 mg/ml. Niacinamide had a value of 112.70 mg/ml at 25 °C which makes it ideal for skin delivery.

E.3.2 The octanol-buffer partition coefficient

According to Donnelly and Sing (2015:13), for a particle to penetrate the skin an optimal log D value between 2 and 3 is required. This demonstrates a particle must possess both lipophilic and hydrophilic properties in order to dissolve both in water and in oil. During this study the log D value of niacinamide was determined to be - 0.43. Therefore, the active might be too hydrophilic to permeate through the lipophilic stratum corneum, since a log D of - 0.43 is not optimal for skin penetration.

E.3.3 Membrane diffusion

The aim of the membrane diffusion studies was to determine whether release of the active from the preparations occurred. In Table E.1 the average flux, median flux, as well as the percentage released after 6 hrs is presented. When comparing the median flux values of the different preparations, the proniosomes (1 163.58 µg/cm^2.h) had the highest median flux value, followed by the niosomes (1 162.58 µg/cm^2.h), then the niosome gel (1 042.08 µg/cm^2.h) and lastly, the

niosome cream (469.72 µg/cm^2.h). The same trend was followed when the average flux values of the different preparations were compared. Although, the median and average flux values did not differ much, the median values are preferred, as they are not affected by outliers (Dawson & Trapp, 2001:30; Gerber *et al*., 2008:190).

Although the pre-formulations cannot be compared to the final formulations, the data indicates that incorporating the niosomes into a gel and a cream had a slightly negative influence on release of the active.

Table E.1: The data obtained from the pre-formulations and final formulations after membrane diffusion studies

Preparation	**Average percentage released (%)**	**Average flux (µg/cm^2.h)**	**Median flux (µg/cm^2.h)**
Pre-formulation			
Niosomes	12.43 ± 0.50	1 162.33 ± 49.27	1 162.58
Proniosomes	12.59 ± 0.47	1 177.32 ± 47.40	1 163.58
Formulation			
Niosome gel	11.30 ± 3.64	1 048.5 ± 133.62	1 042.08
Niosome cream	5.04 ± 1.94	457.06 ± 119.53	469.72

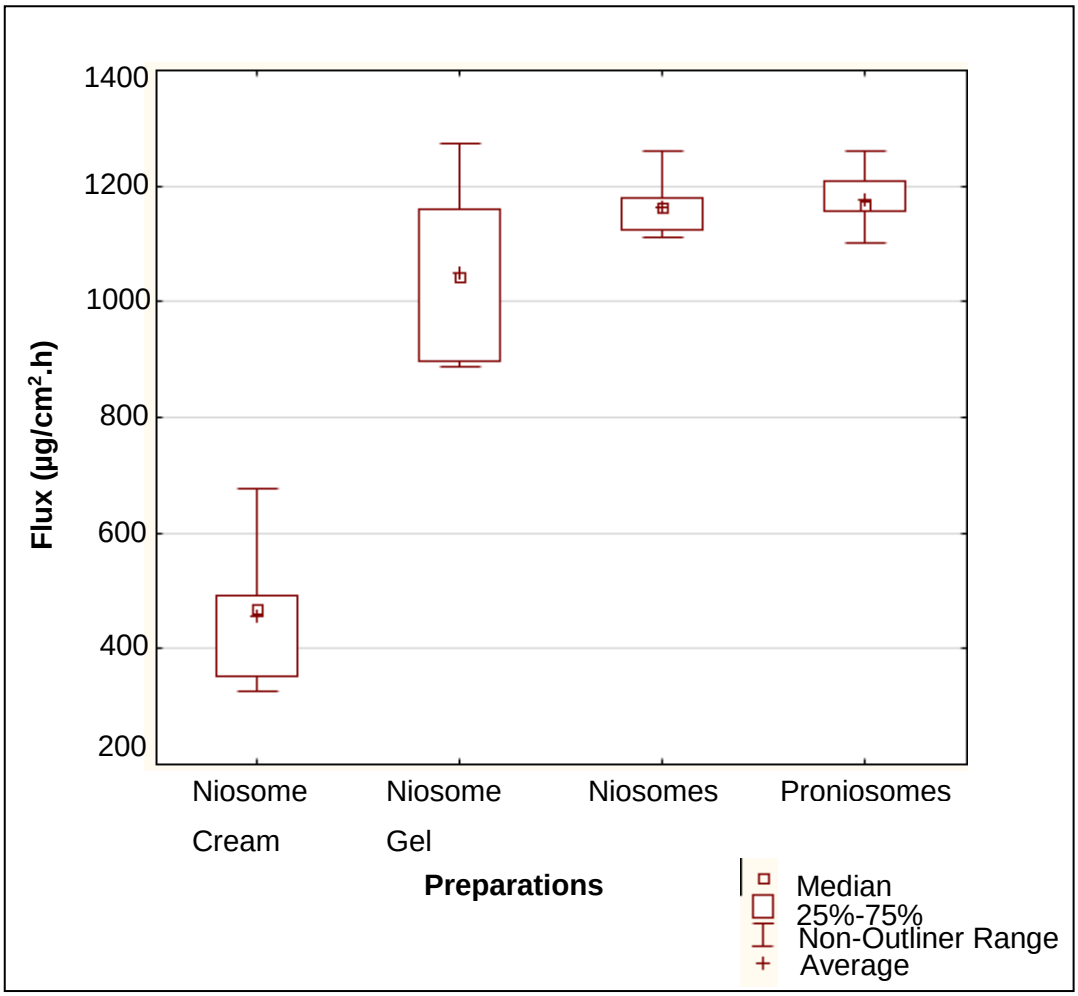

Figure E.3: The box-plots of the membrane diffusion study of all four preparations, illustrating the median (small square) and average flux (plus sign) of niacinamide

Figure E.3 illustrates the diffusion of niacinamide from the four preparations during the membrane release studies. In the box-plot the median (which indicates the centre point) flux and the average (the average value determined by the sum of the data divided by the number of points) flux are presented. The figure also indicates the maximum and minimum flux values.

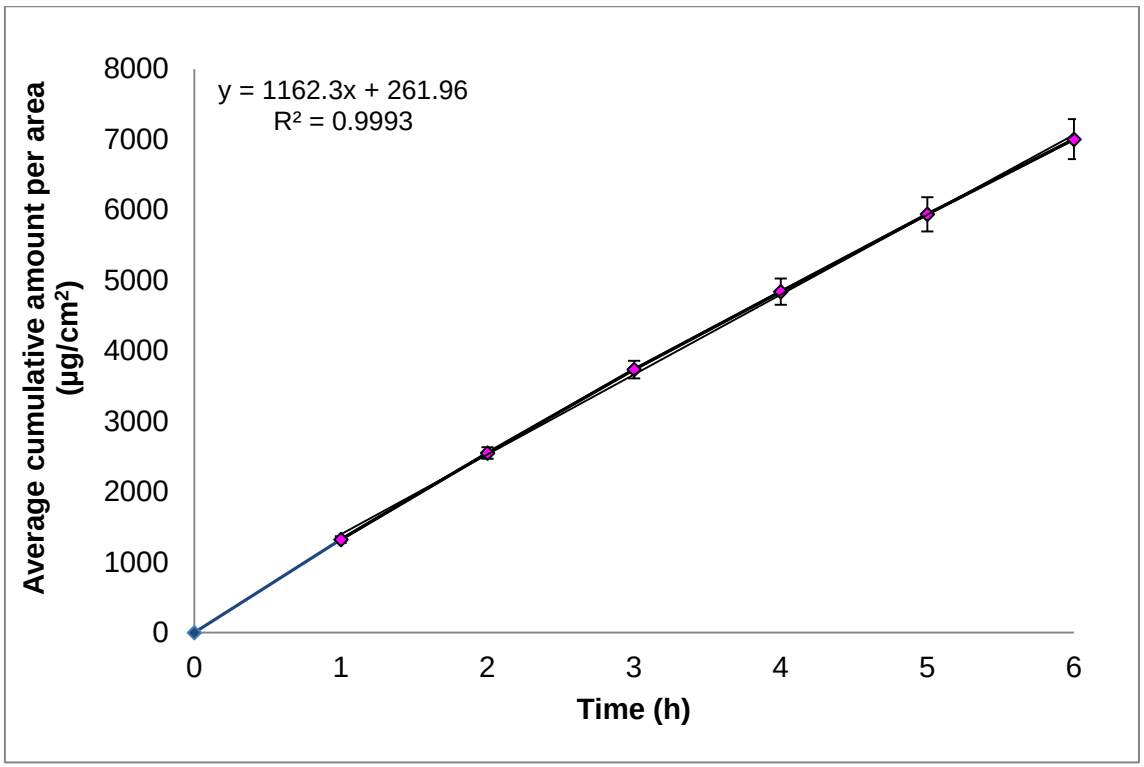

Figure E.4: The average cumulative amount per area (µg/cm^2) of niacinamide released from the niosomes as a function of time (n = 9)

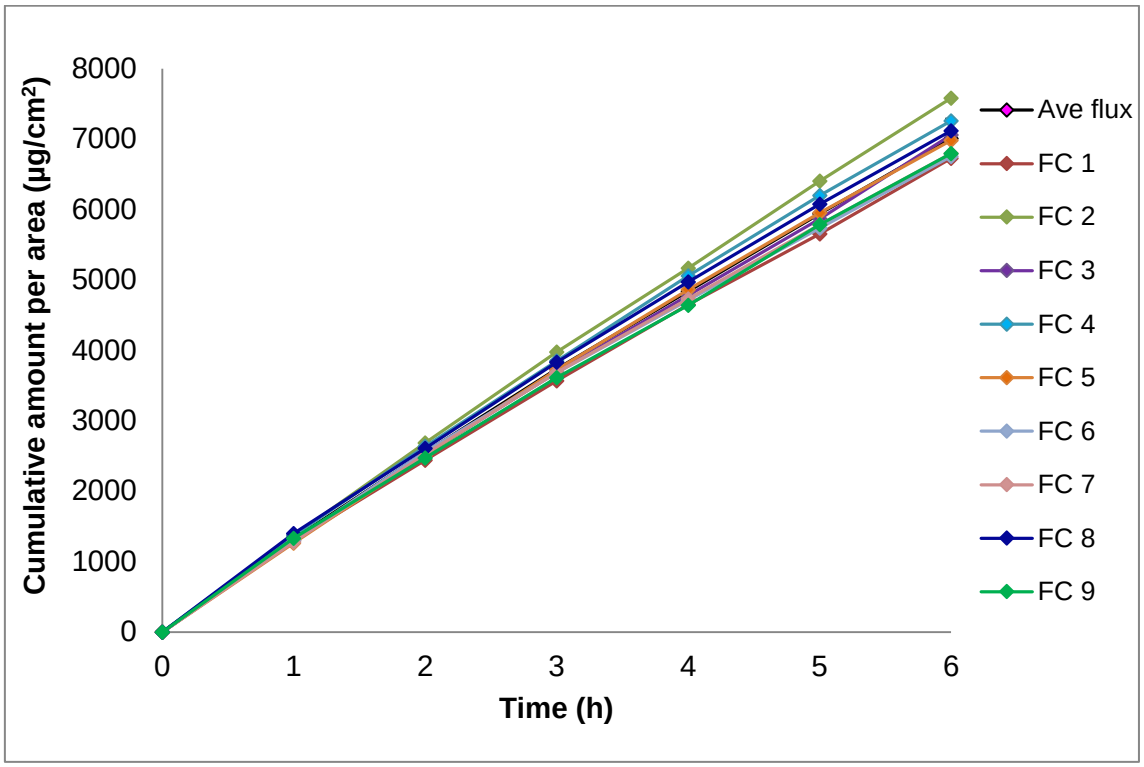

Figure E.5: The cumulative amount per area (µg/cm^2) of niacinamide for each individual Franz cell that was released from the niosomes during the membrane release study (n = 9)

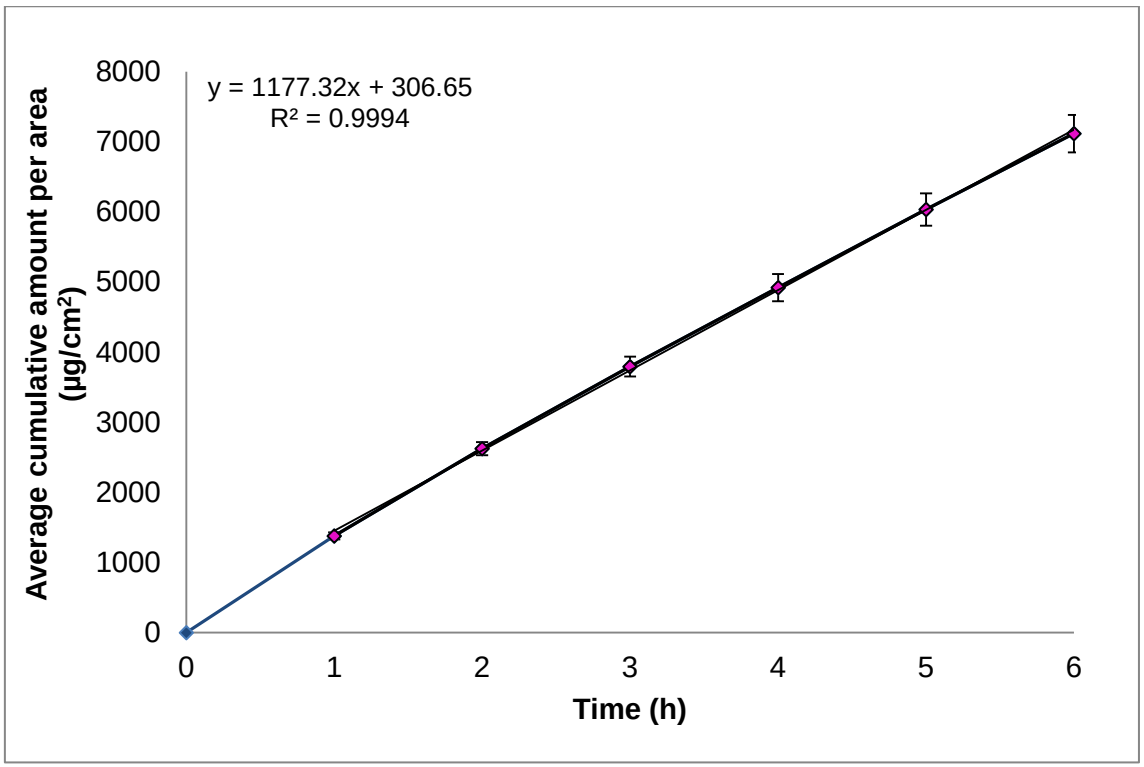

Figure E.6: The average cumulative amount per area (µg/cm^2) of niacinamide released from the proniosomes as a function of time (n = 10)

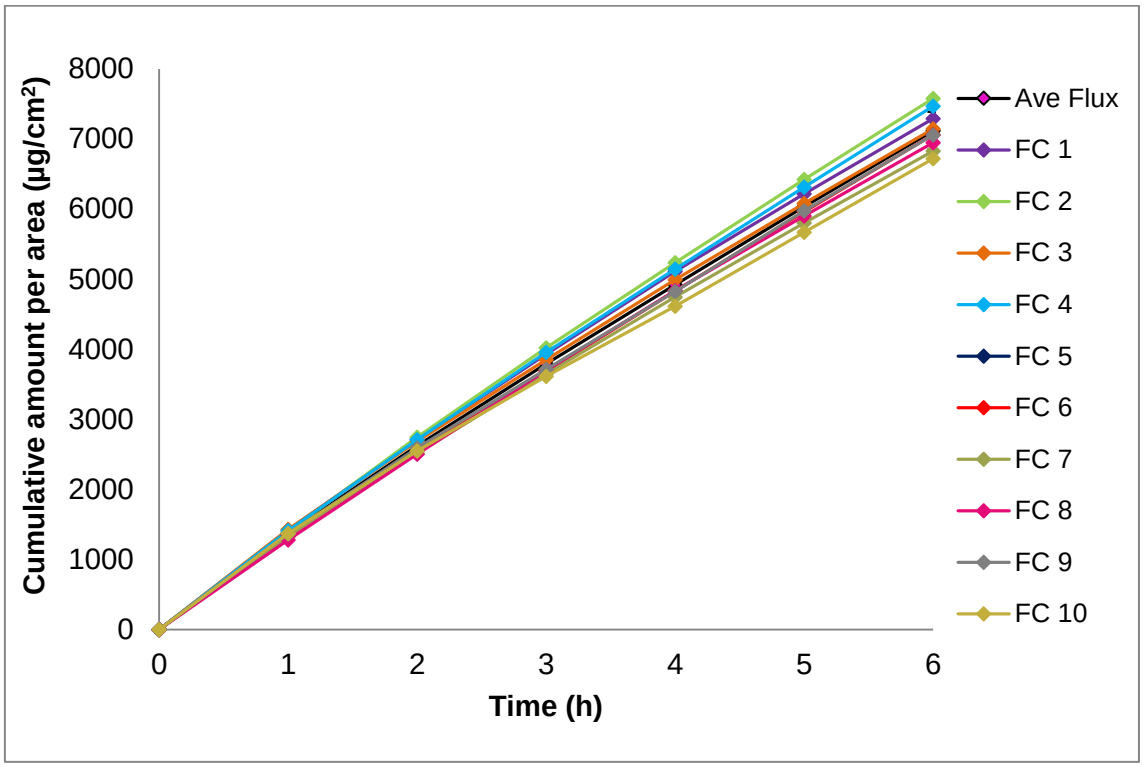

Figure E.7: The cumulative amount per area (µg/cm^2) of niacinamide for each individual Franz cell that was released from the proniosomes during the membrane diffusion study (n = 10)

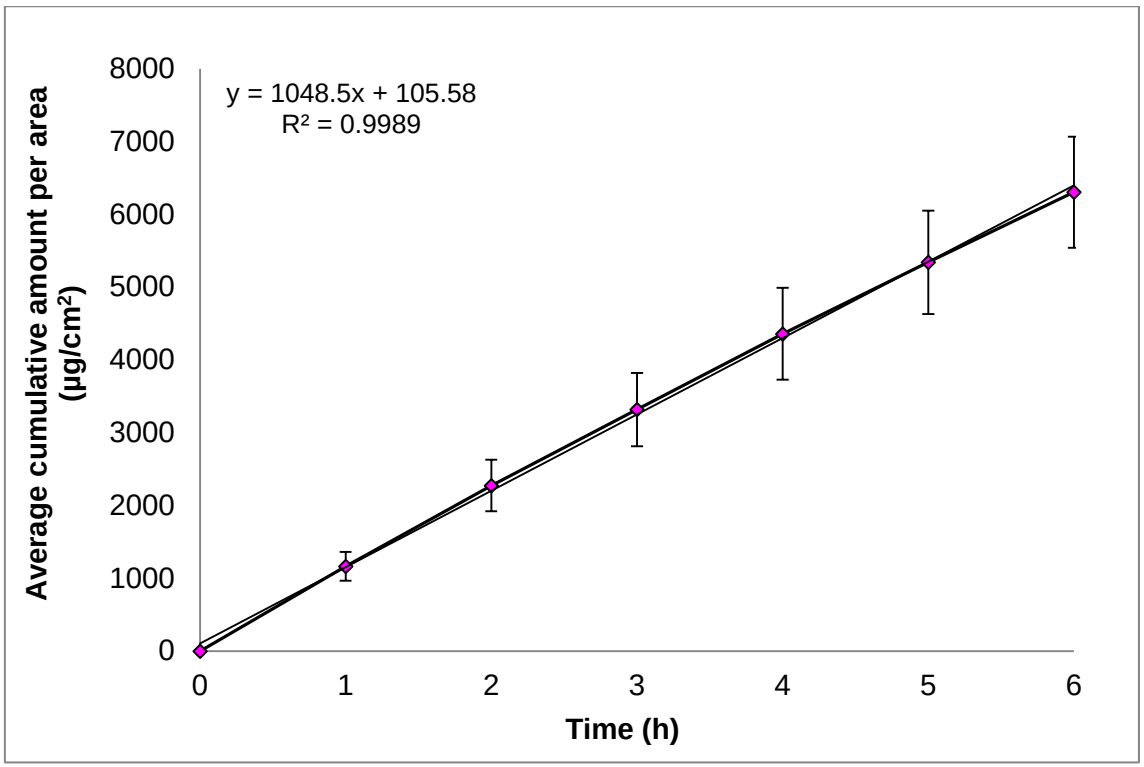

Figure E.8: The average cumulative amount per area (µg/cm^2) of niacinamide released from the niosome gel as a function of time (n = 10)

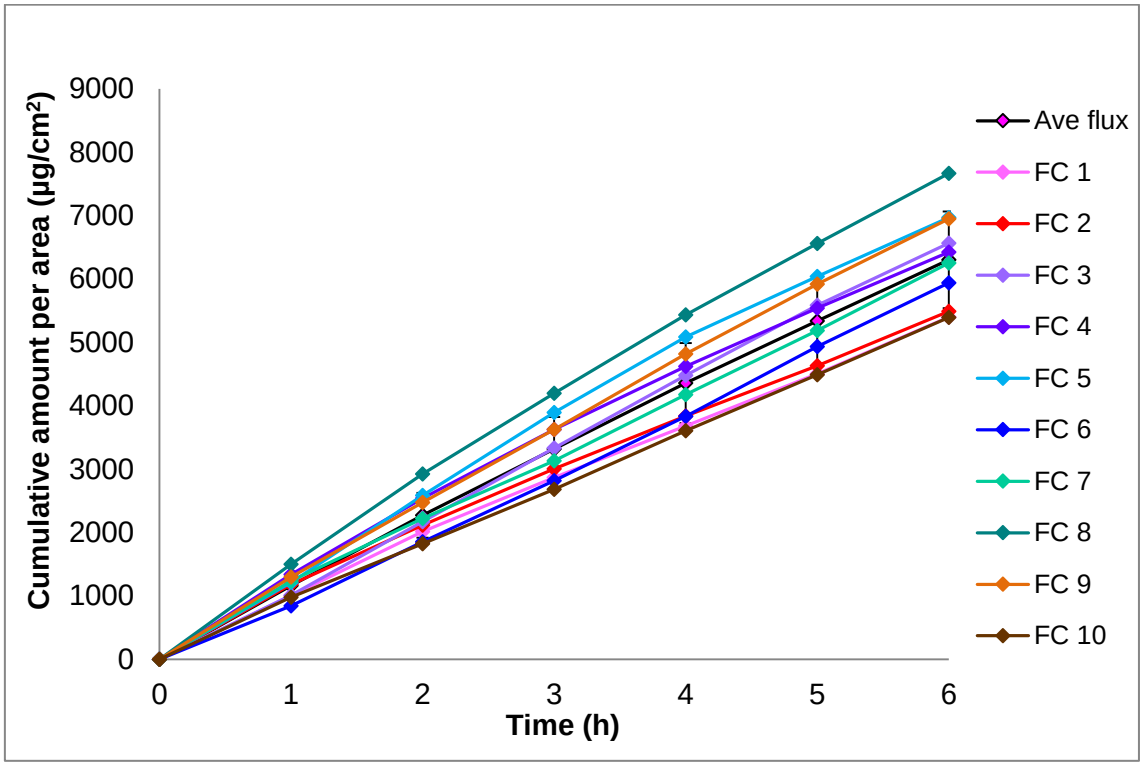

Figure E.9: The cumulative amount per area (µg/cm^2) of niacinamide for each individual Franz cell that was released from the niosome gel during the membrane diffusion study (n = 10)

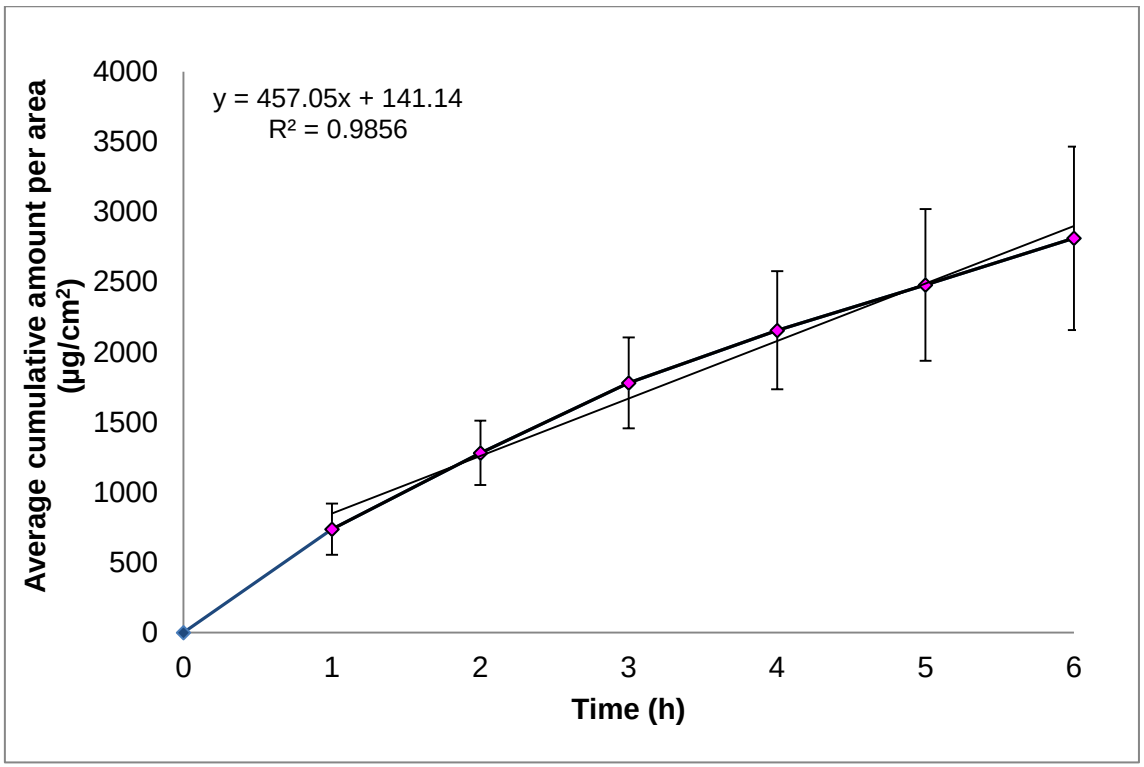

Figure E.10: The average cumulative amount per area (µg/cm^2) of niacinamide released from the niosome cream as a function of time (n = 9)

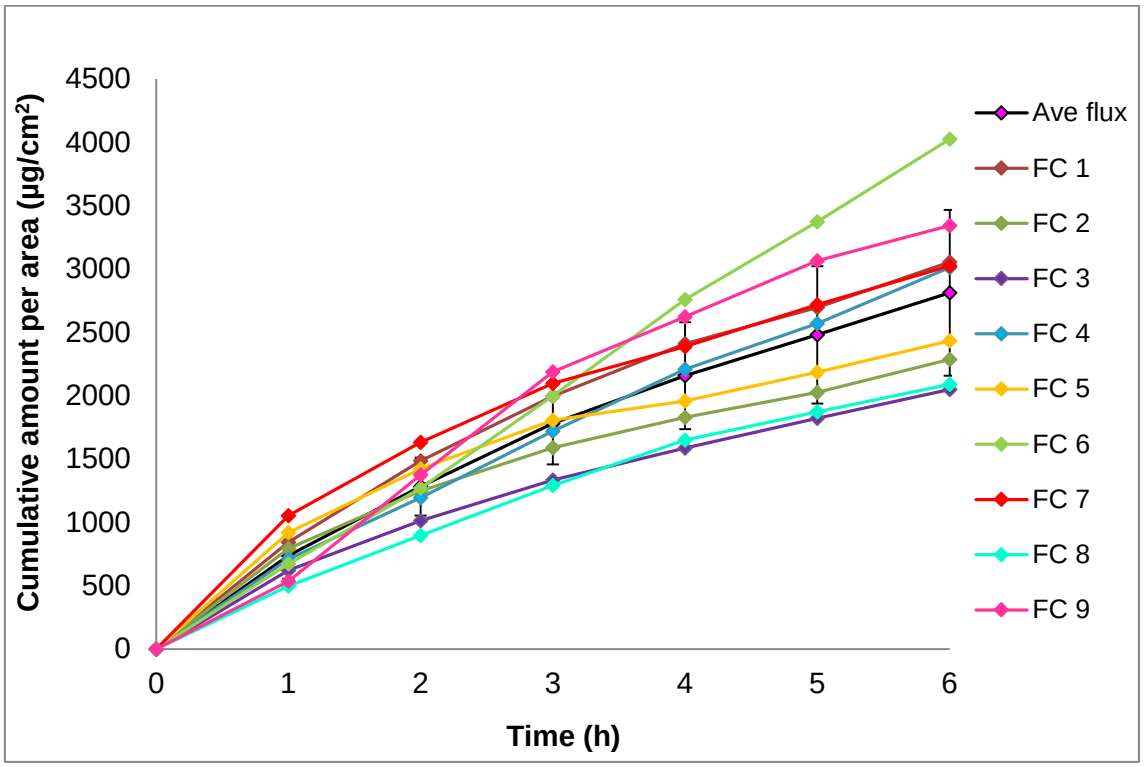

Figure E.11: The cumulative amount per area (µg/cm^2) of niacinamide for each individual Franz cell that was released from the niosome cream during the membrane diffusion study (n = 9)

E.3.4 Skin studies

E.3.4.1 Skin diffusion

The purpose of the skin diffusion studies was to indicate if the active would be delivered topically and/or transdermally. The aim of this study was to deliver niacinamide topically in order to treat skin conditions such as aging, hyperpigmentation, melasma and rosacea (Gehring, 2004:88). During the skin diffusion studies, both the pre-formulations, and the final formulations delivered niacinamide into the receptor phase.

For the skin diffusion study, 10 Franz cells were prepared containing niacinamide and two cells were prepared containing only a placebo. During the HPLC data collection, extremely small peaks were noticed from the samples of the placebo cells. Since these values were above the LOD and LOQ, it had to be brought into consideration. Niacinamide is vitamin B_3 and not naturally found in the body, but may be obtained by the intake of foods such as chicken, beef, pork, fish, nuts and mushrooms (Chen & Damian, 2014:169). Another way of obtaining the active is through daily intakes of supplements, such as multivitamins or injections. These vitamins, especially niacinamide, concentrated in the skin and released into the receptor phase, resulting in the placebos also having niacinamide concentrations.

Due to the aforementioned explanation, a placebo skin diffusion study was executed using 10 Franz diffusion cells containing only PBS (pH 7.4) in both the receptor and donor phases. Extractions were done exactly the same way as for the skin diffusion study, where after tape stripping was performed. The placebo concentrations were analysed and an average was calculated. The average was subtracted from the values gathered from the diffusion studies containing the active. This was done to determine the real amount of niacinamide that diffused from the formulations during this study and to rule out the influence of niacinamide already concentrated in the skin.

Both receptor phases of the pre-formulations and the formulations were extracted as described in Section E.2.9.2. Both the niosomes and the niosome gel had niacinamide concentrations from the first extraction at 20 min, while the proniosomes and niosome cream only presented with niacinamide concentrations after 2 hrs. Franz cells that presented with noticeable leakage were removed from each experiment.

Table E.2: The data obtained from the pre-formulations and final formulations during the skin diffusion study

Preparation	Average percentage diffused (%)	Average flux (µg/cm^2.h)	Median flux (µg/cm^2.h)
Pre-formulations			
Niosomes	0.019 ± 0.006	0.913 ± 0.29	0.844
Proniosomes	0.010 ± 0.003	0.577 ± 0.13	0.462
Formulations			
Niosome gel	0.022 ± 0.016	1.052 ± 0.76	0.692
Niosome cream	0.004 ± 0.004	0.066 ± 0.06	0.172

When the median flux values of the different preparations were compared, it was found that the niosomes (0.844 µg/cm^2.h) had the highest median flux, followed by the niosome gel (0.692 µg/cm^2.h), the proniosomes (0.462 µg/cm^2.h) and lastly, the niosome cream (0.172 µg/cm^2.h). When the average flux values of the different preparations were compared, it was found that the niosome gel (1.052 ± 0.76 µg/cm^2.h) had the highest average flux, followed by the niosomes (0.913 ± 0.29 µg/cm^2.h), the proniosomes (0.577 ± 0.13 µg/cm^2.h) and lastly, the niosome cream (0.066 ± 0.06 µg/cm^2.h). It was observed that the median and average flux values differed, especially for the niosome cream and niosome gel. Hence, the median values were preferred since the median represented the skewed data more accurately and was not affected by outliers (Dawson & Trapp, 2001:30; Gerber *et al*., 2008:190).

The niosomes had a higher flux (average and median) value than the proniosomes; this may be explained by referring back to the characteristics of the vesicle systems. The niosomes entrapped a higher amount of the active than the proniosomes. The niosomes also presented with smaller and more densely packed vesicles than the proniosomes and for this reason the niosomes may have penetrated better through the skin (Csongradi, 2015:191).

As seen in Table E.2, the niosome gel presented with a higher flux (average and median) value than the niosome cream. The reason for the appearance might be the higher water content of the gel, which increased the hydration of the stratum corneum and in this manner, enhanced the transdermal delivery (Bouwastra *et al*., 2003:22).

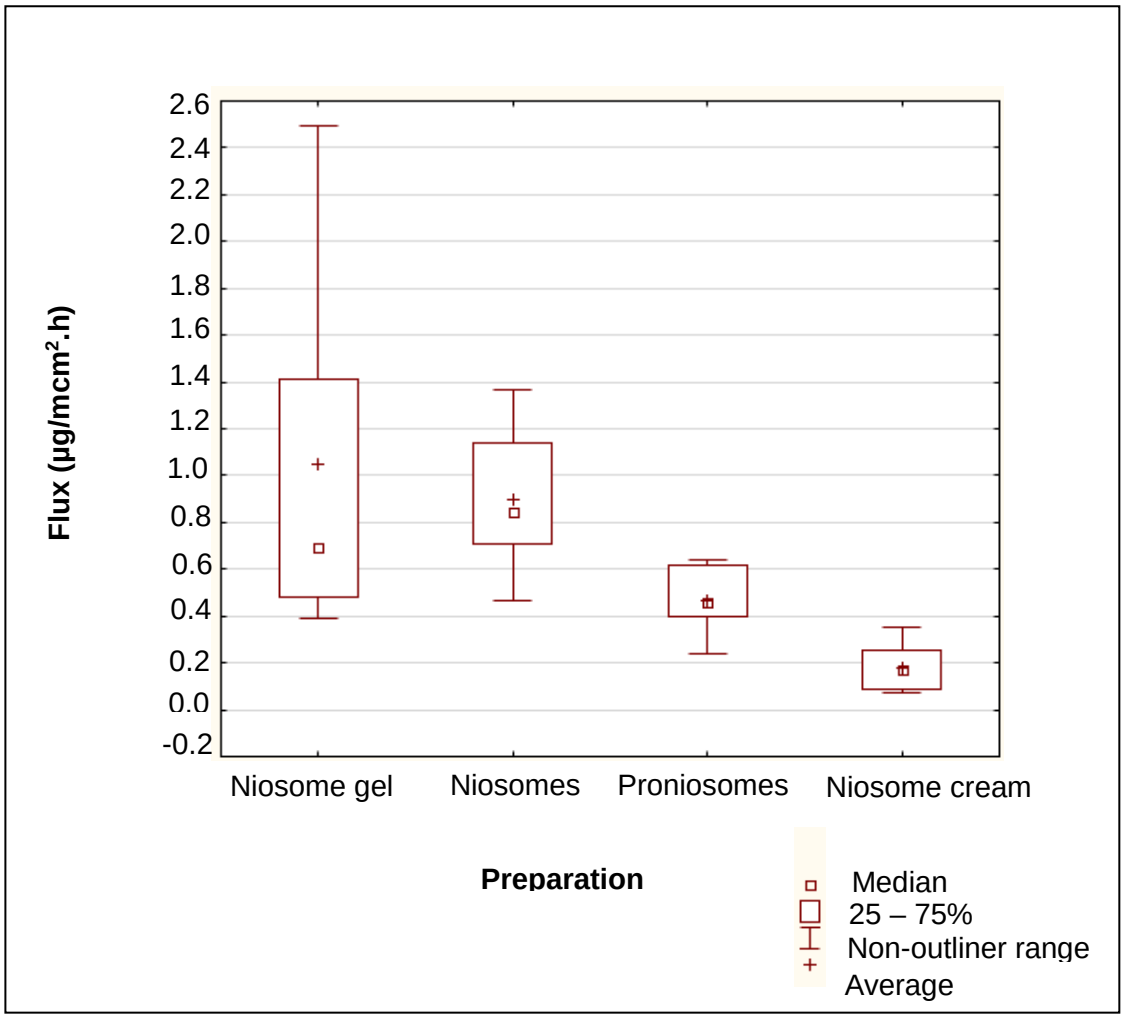

Figure E.12: The box-plots of the skin diffusion study of all four preparations, illustrating the median (small square) and average flux (plus sign) of niacinamide

Figure E.12 illustrates the diffusion of niacinamide from the four preparations after the skin diffusion studies. Both the median (which indicates the centre point of all the data points) flux and the average (which is determined by the sum of the data divided by the number of points) flux are presented. The figure also illustrates the minimum and maximum flux values of each preparation. In Figure E.12 and Table E.2, the mean and average values differ significantly for the niosome gel. This is due to the different way that the average- and mean flux values were calculated as explained in Section E.2.10.

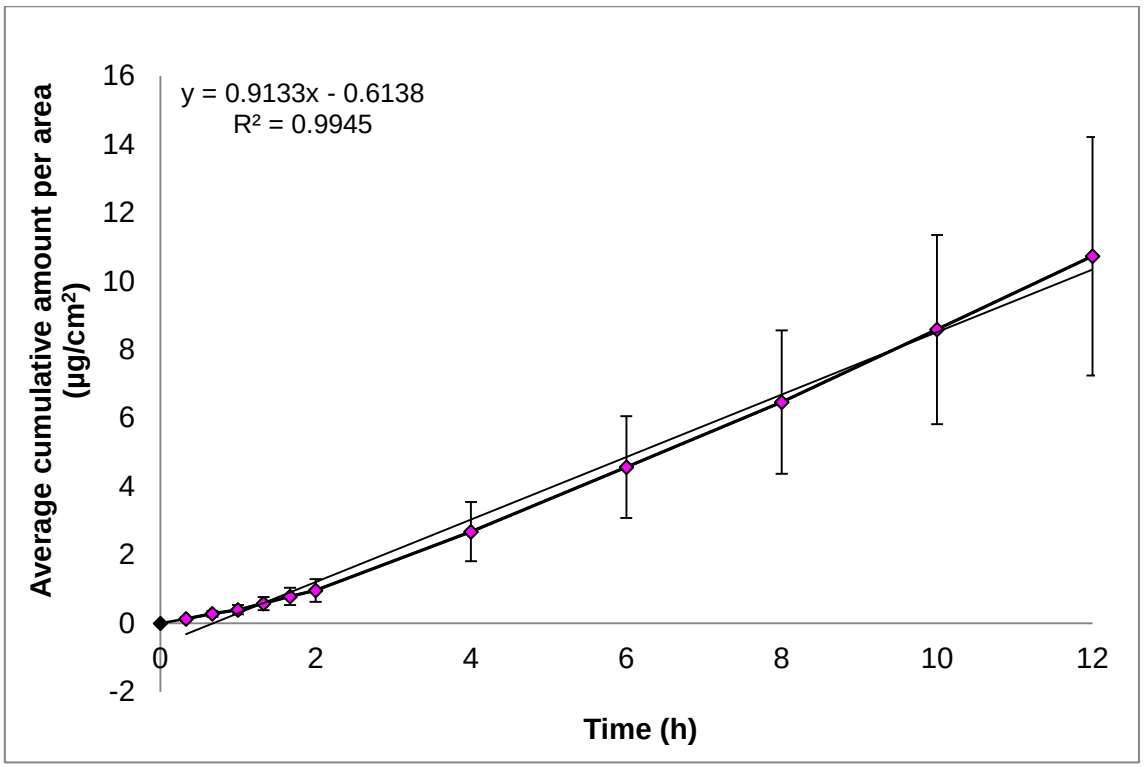

Figure E13: The average cumulative amount per area (µg/cm^2) of niacinamide that penetrated through the skin from the niosomes as a function of time (n = 8)

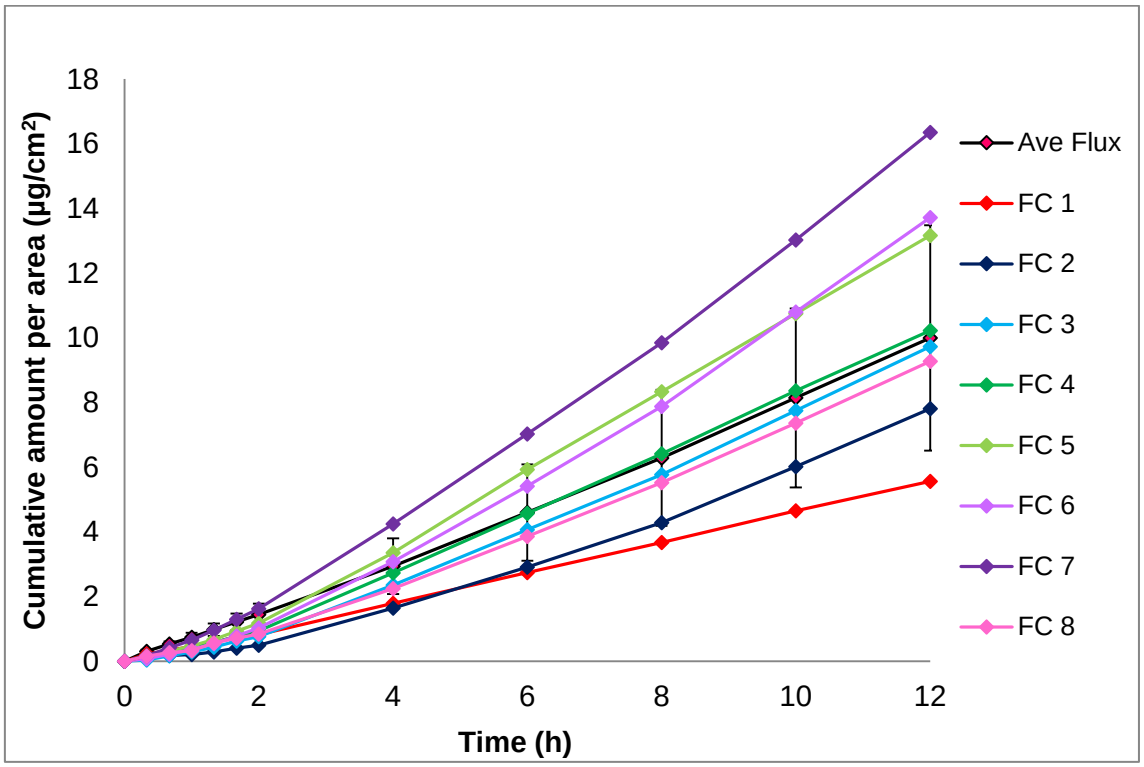

Figure E.14: The cumulative amount per area (µg/cm^2) of niacinamide for each individual Franz call that penetrated through the skin from the niosomes (n = 8)

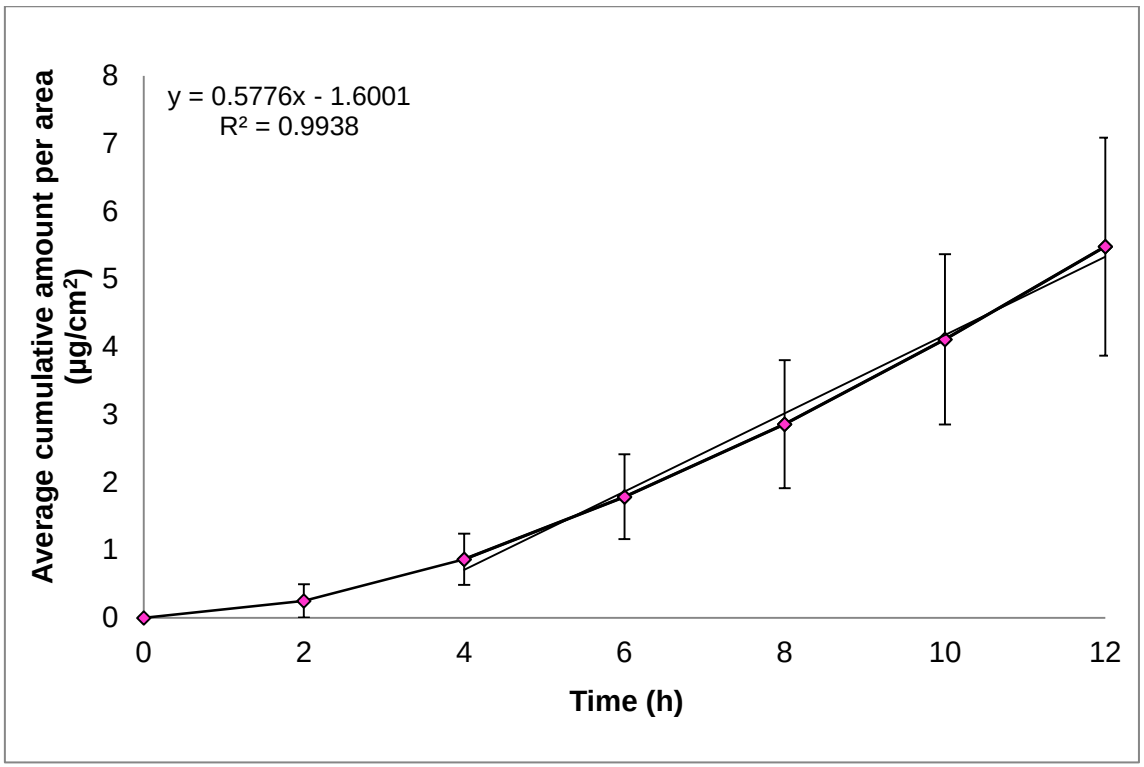

Figure E.15: The average cumulative amount per area (µg/cm^2) of niacinamide that penetrated through the skin from the proniosomes as a function of time (n = 10)

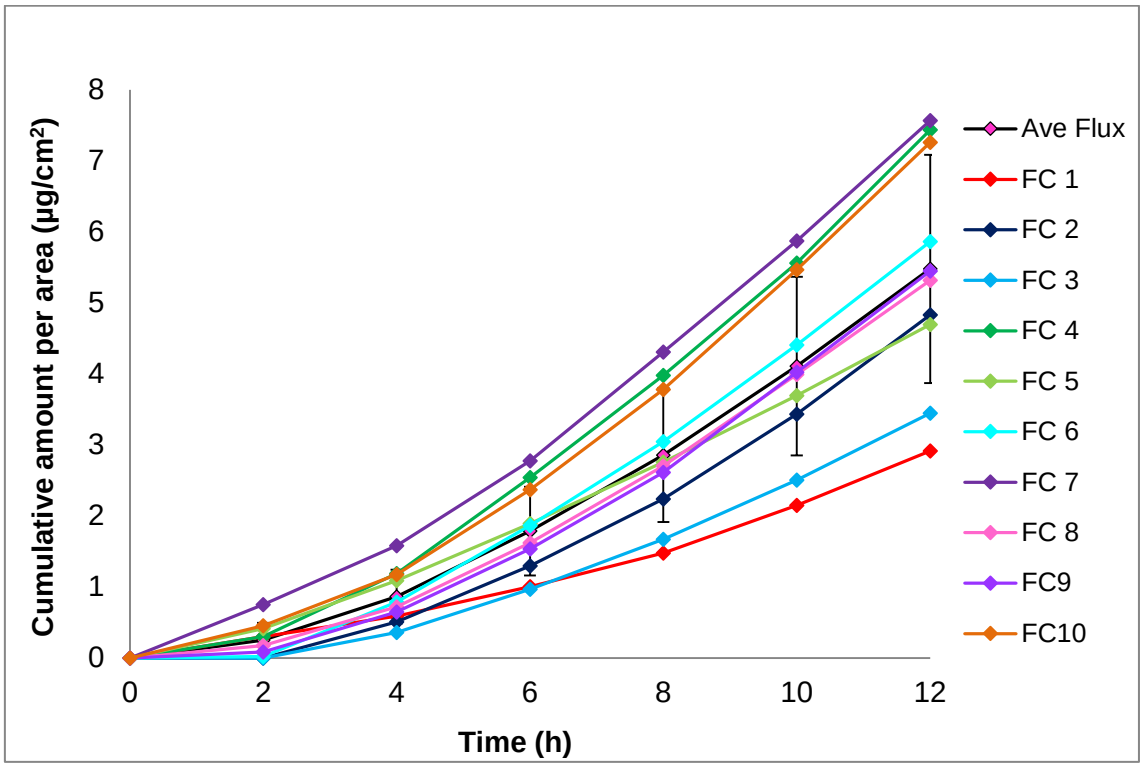

Figure E.16: The cumulative amount per area (µg/cm^2) of niacinamide for each individual Franz cell that penetrated through the skin from the proniosomes (n = 10)

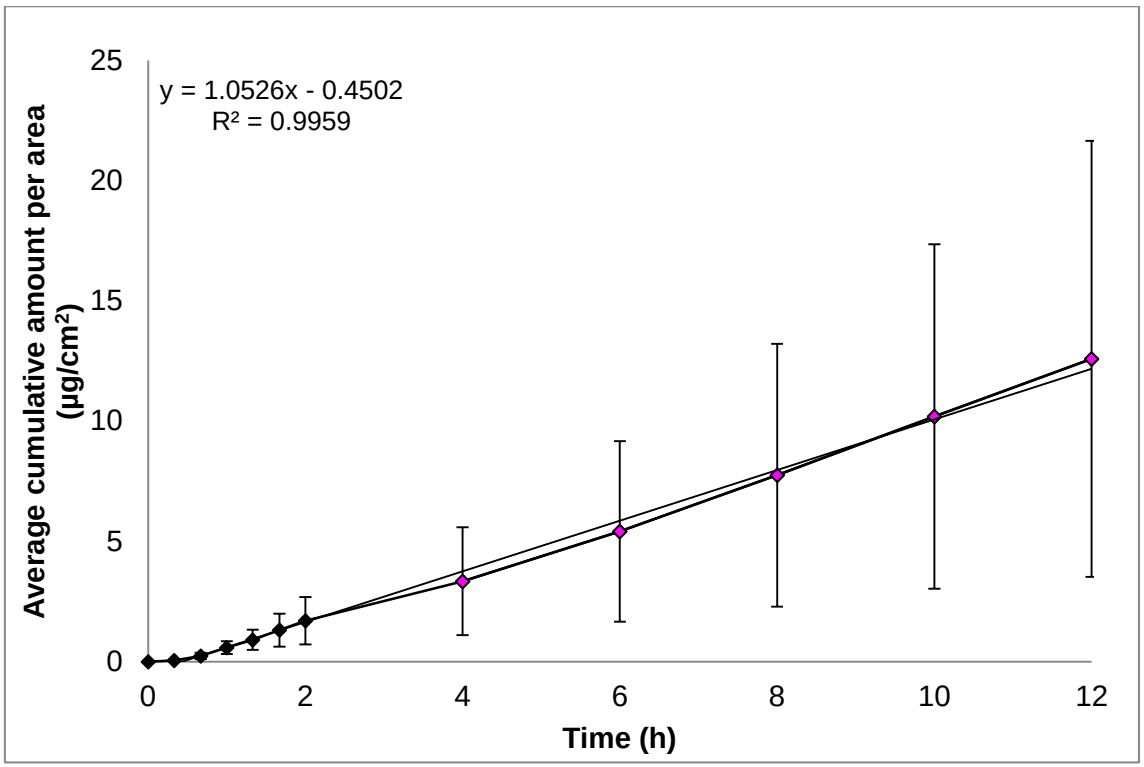

Figure E.17: The average cumulative amount per area (µg/cm^2) of niacinamide that penetrated through the skin from the niosome gel as a function of time (n = 7)

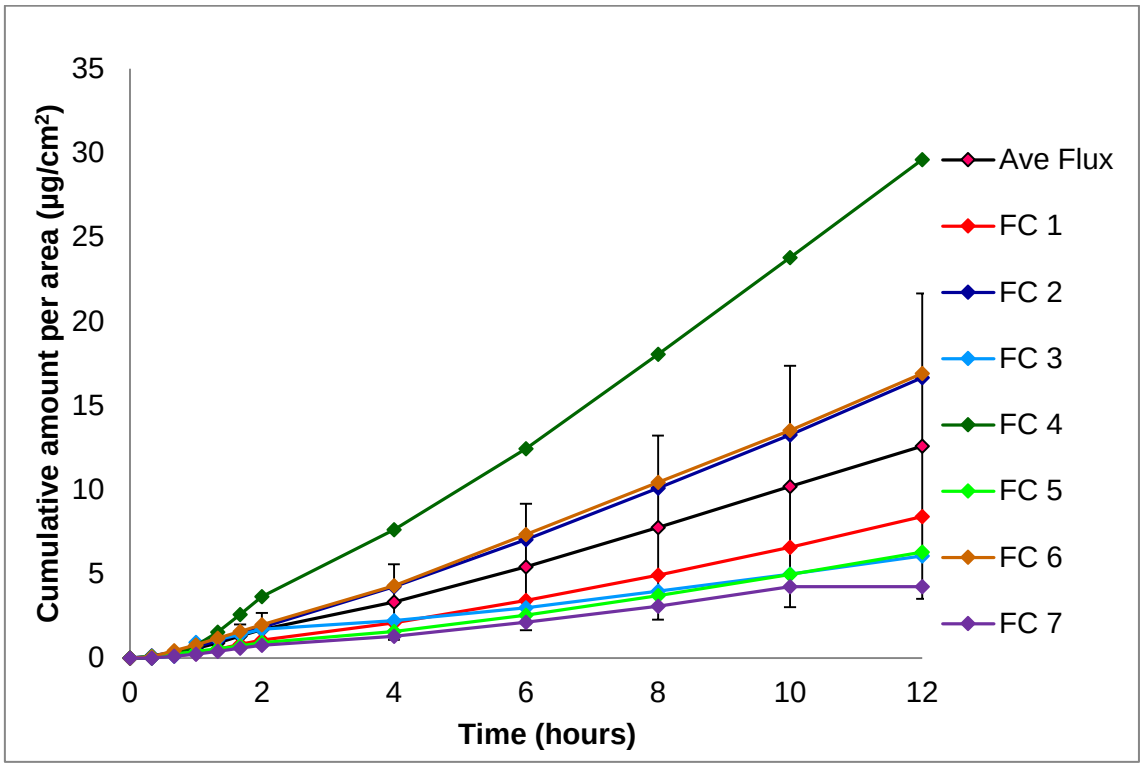

Figure E.18: The cumulative amount per area (µg/cm^2) of niacinamide for each individual Franz cell that penetrated through the skin from the niosome gel (n = 7)

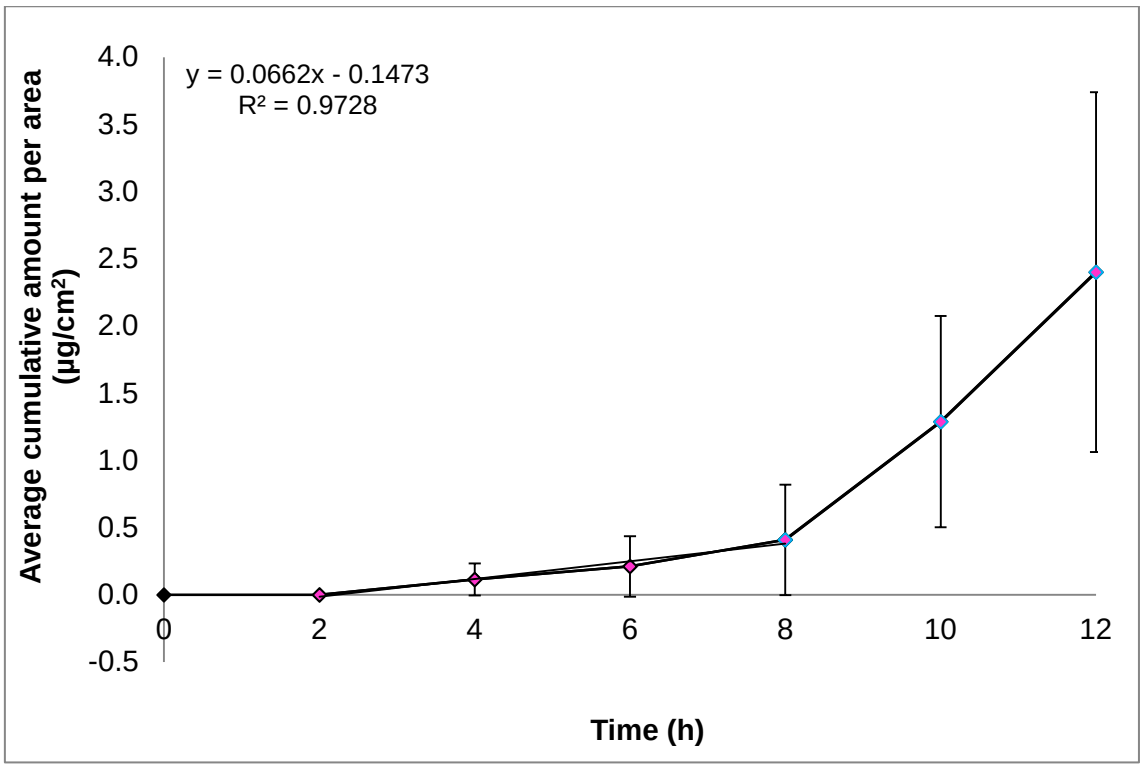

Figure E.19: The average cumulative amount per area (µg/cm^2) of niacinamide that penetrated through the skin from the niosome cream as a function of time (n = 7)

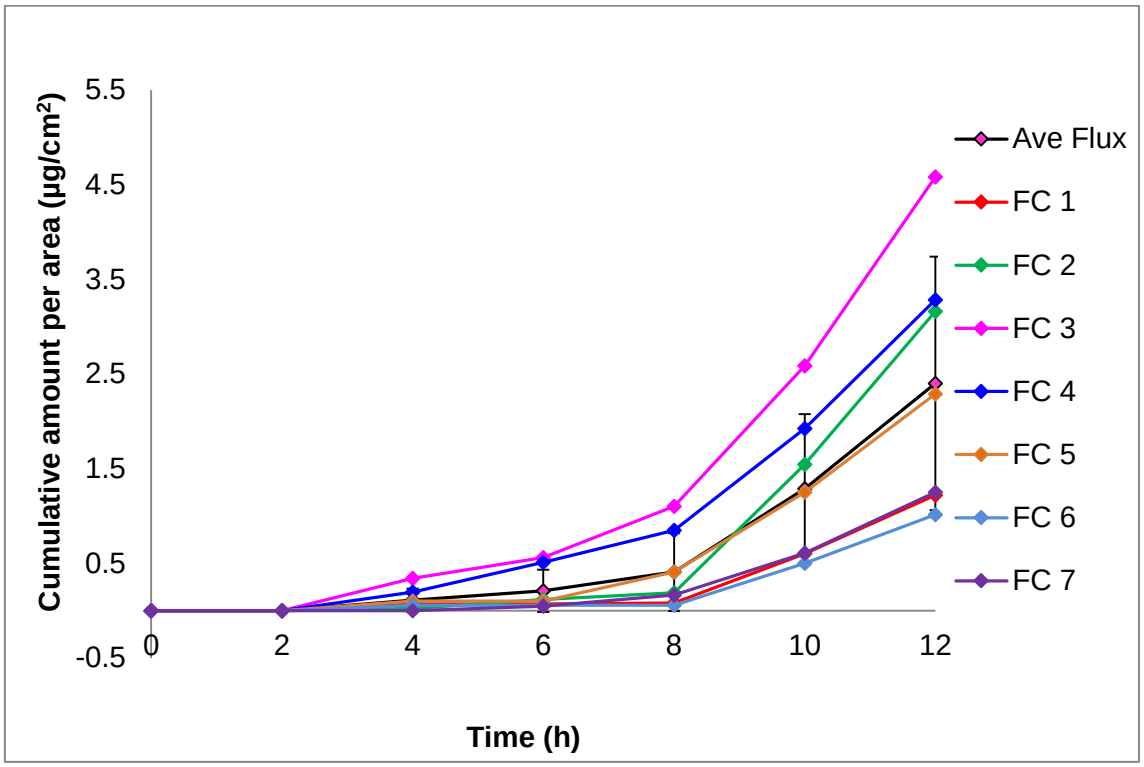

Figure E.20: The cumulative amount per area (µg/cm^2) of niacinamide for each individual Franz cell that penetrated through the skin from the niosome cream (n = 7)

E.3.4.2 Tape stripping

Tape stripping was performed to determine if niacinamide in the different preparations targeted the SCE and ED. The data of the tape strips were also determined by subtracting the placebo in the study. In Table E.3, the results indicate niacinamide did target the SCE. When comparing the median concentrations in the SCE, it was found the niosomes (2.13 µg/ml) had the highest median concentration, followed by the niosome gel (1.94 µg/ml), the niosome cream (1.05 µg/ml) and lastly, the proniosomes (0.71 µg/ml). Comparing the average concentrations of the different preparations; it was found the niosome gel (2.15 ± 1.66 µg/ml) had the highest average concentration, followed by the niosome cream (2.09 ± 2.59 µg/ml), the niosomes (2.08 ± 0.97 µg/ml) and lastly, the proniosomes (0.83 ± 0.47 µg/ml). Although the average concentrations differed from the median concentrations, the median values were preferred; since the median is more accurate and not influenced by outliners in the data (Dawson & Trapp, 2001:30; Gerber *et al*., 2008:190).

Although the barrier function of the stratum corneum complicates topical delivery, the delivery may be eased by hydrating the skin. This increase in hydration causes rheological properties in the skin to change, resulting in an increase in penetration (Donnelly & Sing, 2015:17). During the skin diffusion study, the skin was extremely hydrated, as the experiment was performed in a water bath of 37 °C for 12 hrs. Hydration of the skin causes the lipid organisation in the stratum corneum to change, resulting in better topical delivery of the hydrophilic active (Bouwastra *et al*., 2003:22).

The results obtained from the tape stripping, indicated that the niosomes delivered the highest concentration of niacinamide into the SCE. When comparing the niosomes with the proniosomes it was observed that the niosomes had small and densely packed lipophilic particles, which resulted in close contact with the stratum corneum and in this manner increased the delivery of the encapsulated active (Liu *et al*., 2008:136). When comparing the niosomes with the semi-solid formulations (niosome gel and niosome cream), the niosomes delivered a higher concentration of niacinamide into the SCE. The difference in delivery could be a result of the difference in viscosities, as the semi-solids were more viscous than the niosomes and viscosity might indirectly have an effect on delivery (Ueda *et al.,* 2009:753).

The concentration niacinamide delivered by the niosome gel was higher than that of the niosome cream. It should be kept in mind that the water content of the niosome gel is higher than in the niosome cream, which may have hydrated the stratum corneum and in this manner increased the delivery (Bouwastra *et al*., 2003:22).

Table E.3: The average and median concentrations of niacinamide in the stratum corneum-epidermis after tape stripping

Preparation	Average concentration in SCE (µg/ml)	Median concentration in the SCE (µg/ml)
Pre-formulation		
Niosomes	2.08 ± 0.97	2.13
Proniosomes	0.83 ± 0.47	0.71
Formulation		
Niosome gel	2.15 ± 1.66	1.94
Niosome cream	2.09 ± 2.59	1.05

E.3.4.3 Epidermis-dermis

The aim of this study was to deliver the active, which is encapsulated in a vesicle system, into the ED. In Table E.4, the results indicate that the pre-formulations, as well as the formulations effectively delivered niacinamide into the ED. When comparing the average concentrations of the preparations, the niosomes (4.79 ± 2.71 µg/ml) presented with the highest average concentration, followed by the niosome cream (3.18 ± 2.07 µg/ml), the proniosomes (3.06 ± 2.29 µg/ml) and the niosome gel (1.68 ± 0.61 µg/ml). Considering the median concentrations of the preparations, the niosomes (5.02 µg/ml) had the highest median concentration, followed by the niosome cream (2.86 µg/ml), the proniosomes (2.04 µg/ml) and niosome gel (1.94 µg/ml). Although there were differences between the average and median values; the median values were preferred over the average values; since the median represents the skewed data more and is not affected by outliners in the data (Dawson & Trapp, 2001:30; Gerber *et al*., 2008:190).

The data indicates that incorporating the niosomes into a semi-solid definitely had an influence on the penetration effect of the niosomes, as both the niosome gel and niosome cream had lower concentrations in the ED than the niosomes. The poor delivery of the active from the gel and cream may be a result of the viscosity, which is a rheological property and can influence the delivery (Ueda *et al*., 2009:753).

The possible reason why the niosomes had a higher concentration (average and median) delivered into the ED over the proniosomes may be explained by referring back to the TEM results, the micrographs illustrated the niosomes were more densely packed together whereas the proniosomes formed individually and were less than the niosomes. For this reason, the niosomes could have been in closer contact with the skin, resulting in better delivery (Liu *et al*., 2008:136).

The reason why the niosome cream had a significant amount of niacinamide concentrated in the ED (average and median) over the niosome gel is due to its working mechanism. According to Barry (2007:594), when o/w creams are rubbed into the skin, the continuous phase evaporates, increasing the concentration of the water soluble active in the adhering film. This results in an increase of the concentration gradient across the stratum corneum promoting the topical deposit.

Table E.4: Average and median amount of niacinamide in the epidermis-dermis after tape stripping

Dosage form	Average concentration in ED (µg/ml)	Median in the ED (µg/ml)
Pre-formulation		
Niosomes	4.79 ± 2.71	5.02
Proniosomes	3.06 ± 2.29	2.04
Formulation		
Niosome gel	1.68 ± 0.61	1.94
Niosome cream	3.18 ± 2.07	2.86

E.3.5 Statistical data analysis

The Tukey multiply comparison test indicated that significant differences in the niosomes occurred between the mean concentration of niacinamide in the SCE and ED ($p = 0.03$) and between the mean concentration in the SCE and the receptor phase ($p = 0.003$), but no significant difference between the mean concentrations in the ED and the receptor phase ($p = 0.596$) was noticed. The proniosomes had significant difference between the mean concentration in the SCE and ED ($p = 0.005$), as well as between the mean concentrations of the SCE and the receptor phase ($p = 0.008$). For the proniosomes, no significant difference between the mean concentrations in the ED and receptor phase appeared ($p = 0.982$). The niosomes gel presented with significant difference between the mean concentrations in the SCE and the receptor phase ($p = 0.025$), as well as between the mean concentration in the ED and the receptor phase ($p = 0.013$). No significant difference could be noticed between the mean concentration in the SCE and ED ($p = 0.955$). No significant differences in the niosome cream was noticed in any of the combinations, i.e. between the SCE and ED ($p = 0.560$), SCE and the receptor phase ($p = 0.728$), ED and the receptor phase ($p = 0.195$).

E.4 Conclusion

During the study the physiochemical properties of niacinamide was determined to indicate if niacinamide was a good candidate for topical delivery. The solubility of niacinamide was determined to be 112.70 mg/ml, which predicted good penetration in contrast with the log D value of -0.43, which indicated skin penetration would be challenging.

During the membrane diffusion studies, release of the active occurred from all the preparations, although a noticeable decrease could be observed by incorporating the niosomes into a gel and a cream. The preparation with the highest median flux was the proniosomes.

During the skin diffusion studies the aim was to achieve optimal delivery of the active in the ED. However, transdermal delivery of the active was also achieved. The preparation which had the highest median flux value transdermally was the niosomes. All the preparations delivered niacinamide into the SCE and ED as well.

Additionally, the concentration of niacinamide in the SCE was much lower than in the ED. This is possibly due to the stratum corneum being a lipophilic environment compared to the aqueous environment of the ED, resulting in the water-soluble active to prefer the ED (Abbott, 2012:218). Another reason why the concentration of niacinamide was higher in the ED than in the SCE is because of the vesicle systems used, which may have acted as a penetration enhancer during this study, promoting the topical penetration of the active. Thus looking at the data it can be concluded that all the preparations delivered the active into the target sight successfully. Since the niosomes presented with the highest concentration of niacinamide in the SCE and ED it was the most ideal preparation.

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Appendix F

Validation of the HPLC method for chemical stability assay of Niacinamide, methylparaben and propylparaben

F.1 Purpose of the validation of chemical stability assay for semi-solid dosage forms

The HPLC validation was performed to determine the stability-indicating assay of the semi-solid formulations and done in conjunction with Prof Jan Du Preez at the analytical Technology Laboratory, North-West University, Potchefstroom Campus, South Africa. The purpose of this validation is to detect the changes, over time, of the active ingredient, as well as that of the preservatives. The stability-indicating assay measures the active ingredient as well as the preservatives, without any interference such as degradation, impurities and other excipients (CDER, 2001:6).

F.2 The chromatographic conditions

The chromatographic conditions are presented in Table F.1.

Table F.1: Chromatographic conditions used to analyse the excipients in the semi-solid formulations

Analytical instrument	Agilent 1100 series analytical system, which is equipped with an Agilent 1100 gradient pump, autosampler injection mechanism and diode array detector, was used. It was installed with ChemStation Rev. A10.01 data acquisition and analysis software (Agilent Technologies, Palo Alto, CA, USA). The analysis was performed at room temperature (25 °C)
Column	Venusil XBP C8, 5 µm, 250 x 4.6 mm (Agela Technologies, Newark, DE, USA)
Mobile phase	The mobile phase was used in a gradient. Methanol (A) and 0.075 M octane sulphonic acid (B) buffered to a pH of 3.5 with a few drops of 2 M phosphoric acid
Flow rate	1 ml/min
Injection volume	10 µl
Detection	UV at 212 nm
Retention times	Niacinamide: 3.4 min Methylparaben: 6.9 min Propylparaben: 9.5 min
Stop time	11 min
Solvent	HPLC grade methanol

Table F.2: The gradient table of the mobile phase used.

Time (min)	A%	B%
0.0	45	55
1.0	45	55
2.0	70	30
9.0	70	30
9.7	45	55

F.3 Standard preparation

For the standard preparation, 350 mg of niacinamide was accurately weighed and made up to volume with Milli-Q® HPLC grade water (Merck Millipore, Halfway House, South Africa) in a 10 ml volumetric flask. The methyl- and propylparaben was weighed and placed into a 10 ml volumetric flask and made up to volume with 100% methanol. After the ingredients dissolved completely, 1 ml of the niacinamide, methyl- and propylparaben mixture was diluted with Milli-Q® water and methanol (20:80) in a 100 ml volumetric flask.

F.4 Sample preparation

For the preparation, 1 g of the sample (containing all the excipients) was weighed and placed into a 100 ml volumetric flask. The sample was then dissolved in a 100 ml volumetric flask with Milli-Q® water and methanol (20:80).

F.5 Validation

The parameters for the validation for the purpose of the concentration assay include linearity, accuracy, precision, sensitivity and recovery.

F.6 Validation parameters

The samples were prepared, as described in Section F.4, for all the validation parameters except accuracy. During the determination, a placebo was prepared containing all the ingredients except the niacinamide, methylparaben and propylparaben. The placebo was spiked with known concentrations of the analytes for the determination of accuracy.

F.6.1 Linearity and range

The linearity is the relationship between the response of an instrument and the known concentration of an analyte (CDER, 2001:5). The same equation that was used in Appendix A can be used to describe the linearity.

F.6.1.1 The preparation of the sample for linearity testing

For the linearity testing of the semi-solid dosage form, niacinamide, methyl- and propylparaben was weighed and placed separately in 10 ml volumetric flasks. From each sample, 1 ml was extracted and placed into a 100 ml volumetric flask and made up to volume with Milli-Q® water and methanol (20:80). It was then injected in different concentrations into the HPLC for analysis. The concentration ranges for the active and excipients were as follows: niacinamide 34.80 – 208.80 µg/ml, methylparaben 2.55 – 15.31 µg/ml and propylparaben 0.65 – 3.91 µg/ml.

F.6.1.2 Acceptance criteria

For the linear regression analysis to be acceptable, a correlation coefficient of ≥ 0.99 with six concentration levels is needed (Shabir, 2005:9).

F.6.1.3 Results

The linearity results for niacinamide, methylparaben and propylparaben are represented in Table F.3 and Figures F.1 to F.3. For the linearity to be acceptable the correlation coefficient (r^2) value must be ≥ 0.99 (Shabir, 2005:9). Since niacinamide has a value of 0.999 and both methyl- and propylparaben have a value of 0.997, it shows the method is stable over the concentration range of each analyte.

Table F.3: Linearity results of niacinamide, methylparaben and propylparaben

Niacinamide		**Methylparaben**		**Propylparaben**	
Standard (µg/ml)	**Peak area**	**Standard (µg/ml)**	**Peak area**	**Standard (µg/ml)**	**Peak area**
34.800	12.4	2.553	250.0	0.652	48.8
69.600	29.8	5.106	486.7	1.304	104.3
104.400	58.5	7.659	727.5	1.956	154.6
139.200	115.3	10.212	965.1	2.608	208.6
174.000	173.8	12.765	1211.0	3.260	257.2
208.800	242.3	15.318	1472.0	3.912	308.3
r^2	**0.999**	**r^2**	**0.999**	**r^2**	**0.999**
y-intercept	**92.99**	**y-intercept**	**-0.033**	**y-intercept**	**-0.723**
slope	**76.18**	**slope**	**95.35**	**slope**	**79.312**

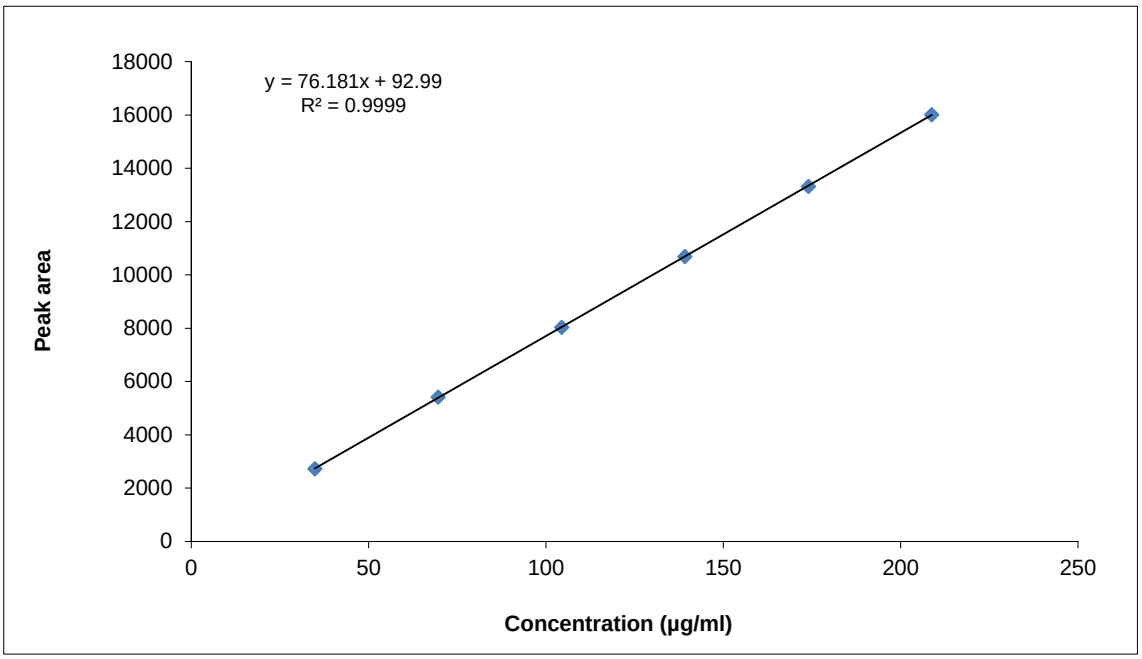

Figure F.1: The line regression graph of niacinamide.

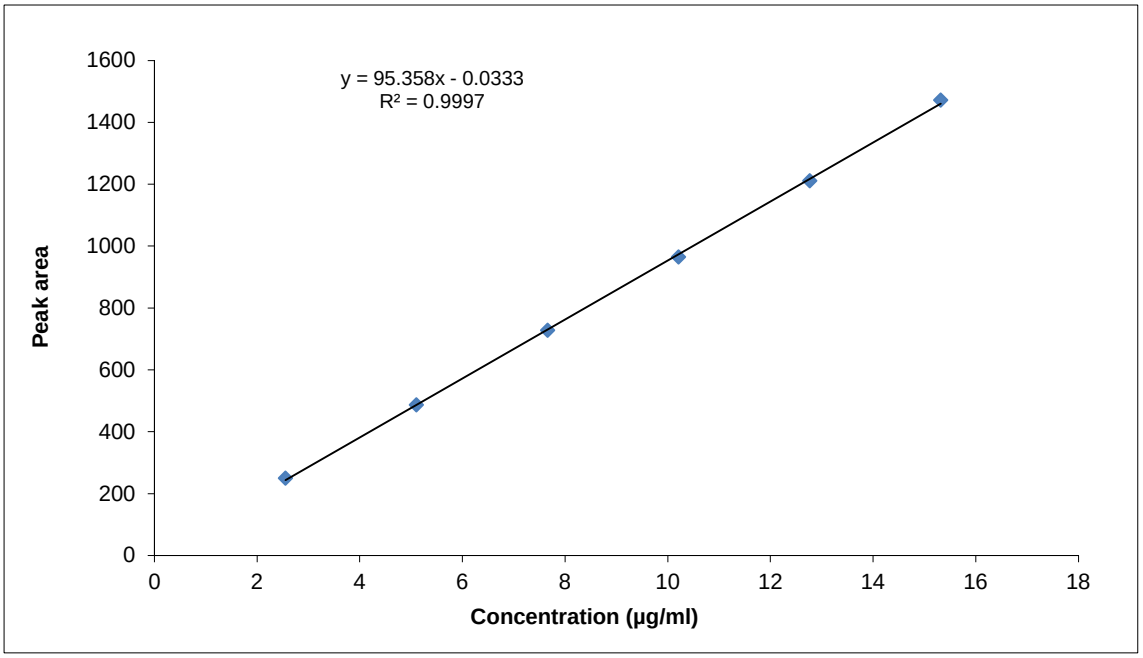

Figure F.2: The line regression graph of methylparaben.

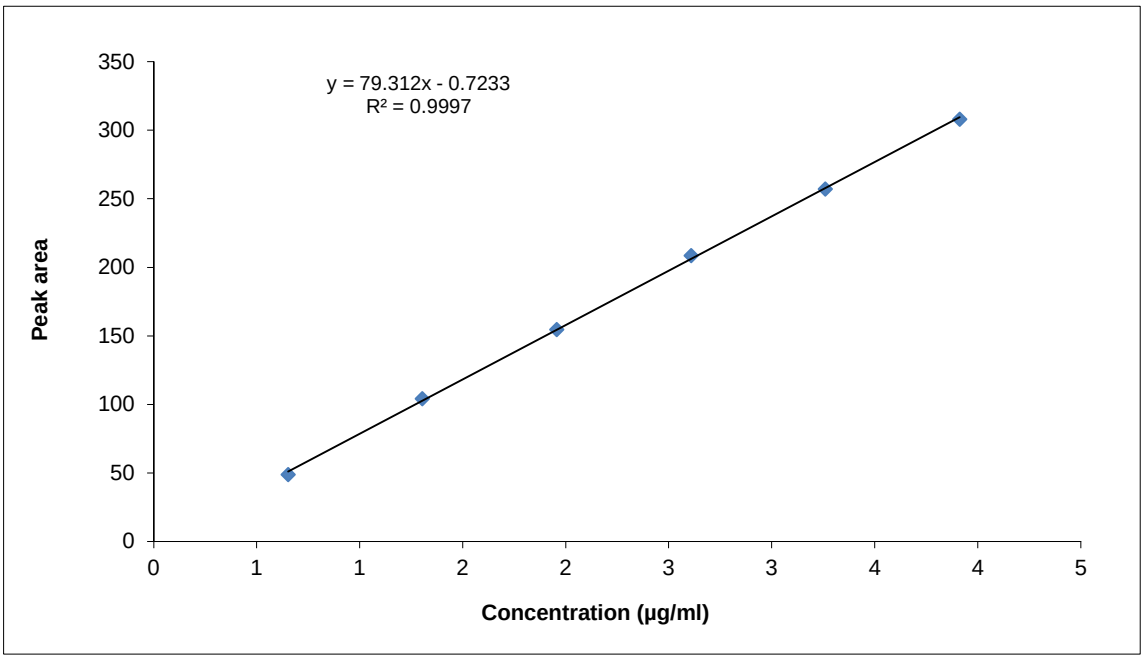

Figure F.3: The line regression graph of propylparaben.

F.6.2 Accuracy

When referring to the accuracy of an analytical procedure, it shows the closeness of the tested concentration in comparison with the true concentration. The accuracy is determined as a percentage of the known amount of the analyte in the sample.

F.6.2.1 The preparation of the standard solution

The standard solution was prepared as described in Section F.3.

F.6.2.2 The preparation of the sample solution

For the preparation of the sample, 0.8 g, 1.0 g and 1.2 g of the placebo niosome gel was accurately weighed in triplicate, 300 mg of niacinamide were dissolved in 100 ml Milli-Q® water and methylparaben (20 mg) and propylparaben (5 mg) were dissolved separately into 50 ml methanol. After all the samples had dissolved, 4 ml of each sample was placed into the 0.8 g placebo niosome gel, 5 ml into the 1.0 g placebo niosome gel and 6 ml into the 1.2 g placebo niosome gel. The samples were made up to volume and placed into HPLC vials for analysis.

F.6.2.3 Acceptable criteria

For the accuracy to be acceptable, the recovery must be between 98 – 102% and the %RSD must be 2.0% or less (Shabir, 2005:9).

F.6.2.4 Results

Table F.4: Accuracy results of niacinamide

Concentration spiked (µg/ml)	Peak area			Recovery	
	1	2	Mean	µg/ml	%
243.1	10064.2	10010.7	10037.5	250.1	102.9
243.1	10000.6	10047.2	10023.9	249.8	102.7
243.1	9965.9	9848.1	9907.0	246.8	101.5
303.9	12441.7	12465.8	12453.8	312.1	102.7
303.9	12497.4	12529.0	12513.2	313.6	103.2
303.9	12472.1	12505.8	12489.0	313.0	103.0
364.8	14802.2	14797.0	14799.6	372.3	102.0
364.8	14812.1	14925.2	14868.7	374.0	102.5
364.8	14759.7	14796.7	14778.2	371.7	101.9
				Mean	**102.0**
				SD	**0.5**
				%RSD	**0.5**

Table F.5: Accuracy results of methylparaben

Concentration spiked (µg/ml)	Peak area			Recovery	
	1	2	Mean	µg/ml	%
16.1	904.8	903.4	904.1	15.6	97.2
16.1	303.6	909.3	906.5	15.7	97.4
16.1	896.9	899.0	898.0	15.5	96.5
19.5	1112.4	1114.8	1113.6	19.3	98.9
19.5	1115.9	1115.2	1115.6	19.3	99.1
19.5	1127.8	1125.0	1126.4	19.5	100
23.0	1351.9	1353.4	1352.7	23.4	101.9
23.0	1352.9	1348.9	1350.9	23.4	101.8
23.0	1331.8	1332.4	1332.1	23.1	100.4
				Mean	**99.2**
				SD	**1.8**
				%RSD	**1.9**

Table F.6: Accuracy results of propylparaben

Concentration spiked (µg/ml)	Peak area			Recovery	
	1	2	Mean	µg/ml	%
3.6	189.3	189.4	189.4	3.6	99.7
3.6	193.5	193.0	193.3	3.7	101.7
3.6	188.4	188.2	188.3	3.6	99.1
4.6	232.7	233.9	233.3	4.4	96.4
4.6	232.7	234.4	233.6	4.4	96.5
4.6	237.5	237.5	237.5	4.5	98.1
5.5	284.6	285.4	285.0	5.4	98.7
5.5	284.2	285.5	284.9	5.4	98.7
5.5	280.3	280.8	280.6	5.3	97.2
				Mean	**98.4**
				SD	**1.6**
				%RSD	**1.6**

The mean recovery of niacinamide, methylparaben and propylparaben was between 98 – 102%, meaning the criteria are acceptable where the %RSD of all the ingredients are less than 2, which is within the requirements.

F.6.3 Precision

The precision of an analyte is the procedure where a series of measurements are collected from a homogeneous sample to be calculated from standard deviations or relative standard deviations. The precision may be measured from the degree of reproductively or the repeatability of the analyte procedure under normal conditions (Johnson & Van Buskirk, 1998:103).

F.6.3.1 Repeatability (intra-day precision)

F.6.3.1.1 Procedure for testing repeatability

The standard solutions were prepared as described in Section F.3. For the sample preparations, 0.8 g, 1.0 g and 1.2 g niosome gel were accurately weighed in triplicate and placed into 100 ml volumetric flasks, where after they were made up to volume. After the gel fully dissolved, it was placed into HPLC vials for analysis.

F.6.3.1.2 Acceptable criteria

According to Shabir (2005:9), the recovery must be between 98 – 102% and a %RSD of 2.0 or less is required.

F.6.3.1.3 Results

Table F.7: Intra-day precision results of niacinamide

Mass (µg/ml)	Peak area			Recovery	
	1	2	Mean	(µg/ml)	%
257.05	10394.9	10463.9	10429.4	260.2	101.2
258.41	10436.2	10522.6	10479.4	261.5	101.2
252.12	10191.7	10219.8	10205.8	254.5	100.9
322.33	13186.7	13257.1	13221.9	331.8	102.9
323.91	13095.1	13224.2	13159.7	330.2	101.9
326.12	13224.8	13258.9	13241.9	332.3	101.9
384.83	15556.2	15737.0	15646.6	394.0	102.4
396.27	16164.1	16188.7	16176.4	407.6	102.9
390.47	15777.1	15783.6	15780.4	397.4	101.8
				Mean	**101.90**
				SD	**0.68**
				%RSD	**0.67**

Table F.8: Intra-day precision results of methylparaben

Mass (µg/ml)	Peak area			Recovery	
	1	2	Mean	(µg/ml)	%
16.40	929.7	934.2	932.2	16.1	98.4
16.50	930.9	937.1	934.0	16.2	98.0
16.27	906.3	913.1	909.7	15.7	96.8
20.40	1183.4	1188.7	1186.1	20.5	100.7
20.50	1176.5	1189.6	1183.1	20.5	100.0
21.04	1186.6	1187.4	1187.0	20.6	97.7
25.30	1398.2	1419.0	1408.6	24.4	96.5
25.57	1455.6	1454.3	1455.0	25.2	98.6
25.19	1414.1	1418.0	1416.1	24.5	97.4
				Mean	**98.2**
				SD	**1.3**
				%RSD	**1.3**

Table F.9: Intra-day precision results of propylparaben

Mass (µg/ml)	Peak area			Recovery	
	1	2	Mean	(µg/ml)	%
4.14	205.4	206.6	206.6	4.1	98.3
4.16	210.0	211.2	210.6	4.2	100.2
4.07	199.7	200.9	200.3	4.0	99.3
5.19	260.6	261.2	260.9	5.0	95.7
5.22	258.7	261.5	206.1	5.2	100.5
5.26	260.8	261.4	261.1	5.1	97.3
6.20	307.0	311.7	309.4	6.0	97.0
6.30	318.9	318.4	318.7	6.1	96.5
6.30	311.3	315.5	313.4	6.1	96.9
				Mean	**98.0**
				SD	**1.6**
				%RSD	**1.6**

Since all of the above %RSD values are less than 2 and the %recovery is between 98 – 102%, the criteria are acceptable for niacinamide, methylparaben and propylparaben.

F.6.4. Reproducibility (inter-day precision)

F.6.4.1 Procedure for the inter-day testing

For the inter-day testing, the standard preparation was prepared as described in Section F.3. For the sample preparation, 1 g of the niosome gel was weighed in triplicate and placed into 100 ml volumetric flasks, made up to volume and placed into HPLC vials for analysis.

F.6.4.2 Acceptable criteria

For the inter-day precision to be acceptable, the mean recovery must be between 98 – 102% and the %RSD must be less than 2% (Shabir 2005:9).

F.6.4.3 Results

Table F.10: Inter-day precision results of niacinamide

	Day 1	Day 2	Day 3	Between days
Recovery (%)	102.90 101.90 101.90	102.70 101.70 102.40	100.80 101.00 101.50	
Mean	**102.23**	**102.27**	**101.10**	**101.87**
SD	**0.47**	**0.42**	**0.29**	**0.07**
%RSD	**0.46**	**0.41**	**0.29**	**0.07**

Table F.11: Inter-day precision results of methylparaben

	Day 1	Day 2	Day 3	Between days
Recovery (%)	100.70 100.00 97.70	100.20 102.00 100.20	96.70 100.20 96.60	
Mean	**99.47**	**100.80**	**98.03**	**99.43**
SD	**1.28**	**0.85**	**1.56**	**0.29**
%RSD	**1.29**	**0.84**	**1.59**	**0.29**

Table F.12: Inter day precision results of propylparaben

	Day 1	Day 2	Day 3	Between days
Recovery (%)	95.70 100.50 97.30	99.50 102.10 100.70	97.80 100.30 97.90	
Mean	**97.00**	**100.77**	**98.67**	**99.09**
SD	**2.00**	**1.06**	**1.16**	**1.92**
%RSD	**2.00**	**10.50**	**1.17**	**1.93**

For the inter-day precision to be acceptable, the mean recovery must be between 98 – 102% of the true value and the %RSD must be ≤ 2%. Since all of the values are in range, the inter-day precision for niacinamide, methylparaben and propylparaben are acceptable.

F.6.5 Ruggedness

The ruggedness of a system reflects the chemical or physical stability of an analyte in a given matrix under certain conditions for a given time interval. Ruggedness may be divided into sample stability and system repeatability (CDER, 2001:7).

F.6.5.1 System repeatability

F.6.5.1.1 The procedure for system repeatability testing

For the repeatability, a sample was prepared as described in Section F.4. The sample contained 3070.80 µg/ml niacinamide, 204.72 µg/ml methylparaben and 51.18 µg/ml propylparaben.

F.6.5.1.2 Acceptable criteria

For this criterion, the peak area and the retention times should have a RSD% of ≤ 2% (Shabir, 2005:10).

F.6.5.1.3 Results

Table F.13: The system repeatability results of niacinamide

	Peak area	**Retention times (min)**
	12789.4 12830.7 12829.9 12871.9 12879.1 12900.5	3.605 3.466 3.473 3.474 3.476 3.473
Mean	**12850.3**	**3.495**
SD	**37.25**	**0.050**
RSD%	**0.29**	**1.417**

Table F.14: The system repeatability results of methylparaben

	Peak area	**Retention times (min)**
	1146.0 1146.8 1148.7 1152.1 1147.4 1149.9	6.936 6.891 6.893 6.899 6.911 6.906
Mean	**1148.50**	**6.906**
SD	**2.05**	**0.015**
RSD%	**0.18**	**0.219**

Table F.15: The system repeatability results of propylparaben

	Peak area	Retention times (min)
	256.2	9.531
	253.0	9.478
	254.1	9.470
	254.5	9.486
	253.4	9.511
	253.9	9.503
Mean	**254.2**	**9.497**
SD	**1.02**	**0.021**
RSD%	**0.40**	**0.219**

Since the %RSD of the retention times are less than 2% for niacinamide, methylparaben and propylparaben, good system repeatability is indicated.

F.6.5.2 Sample stability

F.6.5.2.1 Procedure for sample stability

A sample was prepared as described in Section F.4; containing 3106.8 µg/ml niacinamide, 207.2 µg/ml methylparaben and 51.78 µg/ml propylparaben. This sample was injected over a period of 24 hrs to determine the stability of the sample.

F.6.5.2.2 Acceptance criteria

For an analyte to be stable, the acceptable amount of analyte to undergo degradation is an RSD% of ≤ 2% (ICH, 2005:4).

F.6.5.2.3 Results

Table F.16: The stability test results of niacinamide

Time (h)	Peak area	%Recovery
0	13784.3	100.0
1	13752.0	99.8
2	13758.9	99.8
3	13752.2	99.8
4	13783.6	100.0
5	13758.4	99.8
6	13778.9	100.0
7	13781.5	100.0
8	13784.2	100.0
9	13792.5	100.1
10	13795.7	100.1
11	13799.4	100.1
12	13773.1	99.9
13	13780.7	100.0
14	13789.4	100.0
15	13793.7	100.1
16	13785.8	100.0
17	13789.2	100.0
18	13790.2	100.0
19	13834.4	100.4
20	13790.2	100.0
21	13834.4	100.4
22	13828.9	100.3
23	13815.9	100.2
24	13842.7	100.4
Mean	**13790.8**	**100.0**
SD	**24.13**	**0.18**
%RSD	**0.17**	**0.17**

Table F.17: The stability test results of methylparaben

Time (h)	Peak area	%Recovery
0	1317.7	100.00
1	1309.5	99.4
2	1309.5	99.4
3	1308.7	99.3
4	1310.3	99.4
5	1311.5	99.5
6	1313.7	99.7
7	1325.2	100.6
8	1312.1	99.6
9	1311.3	99.5
10	1312.7	99.6
11	1312.9	99.6
12	1314.2	99.7
13	1315.2	99.8
14	1313.3	99.7
15	1313.5	99.7
16	1313.0	99.6
17	1312.6	99.6
18	1313.5	99.7
19	1312.8	99.6
20	1316.5	99.9
21	1312.8	99.6
22	1316.5	99.9
23	1317.1	100.0
24	131705	100.0
Mean	**1329.6**	**99.7**
SD	**3.37**	**0.26**
%RSD	**0.25**	**0.26**

Table F.18: The stability test results of propylparaben

Time (h)	Peak area	%Recovery
0	287.5	100.0
1	287.2	99.9
2	287.9	100.1
3	287.2	99.9
4	287.6	100.0
5	287.5	100.0
6	288.3	100.3
7	287.9	100.1
8	287.8	100.1
9	288.8	100.5
10	288.5	100.3
11	288.7	100.4
12	288.2	100.2
13	288.1	100.2
14	288.5	100.3
15	288.4	100.3
16	288.1	100.2
17	288.5	100.3
18	288.3	100.3
19	289.4	100.7
20	288.3	100.3
21	289.4	100.7
22	289.3	100.6
23	289.9	100.8
24	289.8	100.8
Mean	**288.4**	**100.3**
SD	**0.74**	**0.26**
%RSD	**0.25**	**0.25**

Since niacinamide, methylparaben and propylparaben have a %RSD of less than 2%; it shows that they are stable over a period of 24 h.

F.6.7 Specificity

The specificity is the ability of an analytical method to detect the analyte in the presence of other components in the test sample (CDER, 2001:4).

F.6.7.1 Preparation of the sample

To test the specificity, distilled water, 10% peroxide, sodium hydroxide (0.1 M) and hydrochloric acid (0.1 M) were used as reagents. A standard solution, as described in Section F.3, was prepared and 1 ml of the reagents was added to 1 ml of standard solution in different test tubes. The samples was left overnight and analysed the following day.

F.6.7.2 Acceptable criteria

For the criteria to be accepted, the degraded samples should not interfere with the determination of niacinamide (ICH, 2005:4).

F.6.7.3 Results

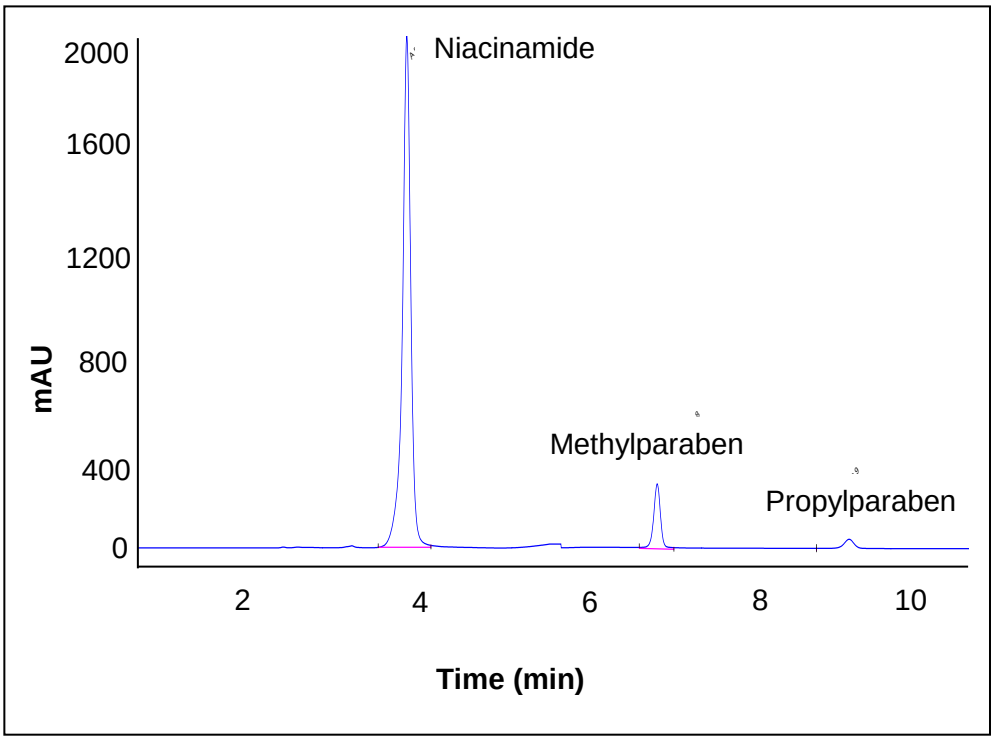

Figure F.4: Chromatogram of niacinamide, methylparaben and propylparaben mixed with distilled water.

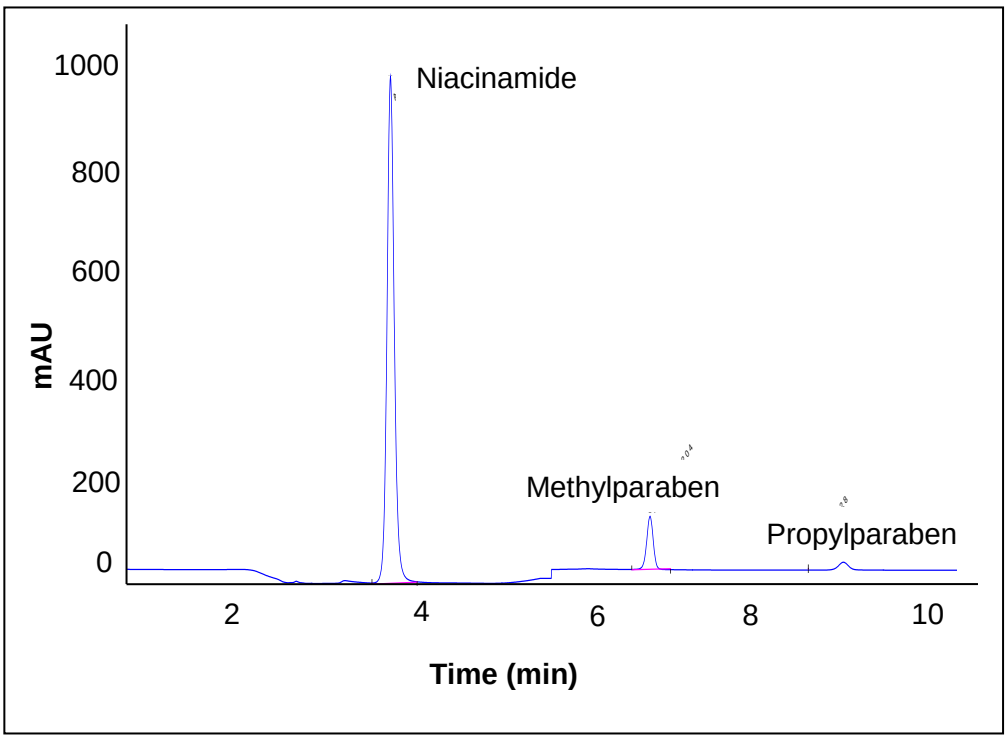

Figure F.5: Chromatogram of niacinamide, methylparaben and propylparaben mixed with 10% peroxide.

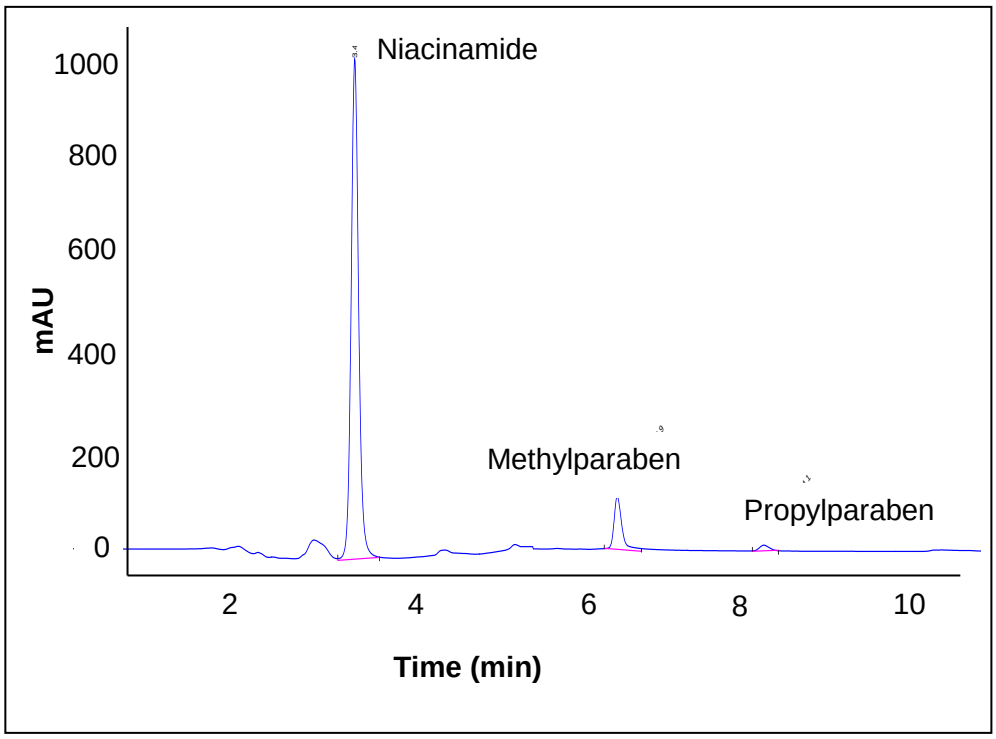

Figure F.6: Chromatogram of niacinamide, methylparaben and propylparaben mixed with sodium hydroxide (0.1 M).

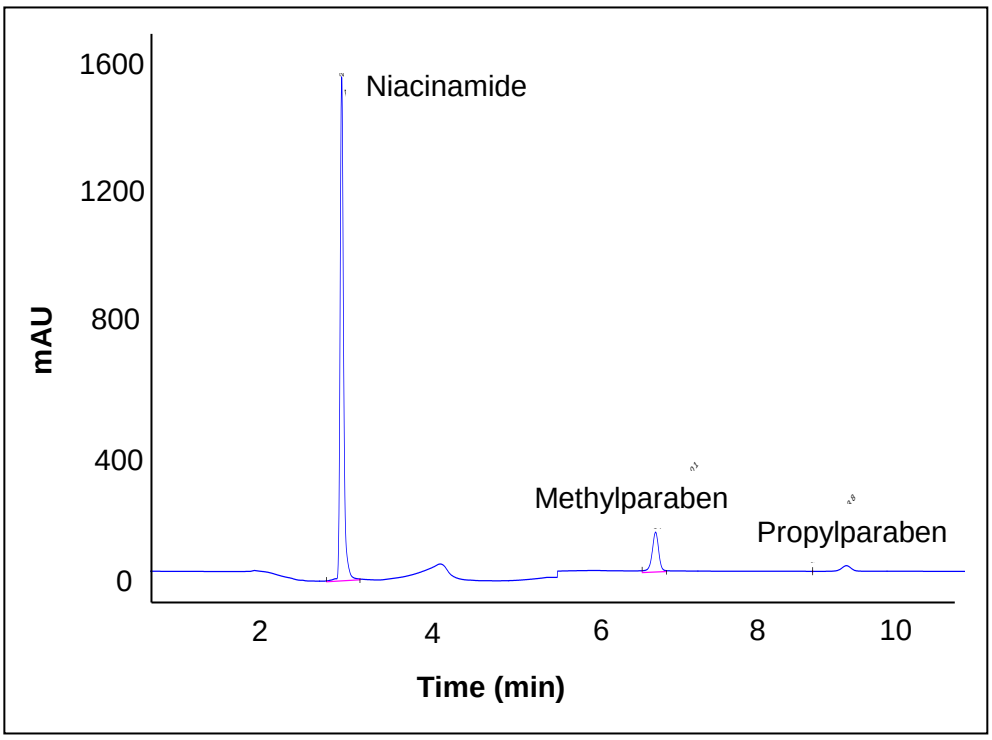

Figure F.7: Chromatogram of niacinamide, methylparaben and propylparaben mixed with hydrochloric acid (0.1 M).

As seen in Figures F.4 - F.7, no remarkable differences could be noticed by adding the reagents. The analyte could still be detected without any interference of the metabolites.

F.6.7 Robustness

The robustness measures the analytical procedure's capacity to remain unaffected by small variations in the method parameters and indicates the ability to produce reproducible results during normal use (Shabir, 2005:11). The variations included changes in the wavelength, injection volume and flow tempo.

F.6.7.1 Preparation of the sample

For the robustness, a sample was prepared as described in Section F.3. The sample was placed into a HPLC vial for analysis.

F.6.7.2 Acceptable criteria

For the criteria to be acceptable, the analyte must remain unaffected by small variations in the method parameters (ICH, 2005:4).

F.6.7.3 Results

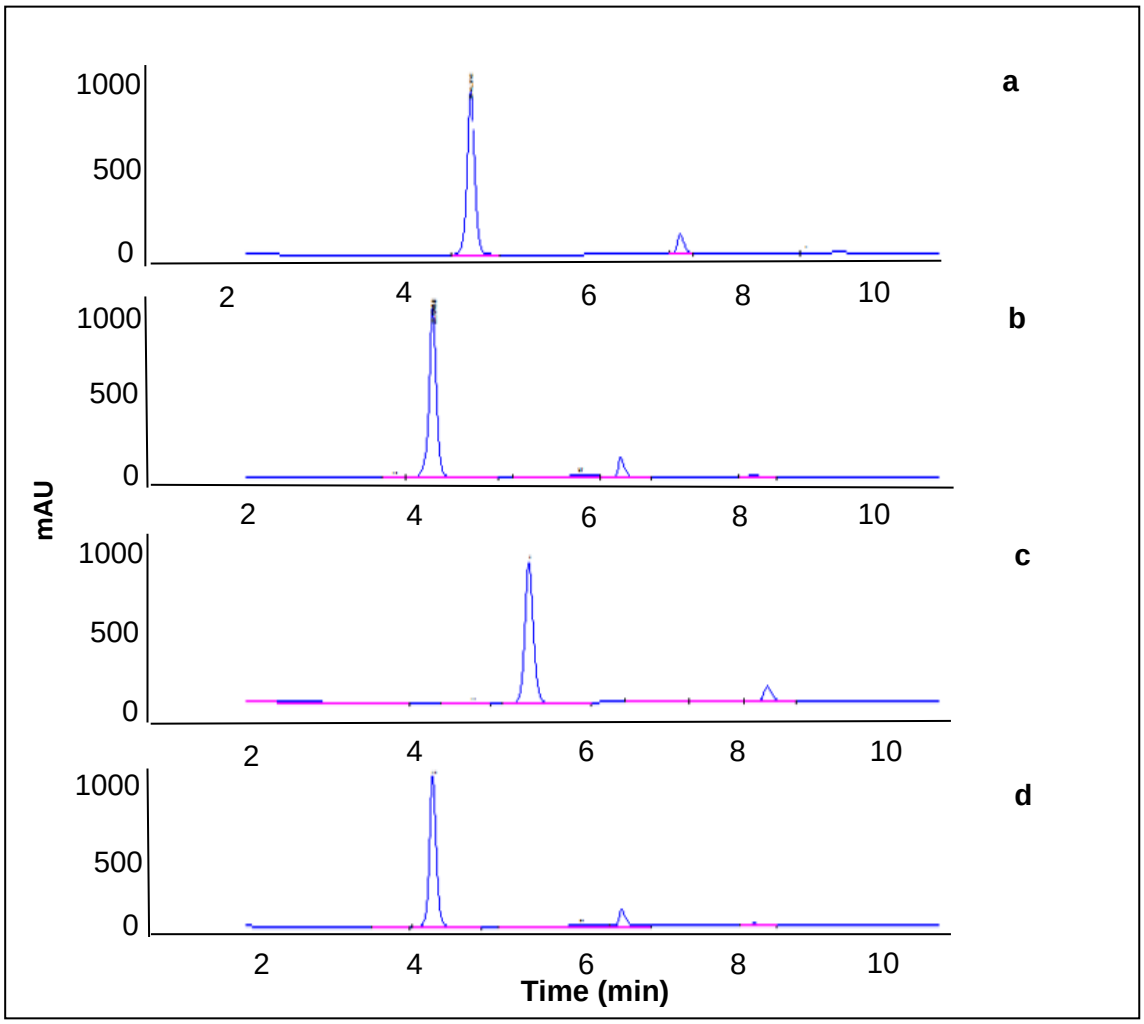

Figure F.8 Chromatogram of niacinamide, methylparaben and propylparaben with small variations in the method parameters: a) standard sample without any changes to method parameters, b) results after increasing the wavelength, injection volume and flow tempo, c) results after decreasing the wavelength, injection volume and flow tempo and d) results after increasing the wavelength and flow tempo and decreasing the injection volume

When comparing chromatograms a, b, c and d (Figure F.8) with each other, the ingredients could still be detected with various changes in the method parameters. For this reason the criteria are acceptable.

F.7 Conclusion

Validation of the HPLC method was performed on niacinamide, methylparaben and propylparaben and found to be acceptable in all the validation parameters. Thus the method may be used to analyse samples for chemical stability assay purposes.

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Appendix G

Stability testing of niosome gel and niosome cream formulations

G.1 Introduction

According to Barel *et al*. (2001:769), stability testing can be defined as the evaluation of a product over a period of time, in order to determine if it stays within the acceptable guidelines. The purpose of this study was to determine whether the stability of the product can stay intact under the influence of environmental factors, including temperature, humidity and light (ICH, 2006:1).

According to Mitsui (1997:191), the first step in stability testing is to determine whether any changes in the physicochemical properties of the formulation occurred, thus both chemical as well as physical changes. Chemical changes can include chemical instability, changes in colour, fragrance and crystallisation, while the physical changes are separation, sedimentation, evaporation and cracking (Barnes 2007:660).

According to Barnes (2007:662), a product must undergo stability testing before it can be marketed, which involves storing a product in cabinets or climate rooms where the temperatures and the humidity are controlled. Samples of the formulations will then be removed at specified time intervals and tested.

In this study, stability tests were performed on the semi-solid formulations, which involved storing the products at different temperatures and humidities for a period of three months. According to the International Conference on Harmonisation (ICH) (2006:3) the product has to be stored at 25 ± 2 °C/60 ± 5% RH (long-term conditions), 30 ± 2 °C/65 ± 5% RH (intermediate conditions) and 40 ± 2 °C/75 ± 5% RH (accelerated conditions).

During the test period the formulations were assessed according to:

- Concentration
- pH and conductivity
- Viscosity
- Light microscopy
- Visual appearance
- Mass loss

- Zeta-potential

The aforementioned tests were performed initially (t=0) as well as after 1, 2 and 3 months in order to determine whether any physicochemical changes occurred.

The ICH (2006:9) defined a significant change as a deviation of more than 5% from the initial value, failure of requirements for appearance and pH and if of the chemical degradation exceeds the acceptance criteria. The deviations were determined as a percentage of the freshly prepared samples (t=0).

G.2 Methods

G.2.1 Concentration of niacinamide, methylparaben and propylparaben

The concentration of niacinamide, methylparaben and propylparaben was determined at predetermined periods by HPLC. The HPLC validation of the semi-solid dosage forms was fully described in Appendix F.

Table G.1: The chromatographic conditions

Analytical instrument	Agilent 1100 series analytical system which is equipped with an Agilent 1100 gradient pump, autosampler injection mechanism and diode array detector was used. It was installed with ChemStation Rev A10.01 data acquisition and analysis software (Agilent Technologies, Palo Alto, CA, USA). The analysis was performed at room temperature (25 °C)
Column	Venusil XBP C_8, 5 µm, 250 x 4.6 mm (Agela Technologies, Newark, DE, USA)
Mobile phase	The mobile phase was used in a gradient. Methanol (A) and 0.075 M octane sulphonic acid (B) buffered to a pH of 3.5 with a few drops of 2 M phosphoric acid (H_3PO_4)
Flow rate	1 ml/min
Injection volume	10 µl
Detection	UV at 212 nm
Retention times	Niacinamide: 3.4 min Methylparaben: 6.9 min Propylparaben: 9.5 min
Stop time	11 min
Solvent	100% Methanol

G.2.1.1 The standard preparation

The standard solution containing 350 µg/ml niacinamide, 20 µg/ml methylparaben and 5 µg/ml propylparaben was prepared as described in Section F.3.

G.2.1.2 The sample preparation

For the sample preparation, 1 g of each dosage form each storage condition was weighed in triplicate. Each sample was dissolved in 100 ml of Milli-Q® water and 100% methanol in a ratio of 20:80. After dissolving all the samples, it was transferred to HPLC vials for analysis.

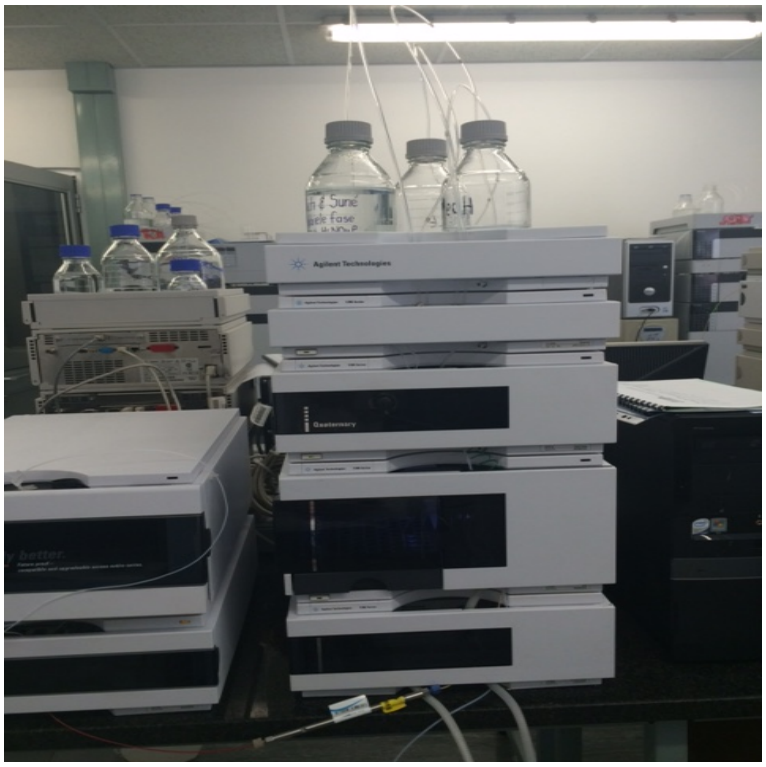

Figure G.1: Agilent 1100 series analytical system

G.2.2 pH and conductivity

The pH was measured using of the Mettler-Toledo pH meter FE20/FG2 (instrument no P16) at room temperature. The pH of each formulation at the different conditions was measured in triplicate.

The conductivity was measured with a GLASS Mettler Toledo In Lab® 731 electrode at room temperature. This was done in triplicate for each formulation at the different storage conditions.

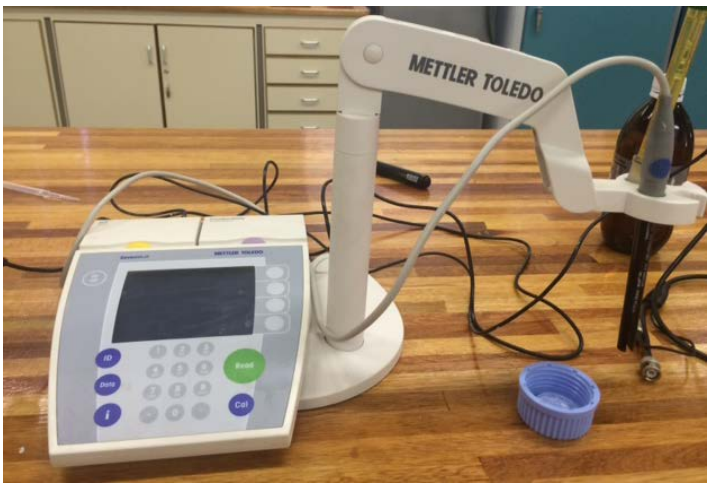

Figure G.2: The Mettler-Toledo pH meter FE20/FG2 (instrument no P16)

Figure G.3: The GLASS Mettler Toledo In Lab® 731 electrode

G.2.3 Viscosity

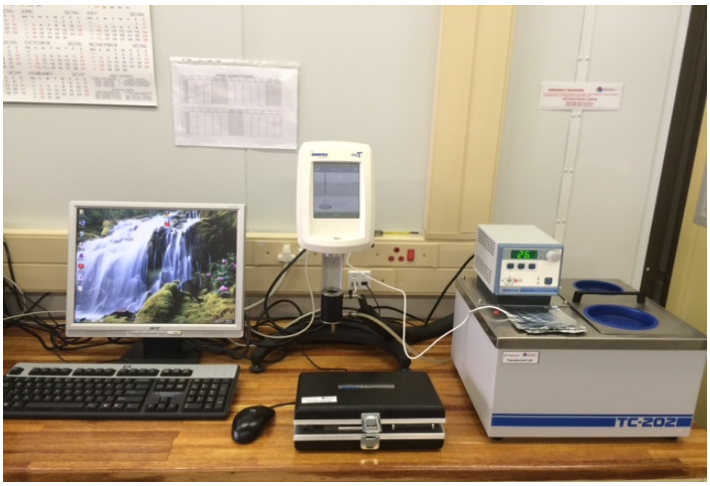

Figure G.4: The Brookfield® Viscometer model DV-III Ultra

The viscosity of each sample was determined using the Brookfield® Viscometer model DV-III Ultra (Middleboro, Massachusetts, USA). At first, the formulations were placed into a water bath to reach a temperature of 25 °C. After the samples reached temperature, 15 ml of each sample was

placed into a chamber. A SC4-25 spindle was placed in the sample and set to rotate at 5 rpm. Multi-point readings were taken every 10 sec for 5 min and an average of 32 readings were gathered.

G.2.4 Light microscopy

Light microscopy was done to determine if any physical changes occurred during the test period. Since the particle size of the niosomes was smaller than 660.20 nm; the influence on the niosomes could not be visualised. A portion of each sample was taken and placed onto a glass microscope slide. The slide was placed onto a Motic microscope (Motic, Hong Kong), equipped with a Moticam 3 camera (Motic, Hong Kong).

Figure G.5: Motic microscope equipped with a Moticam 3 camera

G.2.5 Visual appearance

The visual appearance assessment was performed to determine whether any changes in, separation, sedimentation and cracking occurred (Mitsui 1997; 191). The samples were visually inspected and photos were taken with an iPhone 5 equipped with an 8-megapixile camera.

G.2.6 Mass loss

The mass loss was determined using the Shimadzu (Japan) scale. This test was performed to determine if the formulations had an increase or decrease in mass during the test period. Each sample was weighed in triplicate and the mass was subtracted from the container and determined against initial weight.

Figure G.6: Shimadzu scale

G.2.6 Zeta-potential

The zeta-potential was measured at 25 °C with a Malvern Zetasizer Nano ZS (Malvern instruments, Worcester, UK). This test was done to determine if the different storage conditions had an influence on the zeta-potential of the products. Each sample was diluted (1:20) with Milli-Q® water and placed into a disposable folded capillary cell and measured in triplicate.

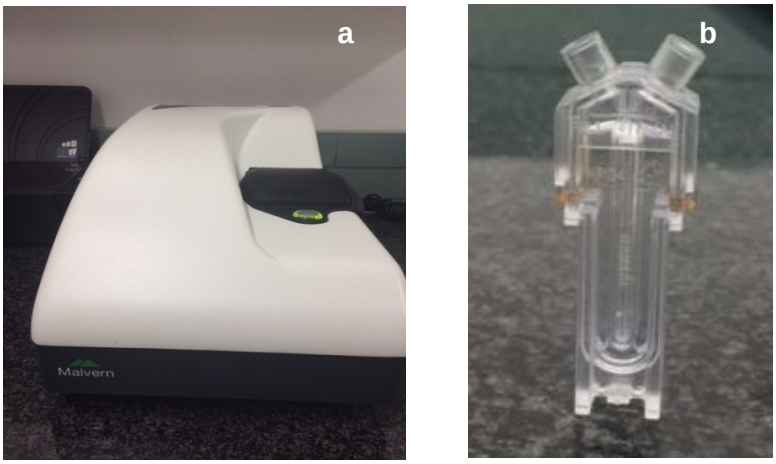

Figure G.7: a) The Malvern Zetasizer Nano and b) the disposable folded capillary cell

G.3 Results and discussion

G.3.1 Concentration assay

The concentration of niacinamide, methylparaben and propylparaben in the niosome gel and niosome cream stored at ± 25 °C/60% RH, ± 30 °C/60% RH and ± 40 °C/75% RH was measured at months 0, 1, 2 and 3. The samples were prepared as described in Section G.2.1.2.

As seen in Tables G.2 to G 7, the formulations were prepared to contain an excess of the ingredients. This was done to increase the shelf life of the product, thus more of the active can be added than declared on the label (Barnes 2007:660). This is called over ageing and is only possible for products where the dose is not critical and the degradation products are not toxic, such as vitamins. According to a USP monograph (2016:6380), a preparation was formulated containing between 95% and 120% of vitamin E, which was still acceptable for use. For this reason the products in this study were formulated containing an excess of the labelled amount.

G.3.1.1 Niosome gel

Table G.2: The concentration of niacinamide in the niosome gel stored at different conditions over a period of three months

	Initial	**Month 1**	**Month 2**	**Month 3**
± 25 °C/60% RH				
Niacinamide (%)	105.15	102.12	103.08	100.95
± 30 °C/60% RH				
Niacinamide (%)	105.15	105.74	104.08	99.56
± 40 °C/75% RH				
Niacinamide (%)	105.15	107.37	103.27	101.28

The concentration of niacinamide in the niosome gel rapidly decreased over a period of three months for the different storage conditions (Table G.2). It was observed that the concentration increased slightly from months 0 to 1 at ± 30 °C/60% RH and ± 40 °C/75% RH; where after a decrease in concentration was depicted from months 1 to 3. The niacinamide had significant break down in the niosome gel stored at ± 30 °C/60% RH. Although the concentration of niacinamide in all the conditions deviated more than 5% from the initial value, the niosome gel still contained between 95 and 120% of the active (USP, 2016:6380), meaning that the niosome gel may still be used for topical effect.

Table G.3: The concentration of methylparaben in the niosome gel stored at different conditions over a period of three months

	Initial	Month 1	Month 2	Month 3
± 25 °C/60% RH				
Methylparaben (%)	111.10	81.65	94.56	83.28
± 30 °C/60% RH				
Methylparaben (%)	111.10	81.53	94.56	76.69
± 40 °C/75% RH				
Methylparaben (%)	111.10	80.25	90.68	51.68

Table G 3 indicates the concentration of methylparaben in the niosome gel. In general, the concentration of methylparaben in the dosage forms stored at the different conditions, displayed an inconsistent pattern throughout the study. The concentration decreased from months 0 to 1; increased from months 1 to 2; then decreased from months 2 to 3. The niosome gel stored at ± 40 °C/75% RH, showed an immense degradation in concentration of methylparaben during the stability test period from the initial month to month 3. Thus, methylparaben stored at ± 40 °C/75% RH, presented with the largest break down during the stability test period. Since the concentration of the methylparaben deviated more than 5% of the initial value, it was not stable during the duration of the test period. A possible reason why the methylparaben were unstable may be since they underwent hydrolysis to form *p*-hydroxybenzoic acid, which consists of little preservative action (Kokoletsi *et al*., 2005:763). Furthermore, the concentration of the active remained stable during the duration of the test period, meaning the methylparaben was effective as a preservative (Haley, 2009:441).

Table G.4: The concentration of propylparaben in the niosome gel stored at different conditions over a period of three months

	Initial	Month 1	Month 2	Month 3
± 25 °C/60% RH				
Propylparaben (%)	108.55	80.77	79.75	78.06
± 30 °C/60% RH				
Propylparaben (%)	108.55	86.25	81.24	107.09
± 40 °C/75% RH				
Propylparaben (%)	108.55	75.45	78.48	71.52

The concentrations of propylparaben (Table G.4), in the niosome gel also presented with an inconsistent pattern during the duration of the test period. In general, the concentration of propylparaben decreased from months 0 to 3, except at ± 40 °C/75% RH; a slight increase was

observed from months 1 to 2 and at ± 30 °C/60% RH; a huge increase was observed from months 2 to 3. The propylparaben concentration stored at ± 40 °C/75% RH had the highest degradation of all storage conditions over the three month period. This inconsistent pattern of the propylparaben may be the appearance of hydrolysis and condensation reactions within the niosome gel (Soni *et al*., 2002:1354). Hydrolysis results in the propylparaben to from *p*-hydroxybenzoic, which have less preservative activity. While the condensation reaction involves the reverse of hydrolysis, resulting in parabens again (Soni *et al*., 2002:1354). Although the propylparaben in the niosome gel deviated by more than 5% from the initial value, it (as preservative) was successful in preventing significant degradation of niacinamide (Haley, 2009:441).

G.3.1.2 Niosome cream

Table G.5: The concentration of niacinamide in the niosome cream stored at different conditions over a period of three months

	Initial	**Month 1**	**Month 2**	**Month 3**
± 25 °C/60% RH				
Niacinamide	103.50	108.04	103.62	104.74
± 30 °C/60% RH				
Niacinamide	103.50	111.48	107.27	105.28
± 40 °C/75% RH				
Niacinamide	103.50	111.36	105.71	103.62

In general, the concentration of niacinamide (Table G.5), in the niosome cream presented with an increase during the stability test period. The concentration of niacinamide in the niosome cream stored at ± 25 °C/60% RH, ± 30 °C/60% RH and ± 40 °C/75% RH increased from months 0 to 1, where after it decreased from months 1 to 2. During months 2 and 3, the concentration of niacinamide stored at ± 25 °C/60% RH increased, but decreased in the formulations stored ± 30 °C/60% RH and ± 40 °C/75% RH. Thus niacinamide stored at ± 30 °C/60% RH, presented with the most deviation. This inconsistent pattern of niacinamide in the niosome cream may be a result of inconsistent mixing during the bulk formulation. Despite the fact that the concentration of niacinamide in all the storage conditions deviated by 5% from the initial value, the concentration in the niosome cream also presented with a concentration between 95 and 120% (USP, 2016:6380) and may therefore be still applied topically.

Table G.6: The concentration of methylparaben in the niosome cream stored at different conditions over a period of three months

	Initial	Month 1	Month 2	Month 3
± 25 °C/60% RH				
Methylparaben	123.71	89.54	99.40	99.92
± 30 °C/60% RH				
Methylparaben	123.71	92.16	102.71	97.89
± 40 °C/75% RH				
Methylparaben	123.71	92.76	99.40	95.65

The concentration of the methylparaben in the niosome cream presented with an inconsistent pattern during the stability tests period. In general, the concentration of methylparaben decreased over the three month test period. In Table G.6, the concentration of the methylparaben stored at ± 25 °C/60% RH, ± 30 °C/60% RH and ± 40 °C/75% RH, decreased from months 0 to 1, but showed an increase from months 1 to 2. From months 2 to 3, the methylparaben concentration decreased for the formulations stored at ± 30 °C/60% RH and ± 40 °C/75% RH and a slight increase in methylparaben concentration was observed for the formulation stored at ± 25 °C/60% RH. Since the concentration of methylparaben deviated by 5% from the initial value; the methylparaben in the niosome cream was unstable during the test period, however, it still prevented the active ingredient from degradation and for this reason it fulfils its purpose (Haley, 2009:441).

Table G.7: The concentration of propylparaben in the niosome cream stored at different conditions over a period of three months

	Initial	Month 1	Month 2	Month 3
± 25 °C/60% RH				
Propylparaben	115.35	81.96	81.71	101.00
± 30 °C/60% RH				
Propylparaben	115.35	84.24	86.15	116.28
± 40 °C/75% RH				
Propylparaben	115.35	85.55	83.03	116.70

Over the three month test period the formulation stored at ± 25 °C/60% RH presented with a decrease in propylparaben concentration, while the formulations stored at ± 30 °C/60% RH and ± 40 °C/75% RH had a slight increase in concentration.. The concentrations of the propylparaben (Table G.7) stored at ± 25 °C/60% RH, ± 30 °C/60% RH and ± 40 °C/75% RH, presented with a decrease in concentration from months 0 to 1, where after

it stayed relatively constant from months 1 to 2. For months 2 to 3, an increase in propylparaben concentration could be observed for all three storage conditions. As seen an inconsistent pattern of the propylparaben may be the result of inconsistent mixing or the presence of hydrolysis and condensation reactions. The propylparaben concentration for all the storage conditions did not deviated by 5% over the three month test period, except for the formulation stored at ± 25 °C/60% RH. As mentioned before, the concentration of niacinamide in the niosome cream was stable; hence, the function of propylparaben was achieved (Haley, 2009:441).

G.3.2 pH and conductivity

G.3.2.1 pH

pH measures the ratio and the concentration of hydrogen ions (H^+) to hydroxide ions (OH^+) of a system (Hach Company, 2010:6). When the pH of a system is 7, the ratio of the [H^+] ions and [OH^+] ions are equal and the system is neutral. Considering pH values lower than 7; the [H^+] ions are greater than the [OH^+] ions, causing the system to be acid. When a system is basic the [OH^+] ions are greater than the [H^+] ions, causing the pH to be higher than 7 (Hach Company, 2010:6).

The pH of the niosome gel and -cream was determined at the different storage conditions of ± 25 °C/60% RH, ± 30 °C/60% RH and ± 40 °C/75% RH on months 0, 1, 2 and 3. The pH was measured in triplicate.

Table G.8: The average pH values of the niosome gel at the different storage conditions

	Initial	**Month 1**	**Month 2**	**Month 3**
± 25 °C/60% RH				
Average pH	6.46	8.30	8.20	7.82
± 30 °C/60% RH				
Average pH	6.46	7.79	7.67	7.53
± 40 °C/75% RH				
Average pH	6.46	8.13	7.91	7.35

The results in Table G.8 indicate that the pH values of the niosome gel increased during the duration of the study. In all three conditions, the pH values increased from months 0 to 1 and then decreased slightly from months 1 to 3.

For the niosome gel formulations stored at ± 25 °C/60% RH, ± 30 °C/60% RH and ± 40 °C/15% RH, the pH increased from months 0 to 3 with 20.97%, 17.09% and 13.71%, respectively. The aforementioned demonstrate that the niosome gel stored at ± 25 °C/60% RH had the most change in pH. As discussed, the pH values of the niosome gel increased during

the test period, which means the initial value of 6.46 represent a formulation containing more [H^+] ions than [OH^+] ions, causing the initial product to be more acid. At the end of the three months period the pH values increased, therefore, the formulation had more [OH^+] ions than [H^+] ions, causing the formulation to be more basic.

Table G.9: The average pH values of the niosome cream at the different storage conditions

	Initial	**Month 1**	**Month 2**	**Month 3**
± 25 °C/60% RH				
Average pH	7.31	6.88	7.00	6.85
± 30 °C/60% RH				
Average pH	7.31	6.88	6.89	6.79
± 40 °C/75% RH				
Average pH	7.31	6.80	6.73	6.70

In general, the pH values of the niosome cream (Table G.9) presented with a decrease during the stability test period for all three storage conditions. The pH values of the niosome cream stored at ± 25°C/60% RH, showed an inconsistent pattern during the duration of the study. Firstly, the pH decreased from the initial month to month 1; where after it increased from months 1 to 2, only to decrease again during the last month. Thus the total decrease in pH values for ± 25°C/60% RH, ± 30°C/±60% RH and ± 40°C/75% RH was 6.31%, 7.16% and 8.35%, respectively. Thus the pH of the niosome cream stored at ± 40 °C/75% RH deviated the most.

When looking at the pH values over the three month period, the pH of the niosome cream formulations decreased at all the storage conditions. The initial value of the niosome cream was 7.31; therefore the formulations at this time were more basic (having more [OH^+] ions than [H^+] ions). After the three months, the pH of the formulations for the different storage conditions varies between 6.70 and 6.85; resulting in more [H^+] ions than [OH^+] ions, causing the formulation to be more acidic.

G.3.2.2 Conductivity

The electrolytic conductivity is the conduction of energy in a solution and is a result of ion movements (Hach Company, 2014:3). Thus the electrical conductivity presents the character of the formulation and indirectly indicates the stability of the system (Ferreira *et al*., 2010:1385).

The conductivity was determined on the niosome gel and niosome cream at the different storage conditions on months 0, 1, 2 and 3. The conductivity was done in triplicate.

Table G.10: The average conductivity (µS/cm) of niosome gel at the different storage conditions

	Initial	Month 1	Month 2	Month 3
± 25 °C/60% RH				
Average conductivity	151.83	489.00	522.66	515.66
± 30 °C/60% RH				
Average conductivity	151.83	404.66	456.66	503.66
± 40 °C/75% RH				
Average conductivity	151.83	498.33	605.33	410.00

The conductivity of the niosome gel had a significant increase over the three month test period. In general, the niosome gel presented with an increase in all the conditions except for the formulation stored at ± 40°C/75% RH. The niosome gel stored at ± 40°C/75% RH presented with an increase in conductivity from the initial up until month 2, where after a decrease appeared from months 2 to 3.

The results in Table G.10 suggest conduction of energy took place in the niosome gel. According to Ferreira *et al.* (2010:1385), conductivity indicates the stability of a product. Since these values deviates more than 5% from the initial value, the niosome gel was unstable according to its conductivity (ICH 2006:9).

Table G.11: The average conductivity (µS/cm) of niosome cream at the different storage conditions

	Initial	Month 1	Month 2	Month 3
± 25 °C/60% RH				
Average conductivity	222.66	245.33	108.73	109.40
± 30 °C/60% RH				
Average conductivity	222.66	187.03	208.33	96.56
± 40 °C/75% RH				
Average conductivity	222.66	59.96	198.86	58.43

The conductivity of the niosome cream stored at the different conditions presented with a decreased over the three month test period. Both the formulations stored at ± 30°C/±60% RH and ± 40°C/75% RH, presented with a decrease in conductivity from months 0 to 1, an increase from months 1 to 2, decreasing again during month 2 to 3. The niosome cream stored at ± 25°C/60% RH, presented with an increase from months 0 to 1, decreasing between months 1 to 2 and increasing again during months 2 and 3.

Consequently, the results in Table G.11 suggests that conduction of energy in the niosome cream also occurred. Although conduction of energy decreased closer to month three, the value still

deviate by more than 5% from the initial value resulting in the niosome cream being unstable according to conductivity.

G.3.3 Viscosity

The viscosity is an important parameter to determine during stability tests of products (Roland *et al.,* 2003:88). This is a rheological measurement and provides the rate of phase separation of a formulation. For this reason it may indicate the stability of a formulation.

The viscosity was determined on the niosome gel and niosome cream on months 0, 1, 2 and 3 as described in Section G.2.3.

Table G.12: The average viscosity (cP) of niosome gel at the different storage conditions

	Initial	**Month 1**	**Month 2**	**Month 3**
± 25 °C/60% RH				
Average viscosity	48 820.67	46 958.75	38 871.09	55 652.81
± 30 °C/60% RH				
Average viscosity	48 820.67	53 121.56	42 194.06	45 585.31
± 40 °C/75% RH				
Average viscosity	48 820.67	36 075.34	30 102.19	28 841.87

The viscosity of the niosome gel had a significant change during the duration of the study. As seen in Table G.12, the viscosity of the formulation stored at ± 25 °C/60% RH decreased from the initial month to month 2; where after it increased again, resulting in a higher viscosity on month 3. The niosome gel stored at ± 30 °C/60% RH had an increase in viscosity from months 0 to 1, where after it decreased from months 1 to 2, to increase again during months 2 and 3. The niosome gel stored in ± 40 °C/75% RH, presented with a decreased during the duration of the test period.

Table G.13: The average viscosity (cP) of niosome cream at the different storage conditions

	Initial	Month 1	Month 2	Month 3
± 25 °C/60% RH				
Average viscosity	149 944.80	234 446.90	97 256.25	85 858.12
± 30 °C/60% RH				
Average viscosity	149 944.80	249 403.10	32 578.75	81 899.62
± 40 °C/75% RH				
Average viscosity	149 944.80	126 165.60	296 390.60	105 606.25

The viscosity of the niosome cream also alternated during the stability test period. As seen in Table G.13, the viscosity of the niosome cream stored at the different storage conditions presented with an inconsistent pattern. The viscosity of the niosome cream stored at ± 25 °C/60% RH and ± 30 °C/60% RH had the same pattern till the end of month 2; both presented with an increase in viscosity from months 0 to 1, and then it decreased from months 1 to 2. During months 2 and 3 the viscosity decreased for the formulation stored at ± 25 °C/60% RH, but increased for the formulation stored at ± 30 °C/60% RH. The niosome cream stored at ± 40 °C/75% RH, presented with a decrease in viscosity from months 0 to 1, where after it increased again from months 1 to 2. From months 2 to 3 the viscosity presented with a decrease again.

As seen in Tables G.12 and G.13, the viscosities of both products had significant change during the duration of the test period. Although the differences in these values are massive, still no changes in the physical appearance could be observed during the test period.

G.3.4 Light microscopy

As described in Section G.2.4, the light microscope was used to determine if any visual changes took place in the niosome gel and niosome cream. Photographs were taken of the samples stored at ± 25 °C/60% RH, ± 30 °C/60% RH and ± 40 °C/75% RH on months 0, 1, 2 and 3. Figures G.8 and G.9 show the changes over a period of three months.

The freshly prepared niosome gel (Figure G.8.a) showed a significant amount air-bubbles. The appearance of the air-bubbles may be an indication that the de-bubbling method during the formulation of the niosome gel was unsuccessful. During the test period less air-bubbles occurred and on month 3, crystals could be observed in the niosome gel stored at ± 40 °C/75% RH (Figure G.8.j). Most likely the crystals observed in the niosome gel could be the active.

In the freshly prepared niosome cream (Figure G.9), small oil droplets could be seen in the formulation at this time. During the duration of the tests period the oil droplets in the emulsion still occurred and no crystals could be observed.

When referring to Figures G.8 and G.9, no outstanding changes could be observed during the duration of the test period and for this reason both formulations were stable according to the light microscope images.

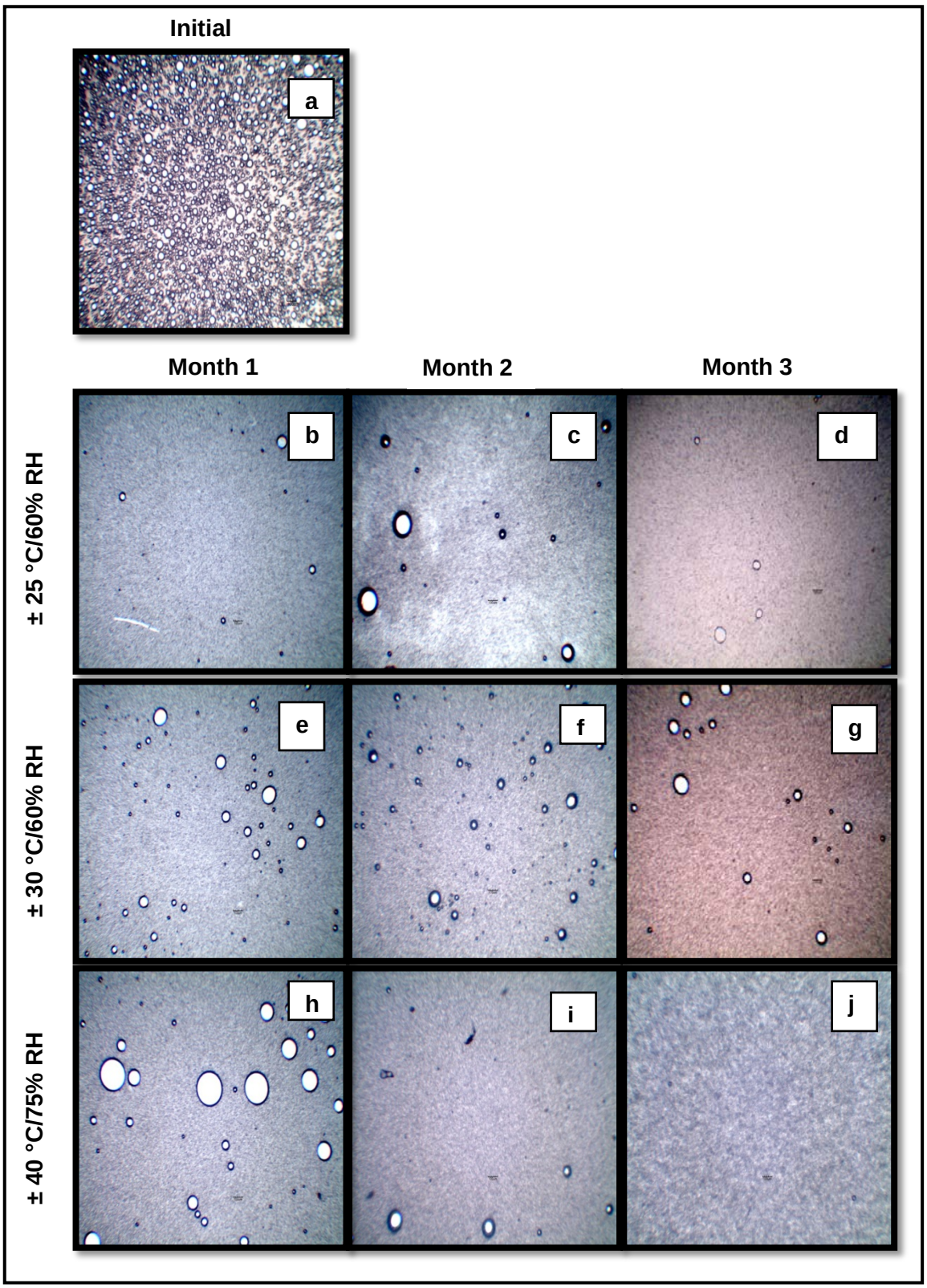

Figure G.8: Light microscope micrographs of the niosome gel over the three month test period

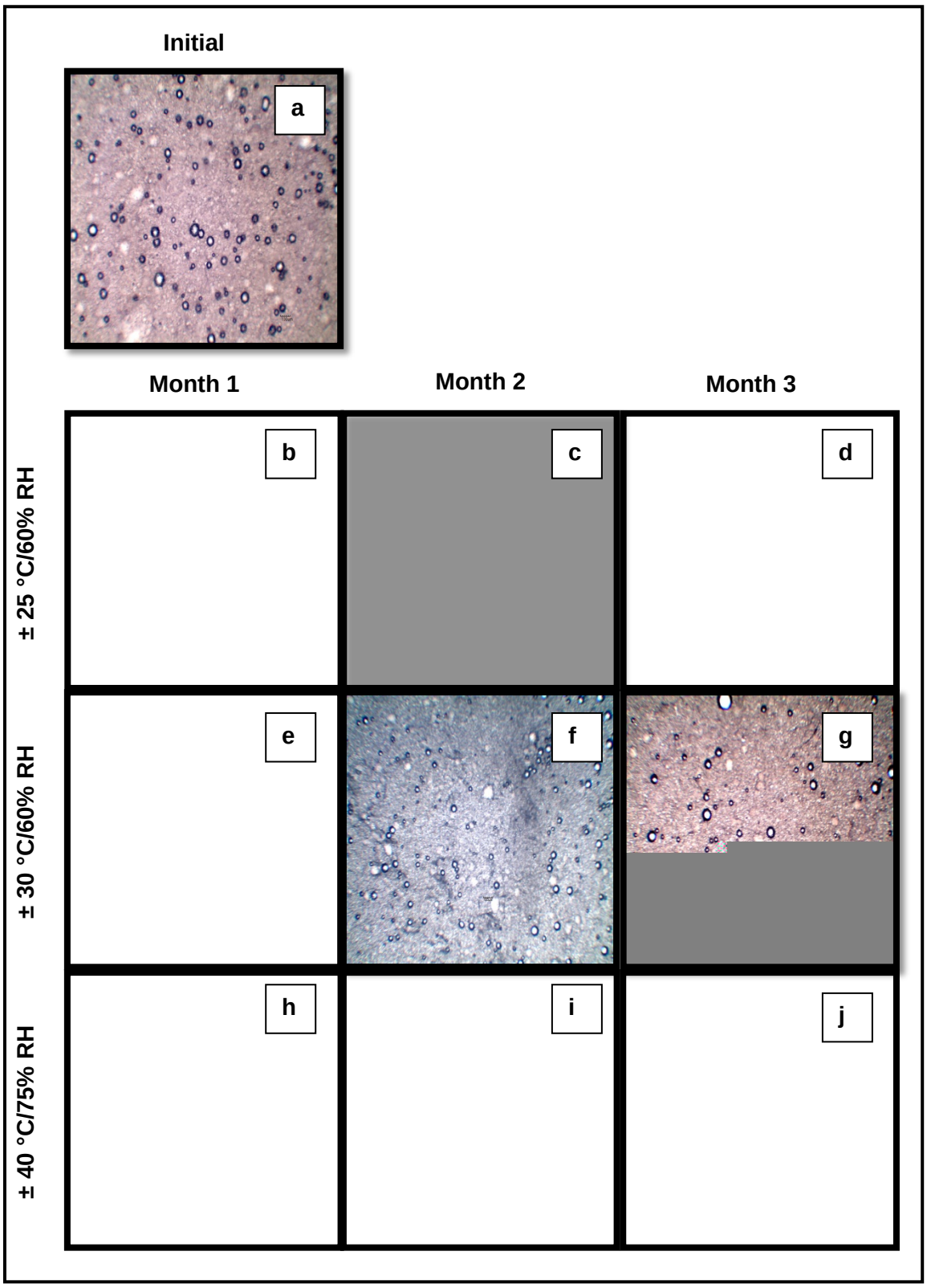

Figure G.9: Light microscope micrographs of the niosome cream over the three month test period

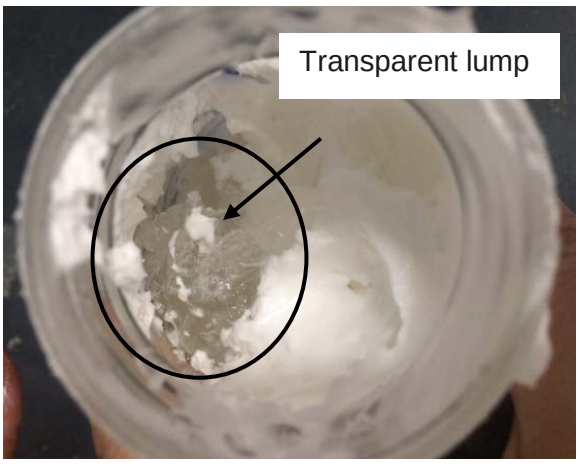

Figure G.11: The transparent lump which occurred in the niosome gel during the test period

The freshly formulated niosome gel had a smooth texture, no unpleasant smell and was white in colour. During the duration of the study, the niosome gel had a few physical changes. Firstly, when comparing the formulations in the different storage conditions over the three month period; it was noticed that air-bubbles formed in the formulations. During this time the volume of the niosome gel also reduced to half the volume of the initial. The air-bubbles that formed together with the volume decrease gives an indication that the de-bubbling method during the preparation of the niosome gel (described in Section D.3.2.1.2) was unsuccessful. Another phenomenon that appeared during the test period was a transparent lump that appeared in the gel (Figure G.11). This phenomenon may be due to not leaving the carbopol to swell completely before the formulation was finalised or, it may be the appearance of phase separation. Furthermore, no colour or fragrance changes could be noticed.

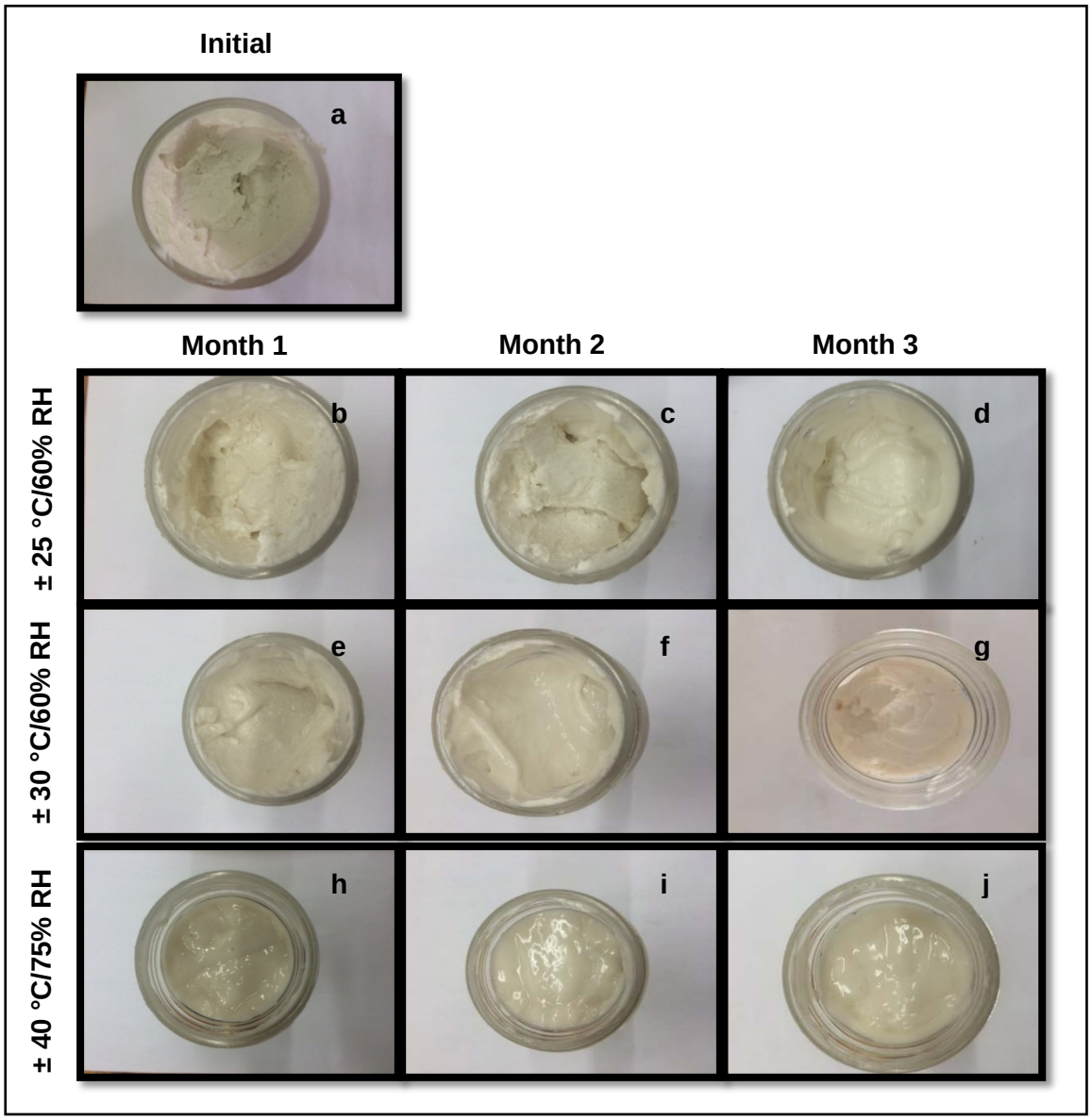

Figure G.12: Photographs of the niosome cream during the duration of the stability test period

The freshly formulated niosome cream did not have an unpleasant smell, was smooth when rubbed between the fingers and an off- white almost beige colour. During the duration of the study no remarkable physical changes in the niosome cream could be observed. The niosome cream stored at ± 40 °C/75%RH, had a more oily appearance when compared to the cream stored at ± 25 °C/60% RH and ± 30 °C/60% RH; no colour or fragrance change could be noticed.

Apart from the small physical changes of the formulations, no significant changes occurred during the duration of the study. Thus, both formulations may be considered as stable according to their physical appearance.

G.3.6 Mass loss

The mass loss of the niosome gel and cream was determined on the initial month and on months 1, 2 and 3. This was done in triplicate on the products stored at ± 25 °C/60% RH, ± 30 °C/60% RH and ± 40 °C/75% RH.

Table G.14: The average mass (g) of niosome gel over a period of three months

	Initial	**Month 1**	**Month 2**	**Month 3**
± 25 °C/60% RH				
Average mass	20.308	20.607	20.578	20.543
± 30 °C/60% RH				
Average mass	20.308	20.574	20.528	20.552
± 40 °C/75% RH				
Average mass	20.308	19.576	19.527	19.513

The mass of the niosome gel (Table G.14) stored at ± 25 °C/60% RH, ± 30 °C/60% RH and ± 40 °C/75% RH, were quite stable during the duration of the study. In general, the mass of the niosome gel stored at ± 25 °C/60% RH and ± 30 °C/60% RH presented with an increase of approximately 1.47% and 1.33%, respectively during the test period. The mass of the niosome gel stored at ± 40 °C/75% RH, decreased with 3.60% over the three month period. The reason for this small alteration in mass may be the release or absorption of moisture from the environment, or as mentioned in Section G.3.5, the transparent lump may have withdrawn moisture from the formulation, swollen more and in this manner increased the mass.

As all the mass stayed within 5% of the initial amount, the criteria are acceptable for the niosome gel. This shows the containers used during this study had the ability to seal the products well enough preventing any evaporation or moisture absorption from the environment.

Table G.15: The average mass of niosome cream over a time period of three months

	Initial	**Month 1**	**Month 2**	**Month 3**
± 25 °C/60% RH				
Average mass	20.678	20.977	18.793	18.583
± 30 °C/60% RH				
Average mass	20.678	20.944	25.321	25.211
± 40 °C/75% RH				
Average mass	20.678	19.944	20.051	19.996

As presented by Table G.15 the mass of the niosome cream stored at ± 25 °C/60% RH and ± 40 °C/75% RH, presented with a decrease, while the niosome cream stored at ± 30 °C/60% RH presented with an increase in mass. During the duration of the study, the niosome cream stored at ± 25 °C/60% RH and at ± 40 °C/75% RH lost approximately 10.11% and 3.26%, respectively. The mass of the niosome cream stored at ± 30 °C/60% RH, increased during the three months period; gaining 22.50% from its initial value.

Since the niosome cream had a deviation of more than 5% during the test period, it leads to the criteria being unacceptable. For this reason the containers used for the cream were insufficient. Despite the fact the same containers were used for both the formulations and exposed to the same conditions; the niosome gel had a smaller variation in mass than the niosome cream. A possible reason why the niosome cream had a significant amount of mass loss/gaining may be since the lids of the containers were not closed properly, causing evaporation or absorption of moisture from the environment.

G.3.7 Zeta-potential

Zeta-potential is a surface property that measures the electric potential on the surface of a particle in a solution. Possessing this property, the zeta-potential gives an indication as to whether the formulation is stable. According to Lamba *et al.* (2015:719), when the zeta-potential of a formulation is greater than - 25 mV, the formulation could be considered stable. This means the Van der Waals attractive forces between the particles are low, resulting in stronger repulsive forces leading to no flocculation of the particles. When the zeta-potential are below - 25 mV, the Van der Waals attractive forces are stronger between the particles, leading to flocculation and aggregation which results in an unstable formulation (Lamba *et al.,* 2015:719).

The zeta-potential of the niosome gel and niosome cream were measured in each storage condition. It was determined in triplicate on the initial month, as well as on months 1, 2 and 3.

Table G.16: The average zeta-potential (mV) of niosome gel over a time of three months at the different storage conditions

	Initial	Month 1	Month 2	Month 3
± 25 °C/60% RH				
Average zeta-potential	-87.03	-67.24	-51.26	-71.80
± 30 °C/60% RH				
Average zeta-potential	-87.03	-69.05	-56.13	-79.73
± 40 °C/75% RH				
Average zeta-potential	-87.03	-67.16	-53.33	-81.13

When considering the zeta-potential values in general, at all the storage conditions the values decreased during months 0 to 2, where after it increased again during months 2 to 3, hence, having a zeta-potential value slightly lower than the initial sample. Although these values deviate by more than 5% of the initial value, the niosome gel stored at ± 25 °C/60% RH, ± 30 °C/60% RH and ± 40 °C/75% RH, may be considered stable. The reason for this might be because all the values presented in Table G.16 were greater than - 25 mV, representing a formulation where the repulsive forces are stronger than the Van der Waals forces, resulting in no flocculation or aggregation of particles in the formulation.

Table G.17: The average zeta-potential (mV) of niosome cream over a time of three months at the different storage conditions

	Initial	Month 1	Month 2	Month 3
± 25 °C/60% RH				
Average zeta-potential	-43.83	-54.04	-51.26	-60.50
± 30 °C/60% RH				
Average zeta-potential	-43.83	-58.32	-56.13	-53.76
± 40 °C/75% RH				
Average zeta-potential	-43.83	-58.46	-53.33	-52.53

The zeta-potential of the niosome cream stored at ± 25 °C/60% RH, ± 30 °C/60% RH and ± 40 °C/75% RH, can also be considered stable during the duration of the stability test period. In general, the zeta-potential had a slight increase during the test period. Despite the fact that these values also deviated by more than 5%; it can still be considered to be stable, since the values presented in Table G17 were greater than - 25 mV, resulting in no flocculation or aggregation of the particles in the formulation.

G.4 Conclusion

In the concentration assay only the parabens were unstable over the three month period and did not remain in the acceptable requirements of the (ICH 2006:3). Although both the methylparabens and propylparabens presented with significant degradation, the degradation of the active were less, meaning both the parabens succeeded in preserving the formulations (Haley, 2009:441).

Although the concentration of niacinamide in both the niosome gel and niosome cream also differed, the products may still be considered as stable. Since the concentration of niacinamide stayed between 95 and 120%, both formulations may still be used. In the data the concentrations of all the ingredients were inconsistent during the test period, which may be the result of inconsistent mixing during the bulk formulation. For the purpose of the niosome gel, the transparent lump as described in Section G.3.5 could have led to water absorption, causing the concentrations of the analytes in the gel to change.

The pH values of both formulations changed over the stability test period. The pH of the niosome gel increased, resulting in a formulation that was more basic at the end of the test period. The pH of the niosome cream decreased during the stability test period causing the cream to be more acidic in accordance to the initial sample. This change in pH reflects that ion exchange in both formulas occurred. Although the pH values of both formulations had deviated more than 5%, both the products had pH values between three and nine meaning no harm would be caused to the stratum corneum and that topical application would still be possible (Barry, 2007:576). Comparing the pH values of the niosome gel and the niosome cream, the niosome cream was more stable. The reason for this is the large amount of water used during the formulation of the niosome gel, causing more ion exchange to take place. Conversely, the niosome cream is an oil in water emulsion, which may affects the ion exchange between the two phases.

The conductivity of the niosome gel increased during the stability test period, which demonstrates water production or hydraulic fracturing in the system. This appearance may be the result of breakage of the polymer hydroxyl group's, which leads to water release (Van der Walt, 2016:81). The niosome cream showed a decrease in conductivity during the duration of the study and the reason should be investigated.

The viscosity of both formulations had significant change over the stability test period. Despite the fact the values differed over the three month period, no change in physical appearance could be noticed. The viscosity of the niosome gel had alternated over the stability test period, which may be due to the water production in the system. According to the results, the niosome cream had deviated less in comparison to the niosome gel. The reason for this phenomenon can be the

lump of carbopol formed in the formulation as described in Section G.3.5. The lump may have caused the deviation in viscosity; since it can absorb or release fluids in to the formulation. Another reason why the viscosity had changed so much, could be due to the conductivity of the system, which changed during the test period. The change in the conductivity indicates that water leakage had occurred in the gel and indirectly influenced the viscosity of the product.

The micrographs of the formulations presented with no significant change during the test period. In the micrographs of the niosome cream oil droplets could be noticed staying unchanged during the test period. In the niosome gel the appearance of crystals become clear, as well as a decrease in air-bubbles. When considering the visual appearance of the formulations, except for the transparent lump formed in the niosome gel and the oily texture of the niosome cream stored at ±40 °C/75% RH, no significant texture, smell or colour changes occurred.

For the mass loss experiment, only the niosome gel stayed within the acceptable criteria of the ICH (ICH 2006:3). The mass of the niosome gel was in the acceptable requirements of the ICH, meaning the packaging during the test period was acceptable (ICH 2006:3). The niosome cream failed to meet the criteria deviating with more than 5% from its initial mass. As explained the packaging of the niosome cream failed; a possible reason may be that the caps of the containers did not close tight enough, resulting in moisture absorption or release into the environment.

The zeta-potential stayed stable during the duration of the stability tests. Both formulations had values higher than - 25 mV throughout the study. This resulted in no flocculation or aggregation in both of the formulations, since the repulsive forces were stronger than the van der Waals attractive forces. For this reason the zeta-potential indicated that the niosome gel and niosome cream were stable.

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Appendix H

Author guidelines: die Pharmazie

H.1 Introduction

The journal **Die Pharmazie** publishes reviews, experimental studies, letters to the editor, as well as book reviews. The following fields of pharmacy are covered: Pharmaceutical and medicinal chemistry, pharmaceutical analysis and drug control, pharmaceutical technology, biopharmacy (biopharmaceutics, pharmacokinetics, biotransformation), experimental and clinical pharmacology, pharmaceutical biology (pharmacognosy), history of pharmacy. Articles are published in English (preferred) or German and are classified as:

- Reviews
- Original articles
- Short communications
- Letters to the Editor

Reviews: A summarizing presentation encompassing the current state of our knowledge and providing comprehensive interpretation with citation of the literature.

Original articles: Publications from all fields mentioned above.

Short communications: Brief publications about the fields mentioned above.

H.2 Conditions

1. For submitted manuscripts, it is the responsibility of the author(s) to demonstrate novelty or a new approach taken in his research. The references should reflect the most recent relevant articles, and the discussion should compare the author's findings with the results of former investigations. For an experimental work, the data have to be determined and classified in a suitable way, problems must be formulated in view of the data, hypotheses should be suggested and/or the author should give possible explanations for any inconsistencies. If possible, the author(s) should perform mathematical or statistical calculations, fit the curves appropriate, and carry out the experiments under controlled conditions. Studies involving animals or human volunteers must include details of ethical approval.

2. Authors are requested to submit all manuscripts online. Paper copy submissions are no longer acceptable. Articles are considered for publication depending on their value and pharmaceutical relevance and with the understanding that they have not been published previously and are submitted exclusively to the journal Die Pharmazie.
3. All manuscripts are subject to experts review. Additional corrections may be done by the editors.
4. A PDF-file of the article is delivered free of charge after the paper has been published in the journal. Please note that, by copyright reasons, this is for personal use of the authors only and *must not* be made available, e.g. by posting on a freely accessible website.
5. Publication charges: Publication fees for publication in Die Pharmazie are 250 € per manuscript (excl. VAT), regardless of type and length. Authors will receive an invoice right after acceptance of their paper which will not be published before the fee has been paid.
6. Delivery of a PDF-file of the article is included in the publication fee. Please note that, by copyright reasons, this is for personal use of the authors only and must not be made available, e.g. by posting on a freely accessible website.
7. The quotation of registered names, trade names, trademarks, etc. in this journal does not imply, even in the absence of a specific statement, that such names are exempt from the relevant laws and regulations and therefore free for general use.

H.3 Preparation of manuscripts

In order to achieve uniform presentation and to avoid unnecessary delays because of further inquires, all authors are requested to observe the following guidelines:

1. Below the title, the surname(s) of the author(s) with initials should be given without academic and professional degrees. The full address of the author for correspondence should appear below author names. Details on the institution where the work was done are requested and should be given above the title.
2. Each manuscript should start with an abstract, containing the most essential results of the study. Extensive review papers and articles for continuous education should be preceded by an outline of topics. Papers should be subdivided into chapters and subchapters according to the decimal system (e.g. 2.1.3.).
3. To achieve clarity and brevity of the presentation, original contributions should be subdivided after the abstract (see 2.) as follows:

3.1. Introduction: This should indicate the question under investigation which is generally based on a brief interpretation of the literature considering the current state of knowledge in the subfield and explaining the necessary theoretical foundations.

3.2. Investigations and results or synthesis of compounds: Methods should only be described generally (see "Experimental"), referring to previous or analogous studies. The presentation of results should be precise, with necessary formulas (numbered in sequence with Arabic numerals), diagrams, tables and figures added separately (together with the legend) to the manuscripts. Numerical values of results should generally be presented either in tables or curves (please mark statistical limits).

3.3. Discussion (unless covered by 3.*2.* As *Investigations, results and discussion*): It should not repeat results already given, but should state the conclusions drawn from the results or provide a theoretical debate and comparison with literature citations.

3.4. Experimental: This part describes briefly the detailed experimental conditions. Unless directions taken from literature have been modified, it suffices to refer to the original source. In the case of well-known inorganic or organic compounds chemical formulae or common abbreviations may be used (e.g. NaCI, H_2SO_4, CH_3OH, C_6H_6: Ac, Eth, Me, Phe, DMSO) under "Experimental". In other parts of the paper this is not desirable. Results of elemental analyses can be omitted if it is stated that all the results were in an acceptable error range.

4. Short communications are published as rapidly as possible. The length of a manuscript is limited to 100 lines (including short summary; subdivisions are not required; the "Experimental" - if there is one - should be marked), up to 15 citations of literature and a maximum of 2 supplementary materials (schemes, figures, tables) are allowed.

5. Only the surnames of authors are given in the text. When there are more than two authors, only the name of the first one is used, followed by *et al*.

6. References in the text have to be cited by author and year, if there are three or more authors, use *et al*. (Miller 1997; Miller and Smith 2000; Miller *et al*. 2001). If the year is the same for several references identify these with a, b, c etc. (Smith 1998a; Smith 1998b etc.) both in the text and in the reference list. At the end of the paper, references are listed in alphabetical order under the first author’s surname. If there are several references to items with the same first author, arrange these chronologically regardless the alphabetical order of the co-authors ("alphabetic-chronological" order). Journal names should be abbreviated according to "Index Medicus" (Medline) or "Chemical Abstracts Service Source Index". Quotations have to follow the following style:

Journal articles:

Lee J (2002) Formulation development of epidermal growth factor. Pharmazie 57: 787 - 790.

Lee EB, Shin KH, Woo WS (1984) Pharmacological study on piperine. Arch Pharm Res 7: 127 - 132.

If each issue of a journal has its own pagination the issue number should be indicated in brackets after the volume number.

Books/Book chapters:

Krishan K, Andersen ME (1994) Physiologically based pharmacokinetic modelling in toxicology. In: Hayes W (ed.) Principles and methods of toxicology, 3rd ed., New York, p. 149 - 187

Only if each issue of a journal has its own pagination the issue number should be indicated in brackets after the volume number.

7. For the identification of pharmaceutical substances, the International Non-proprietary Names (INN) proposed or recommended by the WHO should be used. Registered Trade Marks (usually indicated with R; in an article this sign should only be used when it is first mentioned or used in the summary), trivial names and chemical nomenclature can be added.
8. Nomenclature and spelling should conform to the directions given by IUPAC and IUB.
9. Units of measurement are determined by the directions of the International Units System SI as symbols; M instead of mol/l or mol * l-1 is allowed.
10. Botanical names (species, genus) should be marked in italics.
11. The following abbreviations should be used consequently (except in the title and all subtitles). All other abbreviations have to be explained in the manuscript at first usage, if aforementioned directions are not applicable. Abs. = absolute: anh. = anhydrous; b.p.; b.r. = boiling point, -range; calcd. = calculated; CC = column chromatography; conc. = concentrated; dec. = decomposition, eq. = equation; Fig. = figure; GC = gas chromatography, -chromatogram, HPLC = high performance liquid chromatography, -chromatogram; i.m. = intramuscular; i.p. = intraperitoneal; IR = infrared; i.v. = intravenous; m.p.; m.r. = melting point, -range; MS = mass spectometry, mass spectrum; NMR = nuclear magnetic resonance spectrum; PC = paper chromatography, -chromatogram, % = per cent, percentage, p.o. = peroral; s.c. = subcutaneous; TLC = thin layer chromatography, -chromatogram; UV = ultraviolet.
12. Footnotes must be numbered consecutively and are to be added separately to the manuscript. They are printed following the "Experimental".

13. Dedications (e.g., on the occasion of the 60th or higher birthday) should be inserted between author(s) and summary.
14. Additions to legends of table should be marked by *, **, *** or a, b, c, d, etc.
15. Figures have to be of sufficient quality for reproduction process. Even after size reduction the figures' key has to be easy to read. Manuscripts containing figures of insufficient quality cannot be accepted.