

Formulation and topical delivery of niosomes and proniosomes containing α -lipoic acid

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*I can do all things through Christ who
strengthens me "*

~ Philippians 4:13 ~

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“The dictionary is the only place that success comes before work. Hard work is the price we must pay for success. I think you can accomplish anything if you're willing to pay the price”

~Vince Lombardi~

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~***~

Abstract

α -Lipoic acid, the unique and highly potent antioxidant, is a derivative of octanoic acid that originates endogenously. This compound not only possesses the ability to act as an antioxidant itself by scavenging deleterious reactive species, but also increases the functionalities of other antioxidants, such as vitamins E and C. It is identified as an amphiphilic compound attaining both lipophilic and hydrophilic properties, but is considered to have a higher affinity for lipid environments. Therefore, this compound does not only act against reactive species in the aqueous bloodstream, but also in the lipid compartments of cells as well, deeming this unique compound the universal antioxidant (Biewenga *et al.*, 1997:315; Maczurek *et al.*, 2008:1465; Packer *et al.*, 1995:228). Additionally, this endogenous compound has two active states in which it can present itself, i.e. α -lipoic acid (oxidised state) and dihydrolipoic acid (reduced state), forming a powerful redox couple. This redox couple can regenerate other antioxidants, increase the internal glutathione and coenzyme Q10 levels and ultimately enhance the natural antioxidant defences of the human body (Maczurek *et al.*, 2008:1465; Packer *et al.*, 1995:228; Rochette *et al.*, 2013:116). These antioxidative properties may be potentially useful in the treatment or improvement of the signs of skin ageing. The targeted reactive species cause collagen and elastin degradation by altering gene expression pathways in skin cells (Baumann, 2007:246). However, Masaki (2010:89) stated the topical delivery of antioxidants such as α -lipoic acid might possibly be advantageous if included in the treatment of skin ageing.

Skin ageing is generally defined as the biological phenomenon including two different aspects, namely chronological or intrinsic ageing and photo- or extrinsic ageing. Atypical skin pigmentation, increased laxity, wrinkling and skin sagging are some characteristics of cutaneous aged skin (El-Domyati *et al.*, 2002:398; Jenkins *et al.*, 2002:801; Masaki, 2010:85). Another important ability of α -lipoic acid is the inhibition of certain transcription factors that are considered vital in the natural inflammatory response, plus the attenuation of additional cytotoxic cytokines produced during this inflammatory reaction. These activities performed by α -lipoic acid are attributed to the anti-inflammatory properties of this compound (Lee & Hughes 2002:409; Maczurek *et al.*, 2008:1465); therefore skin inflammatory conditions may be improved by the addition or treatment with antioxidants such as α -lipoic acid. The most commonly known skin condition, induced or influenced by the inflammatory response is psoriasis. Psoriasis affects almost 2 to 3% of the world's population and the possible topical delivery of an antioxidant, such as α -lipoic acid used in this study, may be useful in the treatment of this skin disease (Gelfand *et al.*, 2005:23).

Transdermal delivery is a fast growing researched topic that is fully deserving of the attention it is gaining from scientists. This route of delivery is easily self-administered, is very convenient to use and is of special importance to the cosmetic industry. Advantages of the transdermal route mainly include the painless and prolonged delivery of active ingredients directly to the targeted area, the circumvention of the first-pass metabolism and the non-invasive nature of the administration itself (Brown *et al.*, 2006:177; Liuzzi *et al.*, 2016:295. Shahzad *et al.*, 2015:2).

Although the transdermal delivery route possesses a multitude of advantages, it is not applied widely due to the restricting stratum corneum layer of the skin. This outermost epidermal layer consists of keratinocytes, which are terminally differentiated and known as hydrophilic corneocytes. These flattened corneocytes form a physical barrier with the surrounding lipid medium to keep foreign molecules from penetrating through the skin layers (Kolarsick *et al.*, 2011:205). Aforementioned differences in the solubility of the components are attributed to the barrier function of the skin therefore any potential candidate for topical delivery should possess the necessary solubility properties (Van Smeden *et al.*, 2014:295-296).

According to Mahale *et al.* (2012:47), vesicle systems are formulated for the intended purpose of enhancing the bioavailability of compound and controlling the release of the administered active ingredient. Alexander *et al.* (2012:33) further explained that vesicle systems act as transporters of active ingredients through the skin and also as penetration enhancers. Hence, to deliver α -lipoic acid to the dermal skin layers, niosomal and proniosomal vesicles were formulated during this study to examine their respective characterisation, release and diffusion profiles.

Niosomes are the result of non-ionic amphiphiles assembling themselves to form a closed bilayer structure within an aqueous medium (Sharma *et al.*, 2015:395). For this formation process to initiate a source of energy must be administered, such as an increased temperature or applied mechanical stirring. Niosomal vesicle formation can be done by one of many preparation methods depending on the ingredients' physicochemical properties. This specific vesicle system is considered the preferred choice for cosmetic products due to the fact it causes less skin irritation compared to more conventional vesicle systems (Mahale *et al.*, 2012:47; Sharma *et al.*, 2015:393).

Proniosomes, the dry form of niosomes, has the upper hand on physical stability that makes this type of vesicle system easier to transport and store, and the dosing more accurate (Kumar & Rajeshwarrao, 2011:214). The granular powder is prepared by the slow spray-coating of a water soluble carrier such as sorbitol, which is then hydrated with water prior to administration (Mahale *et al.*, 2012:50-51).

A reliable and accurate high performance liquid chromatography (HPLC) method for the analysis of α -lipoic acid detection was developed and validated. The optimisation and characterisation proved that both dispersions formed large unilamellar vesicles (LUV) for the intended entrapment of α -lipoic acid. Results obtained also revealed acceptable pH and zeta-potential values for both dispersions.

The experimental values determined for the aqueous solubility and octanol-buffer distribution coefficient (log D) of α -lipoic acid was 0.33 mg/ml and - 1.21, respectively. Neither of the determined values indicated favourable permeation through the skin. The membrane release studies revealed desirable amounts of active ingredient released during the 6 h release studies from both vesicle dispersions. Therefore, both the niosomal and hydrated proniosomal dispersions successfully released the α -lipoic acid with an average flux of $467.49 \pm 51.82 \mu\text{g}/\text{cm}^2\cdot\text{h}$ and $332.01 \pm 49.04 \mu\text{g}/\text{cm}^2\cdot\text{h}$, respectively; hence, the average flux value of the niosomal dispersion was slightly higher than that of the hydrated proniosomal dispersion.

Results from the skin diffusion studies confirmed that the niosomal dispersions transported the α -lipoic acid to a better degree compared to the hydrated proniosomal dispersions. Both dispersions delivered the active ingredient transdermally and topically, but to different extents in each individually examined skin layer. Statistical significant differences were identified between the concentrations detected in the epidermis-dermis after the two dispersions were applied to the donor phase. This aforementioned skin layer is the intended target site due to the presence of collagen and melanin, and being the metabolic region in the skin. The average concentrations of active ingredient detected in the epidermis-dermis were $5.077 \pm 1.47 \mu\text{g}/\text{ml}$ for the niosomal dispersion and $2.854 \pm 1.43 \mu\text{g}/\text{ml}$ for the hydrated proniosome dispersion. Keeping the characterisation and diffusion profiles of the separate vesicle systems in mind, the decision was made to execute further clinical efficacy studies on the niosomal dispersion.

After the clinical efficacy studies were conducted on human volunteers, the results obtained were analysed and compared to relative controls. Two studies were done, namely an anti-ageing study that took place over 28 days and an erythema study with a duration of seven days. During the anti-ageing study, α -lipoic acid's effect on skin hydration levels, skin topography (roughness, scaliness, smoothness and wrinkling) and skin elasticity (maximum recovery, elastic recovery, viscous recovery and viscoelastic recovery) were evaluated. The results obtained identified the improvement of several parameters such as hydration level, skin scaliness, maximum recovery, elastic recovery and viscous recovery. Unfortunately, other parameters were negatively influenced by the topical application of a α -lipoic acid formulation, i.e. skin roughness, smoothness, wrinkling and the viscoelasticity of the tested skin areas.

The erythema study was aimed to examine the anti-inflammatory abilities of α -lipoic acid compared to several control groups. The results obtained did in fact identify an anti-inflammatory effect portrayed by the α -lipoic acid formulation. However, the control group showed similar results to that measured from the topical formulation containing α -lipoic acid.

To conclude, both the niosomal and hydrated proniosomal dispersions consisted of vesicles successfully entrapping the α -lipoic acid. The characterisation experiments also indicated the dispersions were stable with acceptable characteristics according to the specific criteria for topically applied substances. The niosomal dispersion delivered the active ingredient more efficiently to the targeted dermal layers and clinical efficacy studies were conducted. Some parameters measured, during the anti-ageing study, improved after treatment with the active test formulation (ATF), whilst other parameters remained the same or even decreased. No statistical significant differences were identified between the ATF and the control group during the anti-inflammatory study. Suboptimal concentrations of α -lipoic acid, compared to the recommended daily dosage, may be an attributing factor to the small therapeutic effects observed during the clinical efficacy studies.

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Wittreksel

α -Lipoësuur, die unieke en hoogs kragtige antioksidant, is 'n derivaat van oktanoësuur wat binne die liggaam self vervaardig word. Hierdie verbinding beskik nie net oor die vermoë om self as 'n antioksidant op te tree nie, maar verhoog ook die funksionaliteit van ander antioksidante soos vitamien E en C. α -Lipoësuur word geïdentifiseer as 'n amfifiliese verbinding wat lipofiliese sowel as hidrofiliese eienskappe besit, maar wat 'n groter affiniteit vir lipied- (vet-) omgewings toon. Daarom kan hierdie verbinding nie net teen reaktiewe spesies in die waterige bloedstroom optree nie, maar ook in die lipiedkompartemente van selle, vandaar die term “universele antioksidant” (Biewenga *et al.*, 1997:315; Maczurek *et al.*, 2008:1465; Packer *et al.*, 1995:228). Daarbenewens het hierdie endogene verbinding twee aktiewe toestande waarin dit kan voorkom, naamlik α -lipoësuur (die geoksideerde toestand) en dihidrolipoësuur (die gereduseerde toestand) wat saam 'n kragtige redokspaar vorm. Hierdie redokspaar kan ander antioksidante regeneer, die interne vlakke van glutatioon en koënsiem Q10 verhoog, en uiteindelik die natuurlike antioksidant-verdedigingsmeganismes van die liggaam verbeter (Maczurek *et al.*, 2008:1465; Packer *et al.*, 1995:228; Rochette *et al.*, 2013:116). Hierdie sterk antioksidierende eienskappe van α -lipoësuur kan potensieel nuttig wees in die behandeling of verbetering van die tekens van velveroudering. Die geteikende reaktiewe spesies veroorsaak dat kollageen en elastien afgebreek word deur die geenuitdrukkingsbane in die vel se selle te wysig (Baumann 2007:246). Masaki (2010:89) sê egter dat die topikale toediening van antioksidante soos α -lipoësuur moontlik as behandeling van verouderende vel voordelig kan wees.

Veroudering van die vel word oor die algemeen gedefinieer as die biologiese verskynsel wat twee verskillende aspekte insluit, naamlik chronologiese of intrinsieke veroudering, en foto- of ekstrasinsieke veroudering. Atipiese velpigmentasie, verhoogde slapheid, plooië en hangende vel is voorbeelde van vel wat verouder (El-Domyati *et al.*, 2002:398; Jenkins *et al.*, 2002:801; Masaki, 2010:85).

Nog 'n belangrike eienskap van α -lipoësuur is die inhiberende effek daarvan op sekere transkripsiefaktore wat noodsaaklik is by die natuurlike inflammatoriese reaksie, asook by die vermindering van addisionele sitotoksiese sitokiene wat tydens hierdie inflammatoriese reaksie geproduseer word. Deur hierdie aktiwiteite in gedagte te hou, kan α -lipoësuur dus gesien word as 'n anti-inflammatoriese verbinding wat moontlik by die behandelingsregime van inflammatoriese veltoestande ingesluit kan word (Lee & Hughes, 2002:409; Maczurek, *et al.*, 2008:1465). Die bekendste veltoestand wat deur inflammatoriese reaksies veroorsaak of

beïnvloed word, is psoriase. Psoriase affekteer byna 2 tot 3% van die wêreld se bevolking en die moontlike topikale toediening van 'n antioksidant soos α -lipoësuur, kan nuttig wees vir die behandeling van hierdie velsiekte (Gelfand *et al.*, 2005:23).

Transdermale toediening is 'n snelgroeiende navorsingsonderwerp wat die aandag wat dit van wetenskaplikes ontvang, ten volle verdien. Hierdie manier van toediening kan maklik self hanteer word, is baie gerieflik om te gebruik, en is van besondere belang vir die kosmetiese bedryf. Verdere voordele van die transdermale toediening is hoofsaaklik die pynlose, nie-indringende aard van die toediening self, die verlengde lewering van aktiewe bestanddele direk aan die teikengebied, asook die vermyding van die eerste deurgangseffek (Brown *et al.*, 2006:177; Liuzzi *et al.*, 2016:295; Shahzad *et al.*, 2015:2).

Hoewel die transdermale toediening 'n magdom voordele inhou, word dit nie noodwendig dikwels gebruik nie, aangesien die stratum corneum-laag van die vel dit beperk. Hierdie buitenste epidermale laag bestaan hoofsaaklik uit keratinosiete wat terminaal gedifferensieerd is en as hidrofiliese korneosiete bekend staan. Hierdie afgeplatte korneosiete vorm 'n fisiese versperring saam met die omliggende lipiedmedium wat verhoed dat indringende of vreemde molekules die vel binnedring (Kolarsick *et al.*, 2011:205). Die beskermende funksie van die vel ten opsigte van hierdie indringende stowwe kan grootliks toegeskryf word aan die oplosbaarheidsverskille tussen die verskeie komponente wat in die vellae self teenwoordig is. Daarom moet 'n potensieële kandidaat vir topikale toediening oor die nodige oplosbaarheidseienskappe beskik sodat die verskeie lae suksesvol gepenetreer kan word (Van Smeden *et al.*, 2014:295-296).

Volgens Mahale *et al.* (2012:47) word vesikelstelsels gevorm sodat die biobeskikbaarheid verbeter kan word, asook om die vrystelling van die aktiewe bestanddeel te beheer. Alexander *et al.* (2012:33) verduidelik verder dat vesikelstelsels as vervoermediums van die aktiewe bestanddele optree en ook penetrasie bevorder. Om α -lipoësuur dus suksesvol by die dermale vellae af te lewer, is niosoom- en proniosoomvesikels tydens die studie gevorm om hulle onderskeie karaktereenskappe, vrystellingsvermoë, asook hulle aflewering in die vellae te bestudeer.

Niosome is die gevolg van die samevoeging van nie-ioniese amfifiele in 'n geslote dubbellaagvesikelstruktuur wat binne 'n waterige medium plaasvind (Sharma *et al.*, 2015:395). Om hierdie samevoegingsproses te laat plaasvind, moet 'n energiebron aangewend word, byvoorbeeld deur die temperatuur te verhoog, of dit meganies te roer. Daar bestaan verskeie metodes om niosoomvesikels te vorm wat afhang van die spesifieke fisies-chemiese eienskappe van die bestanddele wat by die vorming self teenwoordig is. Vir hierdie studie is spesifiek niosoomvesikelstelsels gekies omrede niosome as die verkose draer vir kosmetiese

produkte beskou word. Die rede hiervoor is dat niosoomvesikels minder velirritasie veroorsaak as die meer konvensionele vesikelstelsels soos liposome (Mahale *et al.*, 2012:47; Sharma *et al.*, 2015:393).

Proniosome, die droë vorm van niosome, is fisies baie stabiel met die gevolg dat dit makliker vervoer en gestoor, en akkurate toegedien kan word (Kumar & Rajeshwarrao, 2011: 214). Die korrelrige poeier word voorberei deur die proniosome stadig met 'n wateroplosbare draer soos sorbitol te besproei. Die poeier word dan weer voor toediening met water gehidreer (Mahale *et al.*, 2012:50-51).

'n Akkurate en betroubare hoëverrigting vloeistofchromatografie- (HPLC-) metode vir die ontleding van α -lipoësuur is ontwikkel en geldig verklaar. Die optimalisering en karakterisering van die stelsels het bewys dat beide die niosoom- en gehidreerde proniosoomdispersies groot unilamellare vesikels (LUV) vir die beoogde enkapsulering van α -lipoësuur gevorm het. Die resultate wat verkry is, het getoon dat die pH sowel as die zeta-potensiaalwaardes vir albei dispersies ten opsigte van die geskikte kriteria aanvaarbaar was.

Die eksperimentele waardes wat vir die wateroplosbaarheid en oktanolbuffer-verspreidingskoëffisiënt ($\log D$) van α -lipoësuur bepaal is, was onderskeidelik $0,33 \pm 0,002$ mg/ml en $-1,21$. Albei hierdie waardes was 'n aanduiding van moontlike suboptimale penetrasie van die velle. Resultate verkry vanaf die membraanvrystellingstudies het bewys dat beide vesikeldispersies wenslike hoeveelhede van die aktiewe bestanddeel tydens die ses uur lange studie vrygestel het. Die gemiddelde vloedwaardes van die niosoomdispersie ($467,49 \pm 51,82$ mg/cm².h) asook die gehidreerde proniosoomdispersie ($332,01 \pm 49,04$ mg/cm².h) het die suksesvolle vrystelling van die α -lipoësuur bevestig. Uit hierdie waardes kon gesien word dat die niosoomdispersie se gemiddelde vloedwaardes effens hoër was as dié van die gehidreerde proniosoomdispersie.

Die resultate verkry vanaf die veldiffusiestudies het bevestig dat die niosoomdispersies die α -lipoësuur beter afgelewer het vergeleke met die gehidreerde proniosoomdispersies. Beide het die aktiewe bestanddeel transdermaal asook topikaal afgelewer, maar tot verskillende mates in elke individuele vellaag. Statisties betekenisvolle verskille is geïdentifiseer tussen die konsentrasie van die α -lipoësuur wat in die epidermis-dermis-laag van die vel waargeneem is nadat die twee dispersies in die donorfase toegedien is. Die bogenoemde vellaag is die beoogde teikengebied vir die aflewering van die aktiewe bestanddeel as gevolg van die kollageen- en melanienteenwoordigheid in die laag, asook die hoë metaboliese aktiwiteit in die gebied. Die gemiddelde konsentrasie α -lipoësuur in die epidermis-dermis was $5,077 \pm 1,47$ mg/ml vir die niosoomdispersie, en $2,854 \pm 1,43$ mg/ml vir die gehidreerde proniosoomdispersie. Na aanleiding van die karakteriserings- en verspreidingsprofile van die

afsonderlike vesikelstelsels, is daar besluit om verdere kliniese-effektiwiteitstudies op die niosoomdispersie uit te voer.

Na afloop van die kliniese-effektiwiteitstudies wat op menslike vrywilligers uitgevoer is, is die resultate ontleed en met geskikte kontrolegroepe vergelyk. Twee afsonderlike studies is gedoen, naamlik 'n teenverouderingstudie wat oor 28 dae gestrek het en 'n eriteemstudie wat sewe dae geduur het. Tydens die teenverouderingstudie is verskeie parameters geëvalueer, naamlik hidrasievlakke, veltopografie (grofheid, skubberigheid, gladheid en plooivorming) asook die elastisiteit van die vel (maksimum herstel, herstel van rekking, viskeuse herstel en visko-elastiese herstel). Die effek van topikale behandeling met α -lipoësuur het gelei tot die verbetering van sommige van die bogenoemde parameters, soos hidrasievlak, skubberigheid, maksimum herstel, herstel van rekking, en viskeuse herstel. Ongelukkig is ander parameters deur die topikale toediening van die α -lipoësuurdispersie onveranderd gelaat of negatief beïnvloed. Hierdie parameters sluit in velgrofheid, velgladheid, voorkoms van plooië, en die viskeuse elastisiteitseienskappe van die verskeie veloppervlakke wat getoets is.

Die doel van die eriteemstudie was om die anti-inflammatoriese eienskappe van α -lipoësuur te ondersoek deur dit met verskeie geskikte kontrolegroepe te vergelyk. Die resultate wat verkry is, het wel 'n anti-inflammatoriese effek vir die niosoomdispersie geïdentifiseer, maar ongelukkig is soortgelyke resultate ook deur die kontrolegroepe gelever. Die anti-inflammatoriese aksie kan dus nie aan die teenwoordigheid α -lipoësuur toegeskryf word nie.

Ten slotte, beide die niosoom- asook die gehidreerde proniosoomdispersies het vesikels bevat wat α -lipoësuur suksesvol geënkapsuleer het. Die karakteriseringseksperimente het ook bewys dat albei dispersies stabiel was en aan die spesifieke kriteria vir topikale toediening van aktiewe stowwe voldoen het. Die niosoomdispersie het tydens die veldiffusiëstudies beter aflewering van α -lipoësuur na die teikengebied getoon, en is dus as die geskikste vesikelstelsel vir die verdere kliniese-effektiwiteitstudies aanvaar. Sommige van die parameters wat tydens die teenverouderingstudie geëvalueer is, het na behandeling met die aktiewe toetsformule (ATF) verbeter, terwyl verskeie ander parameters dieselfde gebly of selfs verswak het. Verder is daar tydens die anti-inflammatoriese studie geen statisties betekenisvolle verskille tussen die toetsgroep en die kontrolegroepe geïdentifiseer nie. Suboptimale konsentrasies van α -lipoësuur, vergeleke met die aanbevole daaglikse dosis daarvan, kan 'n moontlike verklaring bied vir die klein terapeutiese effek wat tydens die kliniese-effektiwiteitstudies waargeneem is.

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$J = (K.D)/\Delta C$ Equation 2.1

CHAPTER 3: ARTICLE FOR PUBLICATION IN THE INTERNATIONAL JOURNAL OF PHARMACEUTICS: FORMULATION AND TOPICAL DELIVERY OF NIOSOMES AND PRONIOSOMES CONTAINING A-LIPOIC ACID

$\log D = \text{Concentration a} / \text{Concentration b}$ Equation 1

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CHAPTER 4: ARTICLE FOR PUBLICATION IN SKIN PHARMACOLOGY AND PHYSIOLOGY: CLINICAL EFFICACY OF TOPICAL FORMULATIONS CONTAINING A-LIPOIC ACID IN THE TREATMENT OF SKIN AGEING AND SKIN INFLAMMATION

$\text{Percentage change (\%)} = (T_x - T_0)/T_0 \times 100$ Equation 1

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$Y = mx + c$ Equation A.1

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

APPENDIX D: MEMBRANE RELEASE AND SKIN DIFFUSION STUDIES

$\log D = \text{Concentration a} / \text{Concentration b}$ Equation D.1

APPENDIX E: CLINICAL EFFICACY OF TOPICAL FORMULATIONS CONTAINING A-LIPOIC ACID

$\text{Percentage change (\%)} = (T_x - T_0)/T_0 \times 100$ Equation E.1

List of Abbreviations

ACN	-	Acetonitrile
Alpha-KGDH	-	Alpha-ketoglutarate dehydrogenase
ANOVA	-	Analysis of variance
APVMA	-	Australian Pesticides & Veterinary Medicines Authority
ATF	-	Active test formulation
ATL	-	Analytical Technology Laboratory
AU	-	Arbitrary units
BP	-	British Pharmacopeia
CEL	-	Cosmetic Efficacy Laboratory
dH ₂ O	-	Distilled water
DNA	-	Deoxyribonucleic acid
EE	-	Entrapment efficacy
EEMCO	-	European Group for Efficacy Measurements on Cosmetics and Other Topical Products
GCP	-	Good Clinical Practice
HCl	-	Hydrogen chloride
HPLC	-	High performance liquid chromatography
H ₂ O ₂	-	Hydrogen peroxide
H ₃ PO ₄	-	Phosphoric acid
ICH	-	International Conference of Harmonisation
IL	-	Interleukin

KH ₂ PO ₄	-	Monopotassium phosphate
LB	-	Lamellar bodies
LOD	-	Limit of detection
Log D	-	Octanol-buffer distribution coefficient
LOQ	-	Limit of quantitation
LUV	-	Large unilamellar vesicles
MeOH	-	Methanol
MLV	-	Multilamellar vesicles
NaOH	-	Sodium hydroxide
NF κB	-	Nuclear factor kappa B
NMF	-	Natural moisturizing factor
NRF	-	National Research Foundation
NWU	-	North-West University
OECD	-	Organization for Economic Co-operation and Development
PBS	-	Phosphate buffer solution (pH 7.4)
PDH	-	Pyruvate dehydrogenase
PdI	-	Polydispersity index
PVDF	-	Polyvinylidene fluoride membrane
Q0	-	Maximum recovery area
Q1	-	Elastic recovery
Q2	-	Viscous recovery
Q3	-	Viscoelastic recovery
RH	-	Relative humidity

ROS	-	Reactive oxygen species
RSD	-	Standard deviation
SD	-	Standard deviation
SELS	-	Surface evaluation of living skin
SEsc	-	Skin scaliness
SEr	-	Skin roughness
SEsm	-	Skin smoothness
SEw	-	Skin wrinkling
SLS	-	Sodium lauryl sulphate
SUV	-	Small unilamellar vesicles
TEM	-	Transelectron microscopy
TEWL	-	Trans epidermal water loss
USP	-	United States Pharmacopoeia
UVR	-	Ultraviolet radiation
WHO	-	World Health Organization
WMA	-	World Medical Association

Chapter 1

INTRODUCTION, PROBLEM STATEMENT

AIMS AND OBJECTIVES

The skin is a fascinating organ that consists of three distinct layers each containing different compilations of dermal cells. Conjointly, the skin plays a vital role in our survival due to the important functions it preforms on a daily basis without us even noticing. These functions mainly include chemical and physical protection, with additional thermoregulatory, sensory and endocrine functions. The skin also plays an eminent part in the reproduction process, not to mention its significance in non-vocal communications (Menon, 2002:S4). The main function mentioned, protection, is an intriguing topic for transdermal and topical scientists considering its ultimate limiting effect on drug delivery.

In the past few years, the route of topical drug delivery has been receiving much needed attention from scientists and formulators, especially the field of cosmeceuticals. This is because it has palpable advantages to other routes of administration, particularly its non-invasive feature, direct application on the target site, and the fact that it can easily be self-administered (Prausnitz & Langer, 2008:1261). Another benefit of using this route is the circumvention of the first-pass metabolism in the liver; this amelioration is regarded to be one of the most notable advantages of transdermal drug delivery (Savoji *et al.*, 2014:1). In spite of all these benefits there is only a subset of drugs that make the shortlist for passing through the skin's various layers (Wiedersberg & Guy, 2014:153).

To enhance the efficacy of the topical route, lipid vesicles were formulated to encapsulate the active ingredient and deliver it directly to the targeted site (dermis). Different lipid ingredients and methods of preparation can be used to formulate the desired carrier (Pierre & Costa, 2011:608). Niosomes are formulated as an alternative to the commonly known liposomes, which have better chemical stability and ultimately increases the efficacy of these delivery systems. In addition, niosomes can also act as penetration enhancers due to their flexible nature and by directly influencing the integrity of the stratum corneum (Pierre & Costa, 2011:614). However, physical stability is the main concern with these non-ionic vesicles and

problems such as fusion, leaking and aggregation arise (Hu & Rhodes, 1999:24), hence proniosomes were formulated. Proniosomes are the dry, powdered form of niosomes that aim

to increase the stability and ease the transportation of these vesicles (Pierre & Costa, 2011:615). Therefore, niosomes and proniosomes will be prepared during this study.

The stratum corneum is the layer to overcome for feasible cosmeceutical actives to permeate the skin successfully, due to its sophisticated composition. Therefore, this hydrophobic layer is the ultimate rate-limiting barrier in the diffusion pathway of molecules crossing the skin. Today, it is still the classic simplification of the brick-and-mortar model that we understand best when trying to grasp the detail behind the stratum corneum's composition (McLafferty *et al.*, 2012:37; Menon, 2002:S9; Trommer & Neubert, 2006:106). Owing to the complex, stable and multifunctional structure of the human skin, it remains a challenge to transport active ingredients successfully through or into the skin, which is what this study aims to achieve.

Oxidative stress, caused by an imbalance between pro-oxidant activity and the counteracting anti-oxidant activity in favour of the oxidants, is a major contributor to skin diseases and skin ageing (Kadam *et al.*, 2010:388; Sies, 1997:291; Sohal & Weindruch, 1996:59). Reactive oxygen species (ROS) are naturally generated by-products from aerobic metabolism, but can cause oxidative injury to cells if produced in elevated levels due to increased endogenous and exogenous stressors (Brieger, 2012:1; Sies, 1997:291). Since the skin is frequently exposed to environmental stressors, such as ultraviolet radiation (UVR), it is considered a potential target for these deleterious reactive metabolites (Kadam *et al.*, 2010:388; Kohen, 1999:181; Zhou *et al.*, 2009:891).

The process of skin ageing, both intrinsic and extrinsic, is greatly affected by oxidative stress (Baumann, 2007:241; Binic *et al.*, 2013:2; Rinnerthaler *et al.*, 2015:545). ROS accumulated over time from cell metabolism is the main contributor to cell-senescence and loss of functionality in intrinsic ageing. This natural chronological ageing process clinically manifests as wrinkles, laxity, slackness, xerosis (dry skin) and an array of benign neoplasm appearances (Binic *et al.*, 2013:1). UVR accelerates the ageing process and is the main culprit in extrinsic ageing. Extrinsic ageing, or photo-ageing, presents with hyperpigmentation, freckles, xerosis, inelasticity and areas of purpura due to blood vessels becoming weak (Baumann, 2007:242; Binic *et al.*, 2013:2).

High levels of ROS are also regarded as a contributor to the pathogenesis of various inflammatory skin diseases, such as the hyper-proliferative skin disorder, psoriasis (Kadam *et al.*, 2010:388; Zhou *et al.*, 2009:892). This chronic inflammatory skin disease affects about 2 to 3% of the world's population and is characterised by the scaly, red and raised plaques that are present particularly on the scalp and extensor prominences, but can appear on any part of the body (Kadam *et al.*, 2010:388; Zhou *et al.*, 2009:892).

It is evident that ROS generated oxidative stress can cause a great deal of damage to the skin cells. As mentioned before, the skin has its own internal oxidative stress-eliminating systems that can act against internal ROS levels by using enzymes and low molecule antioxidants (Kammeyer & Luiten, 2015:23; Kohen, 1999:189; Masaki, 2010:89).

By definition, an antioxidant is any substance that when present at low concentrations, compared to those of an oxidisable substrate, significantly delays or prevents oxidation of that substrate (Halliwell, 1995:1341). Therefore, antioxidants act as reducing agents that disarm ROS, but do not completely remove all oxidants due to their respective biological functionality (Kammeyer & Luiten, 2015:23). Despite the production of endogenous antioxidants aimed at maintaining the oxygen homeostasis, some important antioxidants that are required must be supplemented by dietary nutrition or direct topical application (Lademann *et al.*, 2016:42).

α -Lipoic acid, also known 6,8-thioctic acid or 1,2-dithiolane-3-pentanoic acid, is a multifunctional antioxidant derived from octanoic acid (Billgren *et al.*, 2010:181; Moini *et al.*, 2002:85; Dos Santos Pereira *et al.*, 2016:249). Due to its possible biosynthesis in the body, it is not considered a vitamin but can still be supplemented daily like vitamins because of its diverse applications in the body (Bast & Haenen, 2002:230; Packer *et al.*, 1995:227). Not only does α -lipoic acid individually act as an antioxidant that protects against oxidative damage, it also recycles oxidised antioxidants, i.e. vitamin C, vitamin B and glutathione. The ability of α -lipoic acid to recycle other antioxidants comes from the redox couple formed between α -lipoic acid and its reduced form, dihydrolipoic acid, which enables this compound to regenerate used-up antioxidants, hence the term “universal antioxidant” (Biewenga *et al.*, 1997:315; Packer *et al.*, 1995:228; Rochette *et al.*, 2013:116).

α -Lipoic acid is commonly known for its contribution to the treatment of liver diseases, such as cirrhosis, alcohol intoxication, hepatitis, mushroom poisoning and hepatic coma (Bustamante *et al.*, 1998:1023), however, it is mainly acknowledged for being part of the treatment regime for diabetic patients. Some of the symptoms for which it is useful are peripheral neuropathy, autonomic neuropathy, general pain, numbness, tingling, to name but a few (Mitchell, 2007). Type-1 diabetes, caused by the destruction of pancreatic β -cells due to an inflammatory or immunological response, as well as type-2 diabetes can be treated with α -lipoic acid (Packer *et al.*, 1995:238). A syndrome closely linked to type-2 diabetes, metabolic syndrome, can also be treated with α -lipoic acid for numerous reasons (Mitchell, 2007).

Several other conditions are treated with additional α -lipoic acid supplementation, such as glaucoma, cataracts, burning mouth syndrome, conditions involving the degradation of bone density, brain damage in patients after suffering a stroke and metal toxicity. It is also useful in

migraine management by reducing the intensity and frequency of migraine attacks (Mitchell, 2007). α -Lipoic acid is an amphipathic compound making all these diverse applications understandable (Moini *et al.*, 2002:84). It can quench free radicals in both aqueous and lipid environments, meaning it is hydrophilic enough to operate in the aqueous demeanour of the blood plasma, but it is also lipophilic enough to cross the blood brain barrier and reach the brain tissue (Packer *et al.*, 1995:228).

This potent and versatile antioxidant will be used as the active ingredient in this study because it has a broad spectrum of therapeutic applications particularly that of cosmetic products thanks to its acclaimed anti-ageing and anti-inflammatory properties. α -Lipoic acid is claimed to be useful in the treatment of skin ageing and inflammatory skin conditions, such as psoriasis.

The research problem can be attributed to the unique barrier function of the human skin to external influences, due to the complexity of the distinctive layer compositions (Menon, 2002:S4). The solubility differences of these layers, i.e. the lipophilic stratum corneum and the preceding hydrophilic layers, are responsible for withholding molecules (Naik *et al.*, 2000:318). α -Lipoic acid is an amphipathic molecule that is considered more lipophilic and would therefore possibly accumulate in the stratum corneum (Mioni *et al.*, 2002:84). In order to deliver the active ingredient to the dermis and ultimately increase its concentration at the target site, niosomal and proniosomal vesicles will be formulated during this study. Vesicular systems are capable of storing both hydrophilic and amphiphilic substances in their aqueous phase, as well as lipid soluble substances in the vesicle's lipid bilayers (Pierre & Costa, 2011:609). The niosome dispersions will be compared to the more stable dry form, i.e. the proniosomes. The vesicle dispersion with the most satisfactory characteristics will then be tested during clinical efficiency experiments to determine if the formulation containing α -lipoic acid did in fact improve the skins' typography and promote anti-inflammatory processes. By succeeding in delivering the α -lipoic acid to the viable epidermis and dermis, this dispersion could be administered topically for the possible treatment of signs of skin ageing and inflammatory skin conditions, such as psoriasis as mentioned before.

The aim of this study is to formulate two types of vesicle dispersions for the topical delivery of α -lipoic acid. The vesicles that will be formulated are niosomes and proniosomes, to transport the active to the targeted layer of the skin, specifically the dermis. These vesicle dispersions will then be subjected to several studies to determine their characteristics, membrane release and skin diffusion potential, clinical efficacy and possible dermal toxicity. Therefore, this study aims to formulate a vesicle dispersion containing α -lipoic acid that can potentially permeate the skin and is effective against signs of skin ageing and inflammatory processes in the skin.

The objectives of this study include:

- The development and validation of a HPLC method to quantitatively determine the concentrations α -lipoic acid in both vesicle systems.
- The determination of α -lipoic acid's aqueous solubility and octanol-buffer distribution coefficient (log D) values
- The formulation of niosomal and hydrated proniosomal dispersions for topical delivery containing α -lipoic acid.
- The characterisation of both the niosomal and hydrated proniosomal dispersions in terms of formation, droplet size, entrapment efficacy and zeta-potential.
- Conducting membrane release studies on both dispersions to determine the release of α -lipoic acid from each vesicle system.
- The transdermal delivery of the active ingredient in terms of skin diffusion studies.
- Performing the tape stripping method to determine the concentration of α -lipoic acid delivered to the dermal target layers by each dispersion.
- Conducting clinical efficacy studies to evaluate the effectiveness and possible skin reactions to the best suited vesicle system containing the active ingredient compared to placebo formulations.

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Chapter 2

TOPICAL DELIVERY OF α -LIPOIC ACID FOR THE POTENTIAL TREATMENT OF AGEING AND INFLAMMATORY SKIN DISEASES

2.1 α -Lipoic acid

The octanoic acid derivative, α -lipoic acid (6,8-thioctic acid or 1,2-dithiolane-3-pentanoic acid) is a natural occurring coenzyme that plays a vital role in metabolic processes concerning macronutrients in the body (Billgren *et al.*, 2010:181; Dos Santos Pereira *et al.*, 2016:249; Moini *et al.*, 2002:85;). This endogenous compound is known for its unique ability to accumulate in both aqueous and lipid environments, resulting in antioxidant activity in any tissue or cell type. Additionally, α -lipoic acid is active in both its oxidised and reduced states (lipoic and dihydrolipoic acid) and can therefore perform therapeutic actions in either (Figure 2.1). These aforementioned characteristics are unlike any other antioxidant, giving rise to the term generally used to describe this compound, i.e. universal antioxidant. When administered externally, α -lipoic acid is first reduced to dihydrolipoic acid in most cells thereafter the redox couple performs several therapeutic actions in the body. α -Lipoic acid can directly scavenge free radicals that cause oxidative damage to cells and can, indirectly, facilitate an increase in the functionality of other antioxidants. Conjointly, this redox couple can also regenerate some endogenous antioxidants, such as vitamin C and E, and cause an increase the levels of glutathione and coenzyme Q10 ultimately leading to overall ameliorated defences against oxidative stress (Biewenga *et al.*, 1997:315; Maczurek *et al.*, 2008:1465; Packer *et al.*, 1995:228; Rochette *et al.*, 2013:116; Tack, 2016).

Additionally, α -lipoic acid also attains metal chelating abilities that are used for arsenic and mercury poisoning and liver diseases induced by excessive cadmium levels in the body (Bustamante *et al.*, 1998:1023; Maczurek *et al.*, 2008:1465). This happens through the formation of complexes (chelates) between α -lipoic acid and the specific metal ions that ultimately lead to the reduction of the metallic elements in the body. Another important function of α -lipoic acid is its role in energy metabolism. Mitochondrial enzymes such as pyruvate dehydrogenase (PDH) and alpha-ketoglutarate dehydrogenase (alpha-KGDH) are recharged by α -lipoic acid and their availability to produce energy is therefore increased.

Interestingly, this derivative of octanoic acid was also found to be an affective stimulator for the uptake of glucose by muscles and was even described as being comparable with insulin; it is for

this reason that α -lipoic acid is included in the treatment regimens for conditions such as hyperinsulinaemia, insulin resistance and the metabolic syndrome (Maczurek *et al.*, 2008:1466; Packer *et al.*, 1995:238).

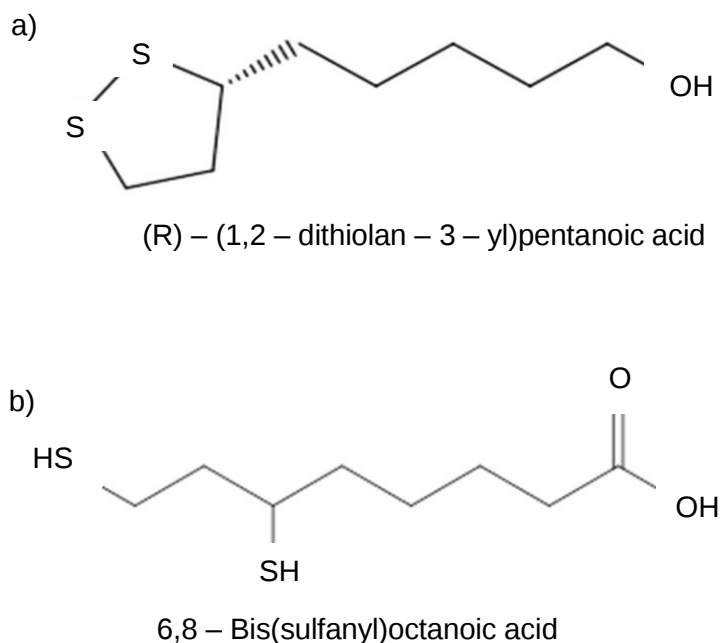


Figure 2.1: a) oxidised and b) reduced forms of α -lipoic acid

According to Lee and Hughes (2002:409), α -lipoic acid possesses the ability to inhibit transcription factors, such as NF- κ B (nuclear factor kappa B), in the inflammatory system, which is responsible for its anti-inflammatory action. In addition to this, the down-regulating effect of this antioxidant on the inflammatory transduction processes also leads to the attenuation of further free radical and cytotoxic cytokine production (Maczurek, *et al.*, 2008:1465). This intriguing compound exhibits anti-inflammatory effects on both chronic and acute inflammatory conditions and is therefore a potentially useful compound in the treatment of several inflammatory skin conditions (Beitner, 2003:847; Lee & Hughes, 2002:409; Odabasoglu *et al.*, 2011:31).

Considering these significant antioxidant, amphiphilic, anti-inflammatory, metal chelating and energy production functions, it is easy to understand why this compound is deemed the universal antioxidant.

2.2 Structure and barrier function of the skin

The human skin is the largest organ in the body's integumentary system and performs many functions vital for our daily survival. The skin's three main functions are protection, sensation and regulation (Kolarsick *et al.*, 2011:203).

2.2.1 Skin anatomy and physiology

Traditionally the skin consists of three layers, which collectively serves as the powerful barrier protecting us from external commodities, accommodates internal processes, such as immunological responses, and plays a vital role in thermoregulation (Kolarsick *et al.*, 2011:203).

The deepest layer, known as the hypodermis (subcutaneous fat layer) mainly consists of fat cells (adipose tissue), macrophages and fibroblasts. It occupies connective fibres of elastin and collagen to bind this innermost layer to the preceding dermis. The hypodermis also links neural and vascular systems in the skin itself and connects these systems to the subjacent muscle (Escobar-Chávez *et al.*, 2012:205; Fenner & Clark, 2016:11; Wong *et al.*, 2015:94).

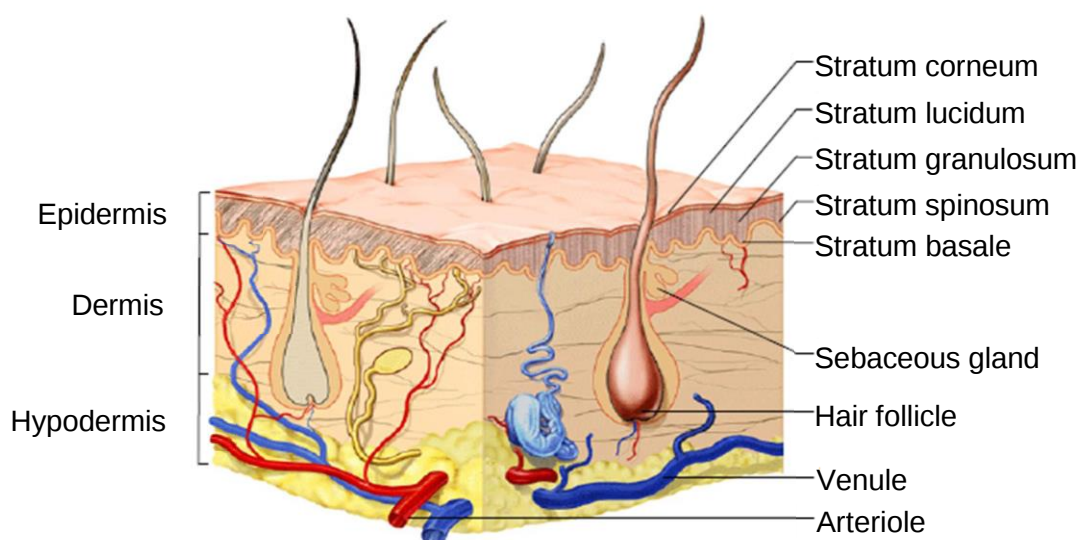


Figure 2.2: Anatomy of the human skin layers (Adapted from Dermnet NZ, 2008).

The dermis, which can vary in thickness from 0.3 mm on the eyelid area to 3.0 mm on the back, is mainly responsible for the nutrition and sensation in the skin, due to the blood vessels and nerves present (Fenner & Clark, 2016:7). Thanks to the array of micro-sized blood vessels, a vascular bed for drug absorption is formed for the transport of therapeutic agents into the systemic circulation (Prausnitz & Langer, 2008:1262). Mostly the dermis consists of elastin fibres, collagen fibres and extra-fibrillar matrix, all formed by the dermal fibroblasts (Fenner & Clark, 2016:8; Wong *et al.*, 2015:93). Proteoglycans are formed in this hydrophilic layer, which contributes to the elastic demeanour of the human skin. The dense network of blood vessels is responsible for several other functionalities in the skin, such as heat regulation, immunological functions, nutritional functions and repair functions (Escobar-Chávez *et al.*, 2012:205).

The epithelial and avascular layer, the epidermis, mainly consists of keratinocytes (95%), melanocytes, Langerhans cells and Merkel cells (Menon, 2002:S4). This layer is also recognised as an extensive metabolising site of the skin with the dominant phase I enzymes (cytochrome P450) present (Zhang *et al.*, 2009:227). The epidermis is about 100 – 150 µm thick and has four distinguishable sub layers, namely the stratum basale, stratum spinosum, stratum granulosum and stratum corneum (Menon, 2002:S4; Rinnerthaler *et al.*, 2015:548). The three deepest sub layers mentioned first are collectively known as the living epidermis and are, like the dermis, hydrophilic in nature (Bolzinger *et al.*, 2012:156).

2.2.1.1 The four sub layers of the epidermal skin layer

2.2.1.1.1 Stratum basale

This innermost layer, also known as stratum germinativum or basal layer, provides functionalities concerning the reduplication and repair of the skin. This is the only layer that accommodates stem cell keratinocytes capable of actively multiplying (Vitorino *et al.*, 2015:2699). According to Jones (1996:141), apart from the terminally differentiated keratinocytes desquamating to the stratum corneum, these basal stem cells also retain a clonogenic ability to ensure the continuity of the stem cell population. Not only does the basale layer contribute to the skins structural integrity, it also incorporates melanocytes and Langerhans cells that synthesise melanin and T-cells, respectively. Merkel cells are also present and act as touch receptors, which is associated with sensorial perception (Vitorino *et al.*, 2015:2699).

2.2.1.1.2 Stratum spinosum

Also known as squamous, spinous or prickly layer, this layer contains an abundant population of desmosomes connecting spinous cells (Kolarsick *et al.*, 2011:204). A marked increase in lipid and protein synthesis is found in this layer, resulting in the two-fold aspects of epidermal differentiation (Vitorino *et al.*, 2015:2700). Lipid-enriched lamellar bodies (LB) or Odland bodies are also seen in this layer in addition to the organelles found in the stratum basale (Vitorino *et al.*, 2015:2700).

2.2.1.1.3 Stratum granulosum

The granular layer is the last layer that contains viable cells before the differentiation process is complete. These cells contain keratohyaline granules that are important for modification and synthesis of proteins concerning keratinisation (Kolarsick *et al.*, 2011:205). The lipid

components present in these granular cells are excreted into the surrounding spaces to eventually assist in intercellular cohesion and barrier function (Venus *et al.*, 2010:469).

2.2.1.1.4 Stratum lucidum

Only present in palmoplantar skin (soles and palms), this layer consists of compact and flattened cells due to the disintegration of their nucleus and organelles (Vitorino *et al.*, 2015:2700).

2.2.2 Stratum corneum - barrier function of the skin

The most superficial layer of the skin, also known as the horny layer, is what all topically delivered substances must traverse to successfully reach the target area. This lipophilic layer contains a mixture of terminally differentiated keratinocytes (corneocytes) that are polyhedrally shaped and flattened to form a physical barrier. Individual corneocytes are linked by corneodesmosomes that form the basic cohesion factor between adjacent corneocytes. The excreted lipid components in the granular layer progressively form the extracellular lipid matrix surrounding the corneocytes. The corneocytes are considered to be dead because of their voided nuclei and organelles during the desquamation process (Kolarsick *et al.*, 2011:205). To fully understand the unique formation of the stratum corneum, the brick and mortar model described by Elias (2005:5) is easiest to comprehend. According to Elias (2005:5), the lipid-depleted corneocytes embedded in the structure resemble bricks and the surrounding lipid matrix is seen as the mortar. The corneocytes assist with water homeostasis, protection against UVR and physical insult. They also serve as a scaffold for the lipid-enriched matrix and generate a natural moisturising factor (NMF) to maintain water balance in the stratum corneum itself (Elias, 2005:5). The continuous lipid matrix (mortar) primarily consisting of ceramides (50%), cholesterol (25%) and free fatty acids (10 - 20%) and is the foundation of the permeation barrier also assisting in antioxidant and antimicrobial protection.

The two phases' of the stratum corneum composition, i.e. polar corneocytes and lipid-enriched matrix, are separated by two individual envelopes. A cornified envelope covers the hydrophilic corneocyte and is composed of proteins such as loricrin, involucrin and filaggrin; a lipid envelope is esterified to the cornified envelope and mounts the continuous lipid layer. Conjointly these two envelopes promote lipid formation and decrease the uptake of foreign substances into the corneocytes, because of their distinct solubility's (Van Smeden *et al.*, 2014:295-296).

Keeping the stratum corneum's unique composition and the solubility differences of the epidermal components in mind, the following conclusions can be made regarding the skin barrier:

- The corneocytes protect against mechanical stressors and UVR, whilst also maintaining optimal hydration levels (Elias, 2005).
- The lipid matrix protects against trans-epidermal water loss (TEWL) from the skin into the direct atmospheric environment, and prohibits the ingress of water-soluble substances into the skin (Baroni *et al.*, 2012:259).
- The skin's surface has an acidic character to protect against infections and bacteria (Baroni *et al.*, 2012:259).

2.3 Transdermal delivery

Apart from the conventional oral delivery of medications, transdermal delivery is the most promising route to deliver several therapeutic agents. Over the last couple of years, this delivery route has been attracting the attention of many researchers hoping to ameliorate the spectrum of drugs that can successfully be transported through the skin. Table 2.1 summarises the ideal properties required for any potential candidate for successful permeation through the skin layers. These properties are considered guidelines for transdermal scientists when researching a novel formulation or reviewing contemporary active ingredients as possible candidates for transdermal delivery. However, even if the active ingredient resembles these characteristics, small amounts should still be sufficiently potent to attain therapeutic activity.

Table 2.1: Ideal properties required for potential compounds to successfully penetrate the skin layers

Properties	Ideal properties ^a	α -Lipoic acid
Daily dose	< 10 mg/day ⁻¹	200 – 600 mg ^b
Solubility in water	> 1 mg/ml ⁻¹	0.127 mg/ml ^c
Molecular weight	< 500 Da	206.33 g/mol ^d
pH of solution	5 – 9	pKa = 4.70 ^c
Oil-water partition coefficient (Ko/w)	10 < (Ko/w) < 1000	3.40 ^c
Melting point	< 200 C	47.5 – 48.0 °C ^c

a) Naik *et al.*, 2000:319; b) Beitner, 2003:847-848; c) Toxnet 2011; d) Perricone *et al.*, 1999:47

There are several different methods of delivering active ingredients through the skin that can be categorised into active and passive methods. Active methods of transdermal delivery include the use of external energy to either reduce the barrier strength of the stratum corneum or act as a driving force to the active ingredient, i.e. iontophoresis and needless injections (Brown *et al.*,

2006:178). Passive methods are usually considered as the conventional means of applying medications to the skin, which includes the use of vesicles systems, i.e. liposomes and niosomes. Recently, modifications have been made to these dosage forms to hopefully increase the permeability of the active ingredients encapsulated in the vesicles and to enhance the driving force of the diffusion through the skin (Brown *et al.*, 2006:176).

The term transdermal delivery actually refers to active ingredients that diffuse through the various skin layers and reach the systemic circulation where the therapeutic effects are applied. Slightly contradictory to that term, the dermal (topical) delivery of active ingredients only describes the targeting of the pathological site within the skin layers, resulting in minimal systemic absorption (Brown *et al.*, 2006:175). In this study, the aim is to deliver α -lipoic acid topically to the dermal layers of the skin, also recognised as an extensive metabolising site (Zhang *et al.*, 2009:227) and once there, to scavenge the deleterious species before any further damage is done to the surrounding organelles that contribute to signs of skin ageing.

2.3.1 Advantages of transdermal/topical drug delivery

The transdermal or topical delivery of active ingredients has the following advantages:

- The circumvention of the first-pass metabolism and other variables that are generally associated with the gastro-intestinal tract, such as enzymes and pH variations (Brown *et al.*, 2006:177; El-Kattan *et al.*, 2000:426; Kornick *et al.*, 2003:953).
- Therapeutic benefits including controlled delivery over a prolonged period of time, ensuring a steady plasma profile and ultimately reducing systemic adverse effects (Brown *et al.*, 2006:177; Thomas & Finnin, 2004:697).
- Painless and convenient administrations leading to improved patient compliance (Brown *et al.*, 2006:177; Delgado-Charro & Guy, 2001:216; Jepps *et al.*, 2012:153).
- The ability to apply medications directly to the target site, e.g. treatment of skin conditions such as psoriasis and eczema (Brown *et al.*, 2006:177).
- Treatment can be rapidly terminated in the event of adverse reaction (Brown *et al.*, 2006:177; El-Kattan *et al.*, 2000:426).
- The large surface area of the skin promotes transdermal absorption (Naik *et al.*, 2000:319).
- Acts as an alternative for other delivery routes in circumstances where a patient is unconscious or suffering from needle phobia (Barry, 2001:105; Brown *et al.*, 2006:177).

2.3.2 Limitations of transdermal/topical drug delivery

The transdermal or topical delivery of active ingredients has the following limitations:

- The effective barrier of the stratum corneum, results in low permeability of drugs (Jepps *et al.*, 2012:153; Lavon & Kost, 2004:671).
- Large molecules (> 500 Da) cannot successfully diffuse across the stratum corneum (Bos & Meinardi, 2000:169; Lavon & Kost, 2004:671).
- Pre-systemic metabolism from enzymes in the skin can possibly reduce the efficacy of the permeant (Steinsträsser & Merkle, 1995:3-25).
- The penetration potential of a compound is difficult to predict due to the complex mechanisms of the delivery pathway (Jepps *et al.*, 2012:153).
- Skin reactions such as irritation or sensitisation can occur (Lavon & Kost, 2004:671).
- Considerable lag time (Lavon & Kost, 2004:671).
- Unpredictable drug levels and inconsistent dosing can be a concern, as microcirculation changes from anatomical sites and skin hydration or age can influence the absorption of compounds (Thomas & Finnin, 2004:700).
- According to Brown *et al.* (2006:177), potential permeates must attain adequate solubility in both aqueous and lipid materials (log P (octanol-water partition coefficient) value between 1 and 3) to successfully diffuse across the different skin layers.

2.3.3 Physiological factors influencing permeation across the skin

Physiological factors that can possibly influence the rate and extent of permeation of active ingredients range from skin age and condition, skin hydration levels, temperature, anatomical site and skin metabolism.

2.3.3.1 Skin age and condition

Permeability across the skin can differ greatly between young and old patients. Older skin undergoes various physiological changes, such as a decrease in elasticity and sebaceous gland activity, increased dryness of the stratum corneum and the destruction of the dermal-epidermal junction, which ultimately results in decreased skin permeability (Ademola & Maibach, 1997:203; WHO, 2010:18). Conversely, infant skin has not fully formed the barrier function and consequently is highly permeable (Ademola & Maibach, 1997:203).

The possibility of overdosing when applying a topical drug on paediatric patients and underdosing in geriatric patients is therefore of concern (Barry, 2002:510). In addition to skin age, skin conditions or the overall state of human skin has a great impact on the penetration potential of chemicals, especially when the stratum corneum's barrier function is altered (Ademola & Maibach, 1997:204; Idson, 1975:909). Broken or inflamed skin has an increased permeability rate, while corns and calluses have the opposite effect (Pefile & Smith, 1997:148).

2.3.3.2 Skin hydration

Increased hydration levels in the skin causes it to soften and swell, resulting in an increased penetration rate through the skin (Barry, 2002:511; WHO, 2010:20). Various creams and ointments form an occlusive layer, which increases skin hydration and reduces the rate of water evaporation from the skin surface (Pefile & Smith, 1997:149).

2.3.3.3 Temperature

According to Pefile and Smith (1997:149), increased temperatures cause the lipid layer of the stratum corneum to become less viscous, resulting in the decrease of activation energy required for diffusion. This means that a rise in temperature on the skin's surface enhances the skin's kinetic action, therefore improving permeability across the skin. Barry (2002:511) stated that a large change in temperature can lead to a tenfold change in penetration rates for compounds.

2.3.3.4 Anatomical site

The thickness of the skin layers, particularly the stratum corneum plays a physiological role in the diffusion rate of compounds across the skin (Lund, 1994:231). The stratum corneum varies in thickness from being the thinnest in the facial regions and the thickest in the palmer and plantar regions. Another aspect that is affected by different anatomical sites is the change in sebum production and the amount of hair follicles and glands present (Amsden & Goosen, 1995:1975). In addition to this, the circulation and microcirculation also vary and can influence the concentration gradient of the dermis by eliminating the drug from the absorption site (Pefile & Smith, 1997:149).

2.3.3.5 Skin metabolism

One of the benefits of transdermal delivery is the reduced enzymatic degradation of drugs however, the skin also has the ability to metabolise drugs by processes such as oxidation, reduction, hydroxylation and conjugation (Amsden & Goosen, 1995:1975). The viable epidermis is the primary site for cutaneous metabolism and metabolites may be transformed into active or inactive forms of the parent-administered active ingredient (Amsden & Goosen,

1995:1975). This region is also the intended target site for the α -lipoic acid due to the large number of reactive species generated by the many metabolic processes taking place. α -Lipoic acid is reduced to dihydrolipoic acid when administered to cells, but as stated before, both the oxidised and reduced states of this compound attain antioxidant activity (Biewenga *et al.*, 1997:315; Maczurek *et al.*, 2008:1465).

2.3.4 Physiochemical factors influencing permeation across the skin

Some physiochemical factors can significantly determine the permeation of a compound into and through the skin. These factors will be discussed with the relative physiochemical properties of the active ingredient evaluated during this study, i.e. α -lipoic acid.

2.3.4.1 pH

The stratum corneum has a pH in the range of 4.8 - 6.0 and can resist pH alterations in a range of 3.0 - 9.0 (Barry, 2002:511; Sznitowska *et al.*, 2001:327). This being said, the diffusion of unionised molecules across the lipid membranes is higher than that of the ionised molecules, according to the pH-partition hypothesis (Aulton, 2007:37; Barry, 2007:576). One reason for this is that unionised molecules attain a higher solubility in lipids and ionised or charged molecules have a better solubility in aqueous environments; therefore, molecules that are ionised face greater resistance due to the lipid nature of the stratum corneum (Zatz, 1993:28). Furthermore, this degree of dissociation of the compound is dependent on the pH, pK_a and pK_b values of that specific compound. α -Lipoic acid contains a carboxylic acid and is considered a weak acid with a pK_a value of 4.7 (Ibrahim *et al.*, 2008:546; Krishnan & Garnett, 2011:3608).

2.3.4.2 Molecular size and shape

There exists an inverse relationship between molecular size and absorption, where larger molecules penetrate the skin progressively poorer than smaller molecules (Barry, 2002:513; Malan *et al.*, 2002:387). According to Abbott (2012:219), molecular size and shape are two properties that determine a drug's diffusion coefficient; molecules that are larger than 500 Da cannot successfully penetrate the corneal layer, whereas smaller molecules will penetrate relatively quickly through this skin layer transcutaneously (Bos & Meinardi, 2000:165; Cevc, 2004:681; Hadgraft & Finnin, 2006:365; Naik *et al.*, 2000:319). Therefore, α -lipoic acid is expected to penetrate the skin to a desirable degree, considering it has a molecular weight of 206.33 g/mol (Perricone *et al.*, 1999:47), which is smaller than the required weight according to Naik *et al.* (2000:319).

2.3.4.3 Aqueous solubility

According to Li and Lim (2016:253), α -lipoic acid is considered a amphiphilic molecule with relatively poor water solubility properties, resulting in low bioavailability of this compound. Toxnet (2011) identified the experimental value of α -lipoic acid's aqueous solubility as 0.127 mg/ml, determined at 25 °C. The skin can be seen as a bi-laminate membrane, meaning it consists of both lipoidal and aqueous layers (Guy & Hadgraft, 1988:753), as previously explained. The aqueous solubility of a compound and the degree of ionisation is directly linked therefore the lipophilicity of a drug is an important aspect to keep in mind during skin permeation studies (Aulton, 2007:37; Behl *et al.*, 1993:240). According to Niak *et al.* (2000:319), the required aqueous solubility of a potential permeate should be > 1 mg/ml for transdermal delivery. Considering the value obtained in literature for α -lipoic acid, the expected skin permeation of this active ingredient may not be ideal.

2.3.4.4 Partition coefficient

The log P value of a compound is indicative of the possible distribution of that compound in two phases, e.g. lipid and water phases (Shargel & Yu, 1993:285) and by determining this value, one can predict the pathway the compound will follow to permeate the skin. If the log P value of a compound is low, the permeability will also be low due to the partitioning of the compound into the skin lipids. Furthermore, if the log P value of a compound is high, the permeability will also be low because of the partition out of the stratum corneum (Thomas & Finnin, 2004:699). The ideal log P value for transdermal delivery is between 1 and 3 (Roberts & Walters, 1998:13). The log P value seen in literature (log P = 2.3) for α -lipoic acid, indicates the topical delivery of this active ingredient may be satisfactory (Cichewicz *et al.*, 2013:818).

2.3.4.5 Diffusion coefficient

The D value of a compound is defined as the transport of matter due to the movement of a given substance within a substrate, and it measures the rate of penetration of a compound under specified conditions (Barry, 2002:512; Rieger, 1993:38). The diffusional speed of a compound is dependent on the state of matter of the specific medium it is diffusing through, for instance, the D value of a compound in air is large due to the void space available to the compound. In a liquid medium, this D value decreases because of the decreased free volume for the component to diffuse within. The diffusivities of the skin gradually decreases and is considered at its lowest within the compact matrix of the stratum corneum (Barry, 2002:512).

2.3.4.6 Drug concentration

Barry (2002:512) stated the concentration gradient is proportional to the flux of the compound across the entire barrier phase. A high concentration is therefore needed in the outer nanometre of the stratum corneum for rapid diffusion to take place (Abbott, 2012:219). According to Rieger (1993:39), the permeation of a compound generally follows Fick's Law of diffusion and can be written as follow (Rieger, 1993:39):

$$J = (K.D)/l \Delta C$$

Equation 2.1

Whereas:

J = flux ($\mu\text{g}/\text{cm}^2.\text{h}$)

D = diffusion coefficient (cm^2/h)

K = partition coefficient

ΔC = concentration gradient ($\mu\text{g}/\text{cm}^3$)

l = membrane thickness (cm)

2.3.5 Routes of drug permeation across the skin

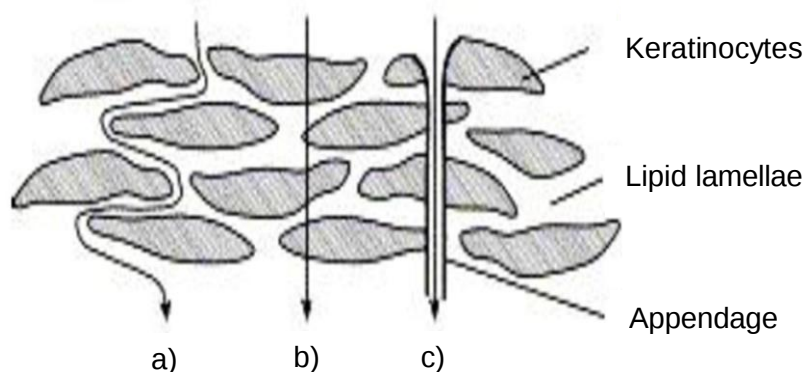


Figure 2.3: Permeation routes through the skin layers: a) the intercellular diffusion route through the lipid lamellae; b) the transepidermal diffusion route through both the keratinocytes and lipid lamellae; c) the transappendageal diffusion route (Adapted from Ho, 2003:50).

There are three possible permeation macro-routes through the skin, namely transappendageal (shunt route), intercellular diffusion route and the transepidermal diffusion route (Figure 2.3). Depending on the penetrant's physiochemical characteristics, especially the solubility aspects, the route followed will be either through the hydrophilic corneocytes, lipophilic matrix or the complete bypassing of the stratum corneum due to hair shafts and sweat ducts.

2.3.5.1 The intercellular route

The intercellular route, dominant and most commonly used route of permeation through the skin, accounts for the diffusion of lipophilic molecules. This pathway consists of the penetrant passing through the spaces in between the skin's cells, and is the potential route of penetration for α -lipoic acid due to the lipophilic nature of this compound.

2.3.5.2 The transepidermal route

The two routes, namely intra- and transcellular, are collectively known as the transepidermal route, thus comprising two different pathways through the stratum corneum (Shahzad *et al.*, 2015:2; Wiechers, 2008:7). Due to the hydrophilic nature of the terminally differentiated corneocytes, polar compounds can be transported through the so-called intracellular, or transcellular, route to the viable epidermis. This route consists of the passive diffusion through corneocytes, but also through the surrounding lipid matrix, which covers each corneocyte. This partially intercellular pathway (lipid lamellae needs to be crossed between corneocytes) can impede and decrease the total flux successfully crossing the stratum corneum (Bolzinger *et al.*, 2012:157).

2.3.5.3 The transappendageal route

The transappendageal route has been side-lined by transdermal researchers due to the minor coverage of these small breaches in the skin's surface. Only 0.1 - 1% of total body surface is considered a functional transport area through this route (Shahzad *et al.*, 2015:2; Wiechers, 2008:7). Although studies have been neglected, it still delivers a swift drug delivery and continues to be a promising route for the transport of large hydrophilic ions and molecules (Williams, 2003:32).

2.4 Vesicles as topical delivery systems

α -Lipoic acid is a poorly water-soluble compound, which is not only largely susceptible to thermolysis and polymerisation due to its low melting point, but also absorbs light readily leading to the decomposition of this compound (Li & Lim, 2016:253). Furthermore, the sulphide present in α -lipoic acid's structure causes this yellowish powder to have a unique, yet unpleasant smell. This matter is of importance, especially because of the potential cosmetic use for this compound. By encapsulating the active ingredient in vesicle systems, the vesicle may act as protection against degradation, as a penetration enhancer, accumulate the active in the target layer, mask the smell of this compound and ultimately, increase the utilisation of α -lipoic acid (Li & Lim, 2016:253)

Mahale *et al.* (2012:47) state that vesicles are lamellar structures that consist of amphiphilic molecules surrounded by an aqueous environment. These systems enhance the bioavailability of compounds and ultimately enable the therapeutic effect to take place over a prolonged period of time by means of controlling the delivery of the compound (Mahale *et al.*, 2012:47). Vesicles have the ability to: 1) act as carriers that deliver compounds across the skin layers, 2) act as penetration enhancers due to the alterations within the stratum corneum, 3) act as a reservoir for sustained release of compounds and 4) can also serve as a controlled release system by its rate-limiting abilities (Alexander *et al.*, 2012:33).

Different types of vesicle systems exist and can be classified according to the principal ingredients used during the preparation (Mahale *et al.*, 2012:47). Niosomal vesicles, opposed to liposomal or transferosomal carriers, have been chosen for this study because of the added advantages it has on its precursors, namely lower toxicity, biodegradability and increased stability, plus it is considered a more economical option (Sharma *et al.*, 2015:393). Furthermore, non-ionic carriers, such as niosomes, are preferred in the cosmetic industry because they are less likely to cause skin irritation (Mahale *et al.*, 2012:47; Sharma *et al.*, 2015:393). According to Buckton (as cited by Mahale *et al.*, 2012:47), the first non-ionic carriers reported were in the application of cosmetics and devised by L'Oreal. As the problem with all vesicle carriers, stability is still a concern and limits their use (Bansal *et al.*, 2012:705). This said, the provesicular systems are delivery systems intentionally formulated to address these stability problems. Provesicular systems include a porous powder that is water soluble and ultimately acts as the carrier in the vesicle system. During the formulation of these provesicular systems an organic solvent is dissolved, thus resulting in a free-flowing granular powder. Problems that arise regarding usage of aqueous vesicle systems, such as liposomes and niosomes, can ultimately be addressed with these provesicular systems (Bansal *et al.*, 2012:706&709).

2.4.1 Niosomes

The primary components of which niosomes (Figure 2.4) consist are non-ionic surfactants, hydration medium and cholesterol (Mahale *et al.*, 2012:47; Moghassemi & Hadjizadeh, 2014:23). Non-ionic surfactants can improve the solubility of compounds such as α -lipoic acid with poor solubility (Kumar & Rajeshwarrao, 2011:209). These vesicles are formed as a result of the self-assembly of the non-ionic amphiphiles in combination with other lipids and aqueous medium (Agarwal *et al.*, 2001:44; Bansal *et al.*, 2012:705-706). Niosomes can encapsulate amphiphilic, hydrophilic and lipophilic compounds and are considered to have better delivery of the compound to the target site compared to other vesicle systems (Bansal *et al.*, 2012:705-706; Mahale *et al.*, 2012:47). Niosomes normally have a particle size of 10 – 1000 nm and are

classified according to the average size of the vesicles in the dispersion. Small unilamellar vesicles (SUV) are 25 – 50 nm, multilamellar vesicles (MLV) are > 50 nm and the large unilamellar vesicles (LUV) are larger than 100 nm (Escobar-Chávez *et al.*, 2012:217; Varun *et al.*, 2012:635).

Due to its lipophilic demeanour and poor water solubility, α -lipoic acid will be entrapped by its partitioning into the lipophilic domain of the vesicle bilayers (Moghassemi & Hadjizadeh, 2014:22; Muzzalupo & Tavano, 2015:23). The successful incorporation of α -lipoic acid into a niosomal carrier may deliver the active to the desired target site, mask its unpleasant smell, ensure sufficient bioavailability and stabilise the formulation against decomposition (Li & Lim, 2016:253).

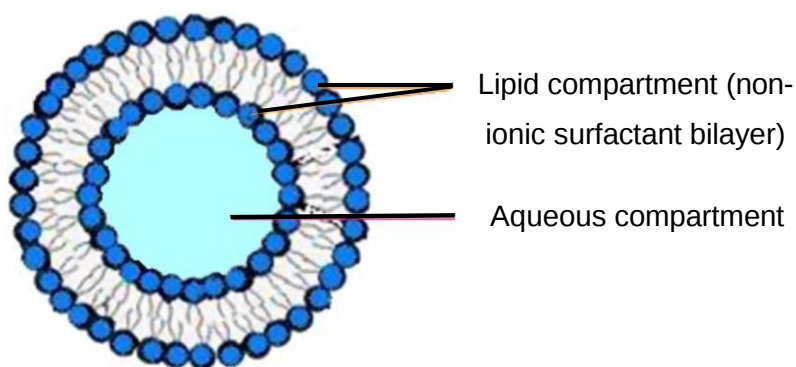


Figure 2.4: Structure of a niosome (Adapted from Kumar & Rajeshwarrao, 2011:209).

2.4.2 Proniosomes

As stated before, provesicular systems can improve the physical stability of vesicles and therefore avoid problems such as fusion, leaking and aggregation (Hu & Rhodes, 1999:24). This dry and free-flowing granular powder is hydrated with water before use; to form a hydrated proniosomal dispersion similar to niosomal dispersions, which are prepared following conventional methods (Bansal *et al.*, 2012:710; Hu & Rhodes, 1999:24; Kumar & Rajeshwarrao, 2011:214).

By coating a water-soluble carrier, such as sorbitol, with a thin film of non-ionic surfactant, cholesterol and chloroform, a dry free-flowing powder is formed. This porous powder can then be hydrated by adding an aqueous phase (e.g. water) with a temperature higher than that of the transition phase (of the active ingredient) for the proniosomes to form (Mahale *et al.*, 2012:50-51).

2.5 Skin ageing

α -Lipoic acid may be of use in the treatment or improvement of signs of skin ageing due to the highly potent antioxidant activity it possesses. This active ingredient can act against damaging reactive species directly and indirectly, resulting in a two-fold antioxidant activity useful in the treatment of ageing skin (Perricone, 2000:217).

Skin ageing is usually conceptualised as a complex biological phenomenon that includes two separate components, i.e. intrinsic ageing and extrinsic ageing. Generally, cutaneous ageing is characterised by increased laxity and wrinkling, with sagging and atypical pigmentation of the skin (El-Domyati *et al.*, 2002:398; Jenkins *et al.*, 2002:801; Masaki, 2010:85).

2.5.1 Intrinsic skin ageing

Intrinsic ageing, or chronological ageing, is by definition an inevitable form of ageing, allegedly not subject to manipulations through human behavioural changes (Baumann, 2007:241). Chronological ageing occurs over time to most internal organs as a consequence of physiological activity and changes that are largely genetically determined (El-Domyati *et al.*, 2002:398; Farage *et al.*, 2008:88; Jenkins *et al.*, 2002:801; Ramos-e-Silva *et al.*, 2013:751). Chronologically aged skin is characterised by fine lines and wrinkles, dry and itchy skin that is thin and transparent, but is still regarded smooth and unblemished when compared to extrinsic aged skin (Baumann, 2007:241; Binic *et al.*, 2013:1; Poljšak *et al.*, 2012:1). Looking at the skin histologically, this type of ageing manifests as epidermal and dermal atrophy, the flattening of epidermal rete ridges and the reduction of mast cells and fibroblasts (Baumann, 2007:241). Free radicals, caused by ROS, are generated endogenously through oxidative cell metabolic processes, and are generally associated with the cell damage and ageing. These radicals are considered reactive because they have unpaired electrons instead of electron pairs normally seen on the outer shells of stable molecules (Giacomoni, 2011:300). Substantial evidence exists illustrating a connection between cellular senescence, mitochondrial oxidative damage and the ageing phenotypes in the skin (Binic *et al.*, 2013:2; Velarde *et al.*, 2012:8).

2.5.1.1 Factors that are related to intrinsic skin ageing

2.5.1.1.1 Ethnicity

The primary effect of ethnical variations in ageing is related to the difference in pigmentation levels. Increased levels of melanin pigmentations are considered a protection mechanism against the cumulative effects of photoaged skin, as seen particularly in African-Americans. Asian skin seems to develop wrinkles to a lesser degree of severity, which also occur later compared to the wrinkles seen in Caucasians. Furthermore, it is accepted that African-

American skin is considered more compact, with an increased amount of intercellular lipids compared to Caucasian skin. This, along with the higher melanin levels seen in darker skin, may add to the resistance to skin ageing (Farage *et al.*, 2008:90; Ramos-e-Silva *et al.*, 2013:751).

2.5.1.1.2 Anatomical variations

Skin ageing becomes more evident in thinner skin regions, such as the eyelids (< 0.5 mm), compared to areas of thicker skin, i.e. the soles on the feet (> 6 mm). It is also known that both lipid distribution and composition differs in various skin areas, which can influence the permeability through the skin on that specific region. The decrease in epidermal thickness that occurs during ageing is another aspect that varies from anatomical site (Farage *et al.*, 2008:90; Ramos-e-Silva *et al.*, 2013:751).

2.5.1.1.3 Hormonal changes

Thyroid, testosterone and especially oestrogens influence processes such as epidermal lipid synthesis and collagen synthesis. Oestrogens also promote water retention, increase the extracellular matrix and affect the synthesis of hyaluronic acid (a glycosaminoglycan that is involved in tissue repair). Low oestrogen levels result in less hydrated and thinner skin, particularly seen in women during menopause (Farage *et al.*, 2008:90; Ramos-e-Silva *et al.*, 2013:751).

2.5.2 Extrinsic skin ageing

Extrinsic ageing or photoageing is considered preventable, by nature and by definition. It is induced by exogenous factors that include smoking, pollution, lifestyle components such as diet and sleeping position, with the main contributor being sun exposure (Baumann, 2007:242; Farage *et al.*, 2008:88; Kinkade, 2011:306). Solar exposure is believed to account for 80% of facial ageing, emphasising the importance of sun avoidance and the use of sunscreens, which are currently the only defence against photodamage (Baumann, 2007:243).

Some clinical manifestations of photoaged skin are visible changes in surface texture, such as dryness, freckles and lentigines, with irregular pigmentation and an increase in skin roughness. Furthermore, the stratum corneum becomes more compact with an increase in thickness of the granular cell layer, but a decrease in the epidermal thickness (Binic *et al.*, 2013:2; El-Domyati *et al.*, 2002:398; Farage *et al.*, 2008:92).

2.5.2.1 Factors that are related to extrinsic skin ageing

2.5.2.1.1 Ambient conditions

Temperature and humidity affects the integrity of the skin through the formation of structural lipids and proteins. Higher temperatures can increase the water evaporation from the skins' surface, whilst the skin stiffens in low temperatures due to a decrease in water loss (Farage *et al.*, 2008:90; Ramos-e-Silva *et al.*, 2013:751).

2.5.2.1.2 Drugs and smoking

Some hypocholesterolemic medications can promote the desquamation of cells and induce xeroderma (dry skin). Furthermore, nicotine is considered one of the biggest contributors to facial ageing, with a greater impact than solar exposure. Smoking can lead to telangiectasia (spider veins), elastosis – a decrease in elastin and also collagen fibres. It causes an increase in the production of free radicals, such as ROS, and can also affect the roughness of the skin promoting physical signs of ageing (Farage *et al.*, 2008:90; Ramos-e-Silva *et al.*, 2013:751).

2.5.2.1.3 Solar exposure

A large spectrum of processes is affected by chronic exposure to sunlight. It is believed that 90% of visible facial ageing is the consequence of chronic exposure to sunlight (Ramos-e-Silva *et al.*, 2013:751). According to Lademann *et al.* (2016:41), solar exposure is the aspect most accountable for the formation of free radicals in human skin. These free radicals affect various cellular processes that lead to skin ageing and cell damage. UVR can damage the skins' integrity by causing a chain of molecular reactions that result in the deterioration of elastin and collagen, both important structural proteins in the human skin. Furthermore, UVR interferes with DNA (deoxyribonucleic acid) repair processes by damaging the involved enzymes and by also affecting the T-cells and Langerhans cells. This ultimately results in a carcinogenic effect, with addition to the preventative action it has on the apoptosis of sun-exposed cells. Therefore, cumulative solar exposure can ultimately lead to premature skin ageing and skin cancer (Farage *et al.*, 2008:90; Giacomoni, 2011:299; Masaki, 2010:86; Zhai & Maibach, 2011:315).

2.5.3 The role of oxidative stress in skin ageing

As mentioned before, oxidative stress is considered the most harmful contributing factor to skin ageing. The imbalance between ROS production and one's endogenous antioxidative defences, tilting toward the pro-oxidants, is defined as oxidative stress (Poljšak *et al.*, 2012:1). According to Masaki (2010:86), ROS can cause mutations in a number of species and

ultimately advances skin ageing, which is characterised by atypical pigmentation with an early onset of wrinkles.

Because skin is a highly metabolic tissue, large amounts of ROS are generated from endogenous and exogenous sources due to various metabolic processes. Internal sources include enzymes, such as xanthine oxidase or nitric oxide synthase that produce superoxide radicals directly in the skin (Kohen, 1999:181). These reactive species can potentially target biological sites such as DNA, lipids and proteins, resulting in cell damage and loss of functionality. Many types of chemical interactions can occur between these metabolites and their potential targets that can ultimately lead to lipid and protein peroxidation (Brieger *et al.*, 2012:1; Kohen, 1999:182). According to Baumann (2007:246), free radicals contribute to the accumulation of elastin emblematic and collagen degradation in photoaged skin by inducing alterations in the gene expression pathways.

To summarise, ROS plays a vital role in metabolic processes and is an essential aspect for living organisms, but when produced in higher levels can become deleterious and cause a great deal of damage to skin cells ultimately promoting skin ageing (Poljšak *et al.*, 2012:1).

2.5.4 α -Lipoic acid and skin ageing

Giacomoni (2011:303) stated that one antioxidant does not solely address all the various free radicals. Considering this, α -lipoic acid can directly scavenge free radicals such as the singlet oxygen, hydroxyl radical and nitric oxide, and when externally administered to the cell it can effectively reduce superoxide radicals in its reduced state (Perricone, 2000:217). Additionally, it can regenerate other endogenous antioxidants, such as vitamin E, vitamin C and glutathione, which significantly decline during ageing. Therefore, this universal antioxidant possesses a dual antioxidant action that can have potential in the fight against signs of ageing (Külkamp-Guerreiro, 2013:709; Maczurek *et al.*, 2008:1465; Perricone, 2000:217).

According to Inoue and Takahashi (2010:397), our natural antioxidative systems that combat endogenous and exogenous oxidative stressors can be classified as:

- Preventative antioxidants
- Radical scavenging antioxidants
- Repair and *de novo* antioxidants
- Adaptation processes, such as induction of all aforementioned antioxidants

α -Lipoic acid is one of the radical scavenging antioxidants that is generated endogenously to stabilise these damaging radicals before they can cause further cell damage. According to

Masaki (2010:89), due to the continuous depletion of natural antioxidant reserves, as a result of UVR exposure, the topical delivery of antioxidants (such as α -lipoic acid) may be advantageous in the prevention or treatment of skin ageing.

2.6 Inflammation of the skin

α -Lipoic acid attains the ability to reduce the production of several transcription factors necessary in the inflammatory responses and therefore acts as an anti-inflammatory agent (Beitner, 2003:847). There are many skin diseases caused or influenced by the body's inflammatory system; one of the most widely recognised skin diseases, characterised by chronic inflammation, is psoriasis (sometimes referred to as the T-cell driven inflammatory disease). The treatment for this lifelong disease is complex and challenging, and some patients may develop psychiatric conditions and other comorbidities, such as psoriatic arthritis, Crohn's disease and metabolic syndrome (Kadam *et al.*, 2010:388).

2.6.1 α -Lipoic acid and inflammation

α -Lipoic acid can reduce the production of certain transcription factors, such as NF- κ B, in human monocytes and can indirectly reduce the gene expression of several inflammatory cytokines, such as interleukins (IL-1, IL-2, IL-6, IL-8) and tumour necrosis factor- α (TNF- α), ultimately giving this endogenous compound anti-inflammatory abilities. Therefore, α -lipoic acid may be useful in treating inflammations in diseases such as psoriasis (Beitner, 2003:847).

2.7 Conclusion

The intended purpose of this study is to successfully deliver α -lipoic acid to the highly metabolic region of the skin and where collagen is present. This may lead to the treatment or improvement of facial signs of ageing and inflammatory skin diseases such as psoriasis.

Transdermal and topical delivery is a convenient alternative for the conventionally used oral route. Considering all the advantages of the transdermal and topical route, it is easy to understand why researchers are intrigued by this alternative. This delivery route is especially important in the cosmetic field, therefore the potential cosmeceutical α -lipoic acid, delivered by means of either niosomal or hydrated proniosomal dispersions will be evaluated in the treatment of signs of skin ageing and inflammation of the skin.

It should be noted that the molecular size of α -lipoic acid (206.33 Da) and the lipophilic nature of the compound are both indicative that the skin diffusion of α -lipoic acid will probably be adequate for the successful accumulation in the epidermal-dermal layers of the skin. For the

potential treatment of ageing signs and inflammation in the skin, the active will need to be present in the epidermis-dermis after tape-stripping analysis.

The characterisation of both the niosomal and proniosomal vesicle systems, encapsulating the active, will be evaluated. After performing release studies and skin diffusion studies on both vesicle systems, the best suited vesicle system will be selected for further clinical efficacy studies. The clinical efficacy of α -lipoic acid in the selected vesicle system will be evaluated in two separate clinical studies to determine if the active ingredient retained its therapeutic activity in the selected vesicle system.

To conclude, two vesicle systems will be evaluated and compared to one another, i.e. niosomal and hydrated proniosomal dispersions, and the most suitable will then be used to evaluate the efficacy of α -lipoic acid in the potential treatment of ageing skin and inflammatory skin diseases, such as psoriasis.

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Chapter 3

ARTICLE FOR PUBLICATION IN THE INTERNATIONAL JOURNAL OF PHARMACEUTICS

This chapter is written in article formal for the purpose of publication in the International Journal of Pharmaceutics. The complete author guidelines for this journal are discussed in Appendix F. To ease the reading of this Chapter; the paragraphs were justified.

Formulation and topical delivery of niosomes and proniosomes containing α -lipoic acid

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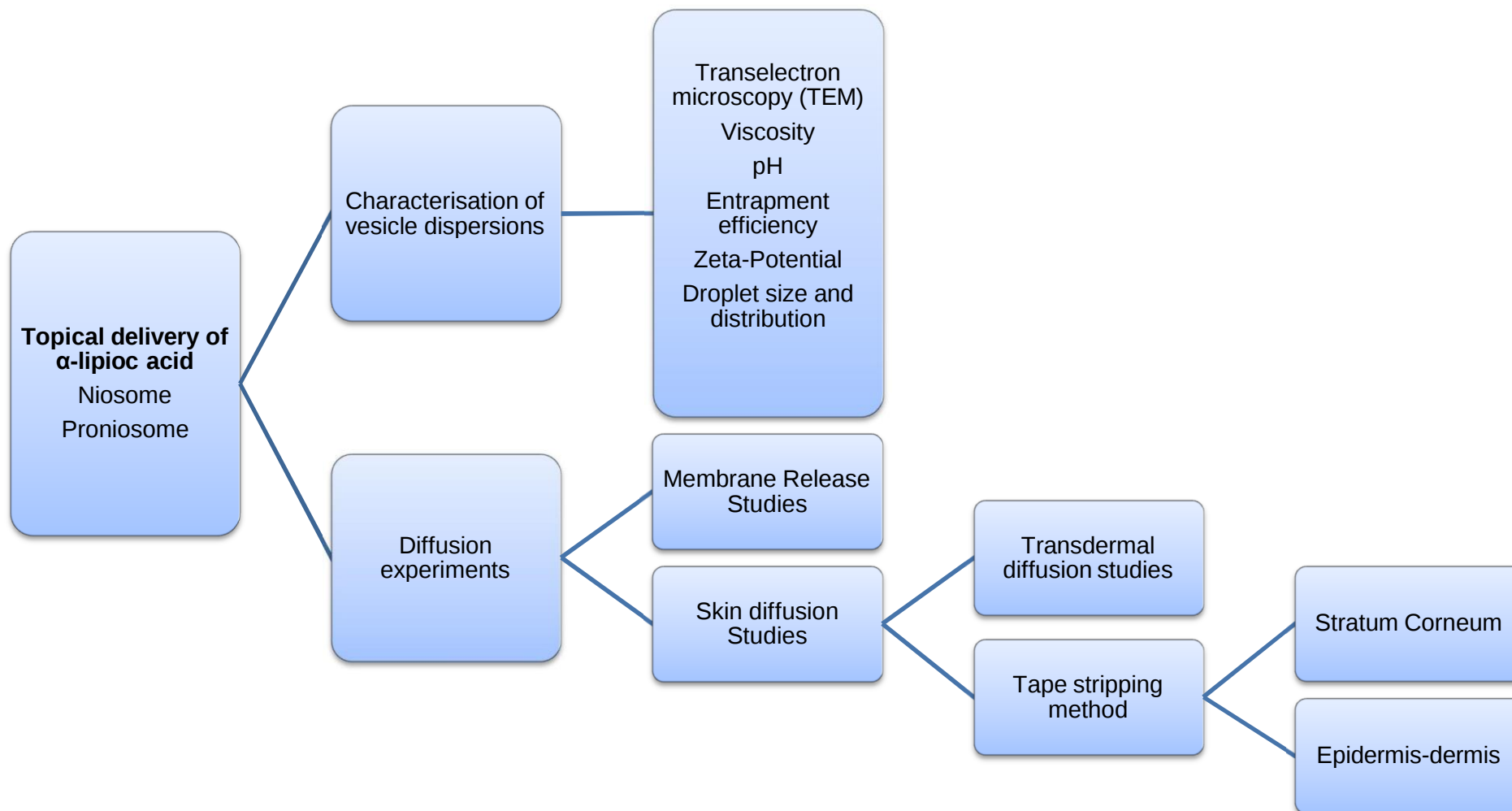
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Abstract

The potent and highly effective antioxidant, α -lipoic acid, is known for its many therapeutic applications. To deliver this endogenous compound to the dermal layers of the skin, certain experiments were performed in order to evaluate the topical delivery potential of different vesicle systems containing α -lipoic acid. The dermal layers are considered the target site for this study, due to the high metabolic activity in this region. The vesicle systems used, i.e. niosomes and proniosomes, underwent characterisation and diffusion experiments to determine the best suited vesicle system for the specific active ingredient. The physical characterisation showed clear and unilamellar vesicles with desired entrapment efficiencies for both the niosomal dispersions (99.6 ± 0.02) and hydrated proniosomal dispersions (92.62 ± 2.76). The zeta-potential results also indicated that both dispersions were considered stable with the niosome dispersion obtaining a zeta-average value of 224.6 ± 8.1 d.nm and the hydrated proniosomal dispersion with a value of 698.3 ± 357.8 d.nm. Furthermore, the membrane release studies revealed good and desirable release from both vesicle systems; however the niosomal dispersion attained better targeted results from the skin diffusion studies. The average concentration of α -lipoic acid that accumulated in the epidermis-dermis after 12 h for the niosomal dispersion was 5.077 ± 1.47 $\mu\text{g/ml}$ and 2.854 ± 1.43 $\mu\text{g/ml}$ for the hydrated proniosomal dispersion.

Keywords: Transdermal delivery, α -Lipoic acid, Antioxidant, Skin diffusion, Niosome, Proniosome.

Graphical abstract



1 Introduction

The largest organ in the integumentary system is the human skin, which plays a vital role in our daily survival. There are three known functionalities of the skin: protection, sensation and regulation (Kolarsick et al., 2011). The skin consists of three distinguishable layers that jointly serve as a powerful barrier against external commodities. The deepest skin layer, the hypodermis, facilitates dermal cells such as fibroblasts, macrophages and adipose cells, and contains the connective fibres of collagen and elastin to bind this layer to the dermal layer (Fenner & Clark, 2016; Wong et al., 2015). The next layer is the hydrophilic dermis, which is responsible for sensation and contains an array of micro-sized blood vessels for absorbing nutrients into the systemic circulation (Prausnitz & Langer, 2008). The outer skin layer, the epidermis, is considered the main site for metabolic processes and can be further sub-divided into four epidermal layers. Most important is the outermost layer, the stratum corneum, which is also known as the ultimate rate-limiting barrier for any substance to potentially permeate the skin; this layer mainly consists of hydrophilic corneocytes that are separated by a lipid-enriched matrix and collectively serve as a powerful skin barrier (Van Smeden et al. 2014).

The transdermal delivery is therefore only acquirable to substances with a specific set of properties, such as a molecular weight smaller than 500 Da, an aqueous solubility of >1 mg/ml and the potential permeant has to attain both hydrophilic and lipophilic properties (Perrie et al., 2012; Naik et al., 2000). Transdermal delivery refers to the transport of substances through the skin layers and into the systemic circulation therefore, the term topical delivery refers to the substance accumulating in the skin layers and circumventing the systemic absorption (Brown et al. 2006).

α -Lipoic acid or 6,8-thioctic acid or 1,2-dithiolane-3-pentanoic acid, is a natural antioxidant derived from octanoic acid, which acts as a metabolic coenzyme and also as a powerful antioxidant against oxidative stresses (Moini et al., 2002; Dos Santos Pereira et al., 2016). Two unique abilities of α -lipoic acid is its amphiphatic nature, being able to scavenge free radicals in both lipid and aqueous mediums and its ability to regenerate other antioxidants, such as vitamins E and C, due to a redox couple formed with its reduced state. In soma cells, α -lipoic

acid rapidly reduces to dihydrolipoic acid, therefore forming this potent redox couple (Perricone, 2000). Previous studies with α -lipoic acid showed the active rapidly penetrated the stratum corneum and was present in both the dermis and subcutis of the skin after 4 h (Beitner, 2003). When α -lipoic acid was present in the skin, it was proven to provide protection against the damaging free radicals generally produced by excessive ultraviolet exposure, and may assist in the treatment of premature ageing (Perricone, 2000). Furthermore, α -lipoic acid effectively inhibits the activation of NF- κ B (transcription factor) giving this compound powerful anti-inflammatory activity. This could mean there might be potential for this unique antioxidant to take part in treatment regimes in inflammatory skin conditions, such as psoriasis (Perricone, 2000).

To conclude, this antioxidant possesses great cosmeceutical potential, also keeping in mind its amphiphilic nature and preferred molecular weight (206.33 Da), the compound should readily penetrate the skin. Therefore, the aim of this study is to determine the characteristics, vesicle release and skin diffusion profiles of two vesicle systems, i.e. niosomes and proniosomes, containing α -lipoic acid. By delivering this active ingredient to the target dermal layers, future inclusion of this antioxidant in the treatment regimens of premature skin ageing and skin inflammation may be beneficial.

2 Materials and methods

2.1 Material

The active ingredient, α -lipoic acid was purchased from Sigma Aldrich. The chloroform, LiChrosolv[®] acetonitrile and D-sorbitol were ordered from Merck-Millipore (Darmstadt, Germany). The cholesterol (Sigma-Aldrich, Steinheim, Japan) and Span[®] 60 (Fluka, Steinheim, Switzerland) were also purchased prior to the experimental work. The PBS (phosphate buffer solution) consisted of sodium hydroxide (uniLAB[®]) and potassium dihydrogen orthophosphate (univAR[®]), which were ordered from Merck Chemicals (Wadeville, RSA). The HPLC grade, Milli-Q[®] water (Milli-Q[®] Academic water purification system, Merck-Millipore, Midrand, RSA) was used throughout the study.

2.2 Methods

All methods used during the formulation, characterisation and membrane release and diffusion studies for both vesicle dispersions are discussed.

2.2.1 Formulation of dispersions

During this study, both niosomal and hydrated proniosomal dispersions were formulated containing the active ingredient α -lipoic acid.

Table 1: List of all ingredients used during the preparation of a niosomal and hydrated proniosomal dispersion (10 ml sample)

2.2.1.1 Niosomal dispersion

All ingredients used during the preparation of niosomes are listed in Table 1. The thin-film hydration method was used to prepare the niosomal dispersions. A lipid mixture (cholesterol and α -lipoic acid) was dissolved in organic solvent (chloroform), where after the solvent was evaporated under a specific pressure using a Büchi[®] rotary evaporator (Switzerland) with an Interface I-100, equipped with a Rotavapor[®] R-100, Vacuum pump V-100 attached to a Heating bath B-100. The resulting thin-film was rehydrated using deionised Milli-Q[®] water, while a magnetic stirrer constantly agitated the dispersion to remove all residual film still stuck to the walls of the flask. A Hielscher Ultrasonic Processor UP200St was used to apply sonic energy to the vesicles to ultimately reduce the average vesicle size (Dua et al. 2012; Kailash et al. 2013; Moghassemi et al. 2014; New, 1990).

2.2.1.1 Hydrated proniosomal dispersion

To prepare the proniosomes, the slow spray-coating method was used (all ingredients seen in Table 1). The method includes the continuous spraying of a lipid mixture (containing the surfactant, active and organic solvent) upon the carrier (sorbitol). The organic solvent is dissolved under a specified pressure by using the same Büchi[®] rotary evaporator (Switzerland) as previously described in Section 2.2.1.1 for the niosomal preparation. The proniosomes were hydrated with deionised Milli-Q[®] water and sonicated, using a Hielscher Ultrasonic Processor

UP200St, to resize the vesicles by means of sonic energy (Kakar et al. 2010; Vasthist et al. 2015).

2.2.2 Analysis of α -lipoic acid

All samples were analysed by means of high performance liquid chromatography (HPLC). An Agilent 1100 Series (Agilent Technologies, Palo Alto, Canada), fitted with a G1311A quaternary pump, G1315A diode array detector, G1322A vacuum degasser, G1313A autosampler injection mechanism and HP ChemStation Software, was utilised. The same chromatographic conditions (as seen in Table 2) were continuously used throughout the study.

Table 2: Chromatographic conditions used during HPLC analysis of samples containing α -lipoic acid

2.2.3 Standard preparation

Accurate amounts of α -lipoic acid were weighed and transferred to a 100 ml volumetric flask before adding 50 ml methanol. The solutions were then made up to volume with PBS (pH 7.4). Two standard preparations were prepared with varying concentrations and injected into the HPLC at different volumes (5, 10, 15 and 20 μ l).

2.2.4.1 Aqueous solubility

Three test tubes, filled with 3 ml PBS (pH 7.4), were used to determine the aqueous solubility of α -lipoic acid. α -Lipoic acid was added to all three test tubes in excessive amounts to supersaturate the solutions, and placed in a Stuart Shaking water bath for a period of 24 h. The water bath maintained a controlled temperature of 32°C to mimic the skins' temperature during the diffusion studies (Williams, 2003). Prior to HPLC analysis, the samples were filtered through 0.45 μ m filters.

2.2.4.2 Octanol-buffer distribution coefficient (log D)

The distribution of α -lipoic acid between an aqueous phase (PBS, pH 7.4) and organic solvent (n-octanol) was determined after both phases were co-saturated. Both liquids were separated and the n-octanol was transferred into three test tubes, each containing 3 ml of the liquid. α -Lipoic acid was added to test tubes and placed in a heating bath at 32°C for 10 min. Equal

amounts of the pre-saturated PBS (pH 7.4) were also added to the three test tubes and allowed to rotate for a further 45 min. Thereafter, the samples were centrifuged using a Beckman Coulter Optima L-100 XP ultracentrifuge, equipped with a 50.2Ti fixed rotor (Beckman Coulter, South-Africa), for 20 min at 4000 rpm to separate the two phases. Before HPLC analysis, the two liquids were carefully separated and filtered through 0.45 µm PVDF (polyvinylidene fluoride) filters. Equation 1 was used to calculate the log D value for both dispersions (Andrés et al., 2015).

$$\text{Log D} = \text{Concentration a} / \text{Concentration b} \quad \text{Equation 1}$$

Concentration a – Refers to the concentration of active in n-octanol

Concentration b – Refers to the concentration of active in PBS (pH 7.4)

Characterisation of 5% niosomal formulations

Both vesicle systems were characterised by means of transmission electron microscopy (TEM), viscosity, pH, entrapment efficiency (EE%), zeta-potential and size distribution experiments.

2.3.1 Transmission electron microscope

The morphology and formation of the niosomal and hydrated proniosomal dispersions were examined using the FEI Tecnai G2 TEM (FEI, Holland) equipped with a Gatan bottom mount camera was used to view the particles and their respective size at 200 kV. Dispersions were viewed containing no active ingredient due to the sensitivity of the microscope.

2.3.2 Viscosity

The viscosities of both dispersions were measured using a Brookfield® Viscometer model DV III Ultra (Middleboro, Massachusetts, United States). Approximately 10 ml of both dispersions were transferred to a cylindrical chamber enclosed in an adapter and flow jacket. The viscosity was measured using a T-bar spindle 18 (SC4-18). Measurements were made in triplicate and the average value calculated.

2.3.3 Potential of hydrogen (pH) determination

The Mettler Toledo SevenMulti™ pH/conductivity meter, equipped with a glass Mettler Toledo InLab® 410 electrode (Schwerzenbach, Switzerland) was utilised to measure the dispersions' pH values. The pH meter was calibrated prior to measurements using Mettler Toledo pH buffer

solutions (pH 4, 7 and 10). Measurements were taken in triplicate and average values calculated.

2.3.4 Entrapment efficiency percentage (EE%)

A Beckman Coulter Optima L-100 XP ultracentrifuge, equipped with a 50.2Ti fixed rotor (Beckman Coulter, SA) was used to separate the formed vesicles from the remaining free active ingredient in the dispersions. The samples were centrifuged at a temperature of 4°C for 30 min at 25000 rpm. Equation 2 was used to calculate the EE% of both dispersions (Abdellatif & Abou-Taleb, 2016; Kurakula et al., 2012).

$$EE\% = (\text{Concentration t} - \text{Concentration f}) / \text{Concentration f} \times 100 \quad \text{Equation 2}$$

Concentration t is the total concentration of α -lipoic acid present in the sample and concentration f, refers to the concentration of free un-encapsulated α -lipoic acid.

2.3.5 Zeta-potential

The Malvern Zetasizer Nano ZS (Malvern Instruments, Worcester, United Kingdom) was utilised to measure the surface charges of the colloidal dispersion. Prior to the measurements, the sample was diluted by adding 1 drop of sample into 20 ml Milli-Q® water. The zeta-potential measurements were performed in triplicate and the average sizes determined.

2.3.6 Droplet size and distribution

To determine the size and distribution of the dispersion, a Malvern Zetasizer Nano ZS (Malvern Instruments, Worcester, United Kingdom) was used. Samples were diluted by adding 1 drop of niosomal dispersion to 25 ml Milli-Q® water. Measurements were taken in triplicate and resulting average values calculated.

2.4 Diffusion experiments

2.4.1 Membrane release studies

Membrane release studies were done to determine if the active was released from the vesicles and was available for diffusion. Vertical Franz diffusion cells (n=10) were used. The donor (with a diffusion area of 1.075 cm² and a capacity of 1 ml) and the receptor (± 2 ml capacitance volume) compartments were mounted together and greased with vacuum grease to avoid any leakage. Inside each receptor compartment a magnetic stirrer was placed with a hydrophilic

PVDF membrane (FP Vericel™, 0.45 µm, 25 mm, Pall®) placed securely on top. The two compartments with membrane in-between were fastened together with a horseshoe clamp.

170 Parafilm® was used to cover the donor compartment to avoid any possible evaporation of the constituents. The receptor compartments were filled with PBS (pH 7.4) (BP, 2013) and the individual dispersions were used to fill the donor compartments. All Franz cells were kept in a heating bath maintaining a temperature of 37°C. For a period of 6 h, the receptor phases were extracted hourly and refilled with PBS (pH 7.4) (BP, 2013) using a suitable syringe. The
175 samples were analysed by means of HPLC to determine the release rate and concentration of the α-lipoic acid that permeated through the PVDF membrane.

2.4.2 Skin preparation

Caucasian abdominal skin (Ethical approval reference number: NWU-00114-11-A5), collected after abdominoplasty surgery, was used during the skin diffusion studies. The skin was kept at -
180 20°C, prior to being dermatomed using a 2.5 cm width plate of the Zimmer™ electric dermatome model 8821, and placed on Whatman® filter paper to dry. Thereafter, the skin was wrapped in aluminium foil and again stored at -20°C until the commencement of the diffusion studies.

2.4.3 Transdermal diffusion

185 The method described in Section 2.4.1 was also used for the skin diffusion experiments, the only difference being the skin sample substituting the PVDF membranes, with the stratum corneum facing upwards. The study period also differed to that of the release studies, being 12 h with 2 hourly extractions. The concentrations of active present in the receptor compartments were analysed by means of HPLC to determine the permeation of α-lipoic acid through the skin.

190 2.4.4 Tape stripping

After final sampling, the Franz cells were dismantled and all skin samples were carefully placed on Parafilm® and excess amounts of the dispersion were removed from the skin's surface using dry tissue paper. 3M Scotch® Magic™ tape was cut into suitable sizes to cover the diffusion area (≈11.7 cm²) of the Franz cells; the first strip was discarded and the following 15 strips were
195 placed in a polytop containing 5 ml PBS (pH 7.4). The remainder of the skin sample was cut

into smaller pieces and also placed in a polytop containing 5 ml PBS (pH 7.4). The polytops were stored overnight at a temperature of 2-5°C, before HPLC analysis.

2.5 Data analysis

Data analysis of the membrane release studies included the calculation of the amount of α -lipoic acid released (%) from the vesicles (niosome and hydrated proniosome) and the average concentration ($\mu\text{g}/\text{cm}^2$) that permeated the membrane. For the diffusion studies, the amount of active diffused (%) together with the average concentration ($\mu\text{g}/\text{cm}^2$) of α -lipoic acid that permeated the skin layers was calculated. Average flux values ($\mu\text{g}/\text{cm}^2.\text{h}$) were calculated for both studies and compared for correlation purposes.

Descriptive statistical analysis was conducted on the membrane release studies, the two skin individual skin layers (epidermis-dermis and stratum corneum-epidermis) and on the concentration of α -lipoic acid present in the receptor compartments. Two-way ANOVA (analysis of variance) tests were used with the dispersions and skin layers to determine if the interactions were statistically significant. Where statistical significant differences ($p < 0.05$) were identified, further comparative tests were conducted.

T-tests were performed on each dispersion and skin layer to determine the targeting ability of both the niosomal and hydrated proniosomal dispersions. For validation purposes the results were analysed by performing the non-parametric Mann-Whitney U-test on both dispersions.

3 Results and Discussion

3.1 Formulation of dispersions

3.1.1 Niosomal dispersion

The niosomal dispersion had an off-white to a slight yellowish colour with a particular smell and also appeared homogenous with a creamy texture.

3.1.2 Hydrated proniosomal dispersion

Prior to the hydration of the proniosomes, the formulation appeared as free flowing granules with a whitish colour and a particular smell. After rehydration and sonication, an off-white milky dispersion was produced with a creamy texture.

3.2 Physicochemical properties

3.2.1 Aqueous solubility

The aqueous solubility of α -lipoic acid in PBS (pH 7.4) was determined as 0.33 ± 0.002 mg/ml (32°C), which is similar to a value (0.224 mg/ml) stated in literature by Drugbank (2016). The slight increase in the value may be attributed to the different mediums (water and PBS pH 7.4) used or variations in temperature. The ideal aqueous solubility of a permeant is >1 mg/ml (Naik et al., 2000; Williams, 2003), therefore it is expected that α -lipoic acid may not attain optimal permeation.

3.2.2 Log D

The determined log D value for α -lipoic acid was -0.21 ± 0.03 , which is indicative that the active in question may have difficulties permeating the skin layers, because the ideal log D range for permeation is 1 to 3 (Williams, 2003).

3.2.3 Characterisation of semi-solid formulations

3.2.1 Transmission electron microscope

Both the niosomal and hydrated proniosomal dispersions successfully formed vesicles. Niosomes can range in sizes of between 10 and 1000 nm. Both dispersions formed large unilamellar vesicles with visible dark edges, representing the membranes strength (Arora et al., 2012; Navya et al., 2014). Figure 1 represents a TEM image of the niosomal dispersion, showing the more uniformly shaped membrane that appeared more spherical in shape compared to the hydrated proniosomal vesicles shown in Figure 2.

Figure 1: TEM micrograph of the niosomal vesicles with a size of 277.19 nm

Figure 2: TEM micrograph of the hydrated proniosomal vesicles with a size of 200.68 nm

3.2.2 Viscosity

The hydrated proniosome dispersion had an overall higher viscosity (109.12 ± 19.39 cP) compared to the niosome dispersion with a viscosity of 11.31 ± 0.09 cP. This could be due to the sorbitol added in the formulation process of the proniosomes to achieve dry granules. Higher

viscosity values of dispersions cause increased resistance to the diffusion of the active component through that dispersion, therefore resulting in decreased penetration through the skin (Cross et al., 2001). Consequently, keeping this in mind, the hydrated proniosomal dispersion may not achieve such good diffusion results compared to the less viscous niosomal dispersion.

3.2.3 Potential of hydrogen (pH) determination

The niosomal dispersion had an average pH value of 4.681 ± 0.01 , which was slightly lower than that of the hydrated proniosomal dispersion attaining an average value of 4.736 ± 0.07 . Both dispersions' pH values were within the tolerable range (pH 4.2-5.6) of topical formulations and are therefore considered acceptable (Kharat & Bathe, 2016).

3.2.4 Entrapment efficiency percentage

The EE% for both the niosomal and hydrated proniosomal dispersions were calculated using Equation 2. Results indicated that both dispersions encapsulated α -lipoic acid successfully, but to different extents. The average EE% for the niosomal dispersion was 99.96 ± 0.02 which was higher than the average EE% obtained from the hydrated proniosomal dispersion with a value of 92.621 ± 2.76 .

3.2.5 Zeta-potential

Both the niosomal and hydrated proniosomal dispersions attained high zeta-potential measurements. It is considered that high zeta-potential (positive or negative), typically greater than 30 mV, will maintain a stable dispersion (Honary & Zahir, 2013). The average zeta-potential value of the niosomal dispersion was determined to be -34.7 ± 2.5 , and -43.2 ± 1.3 for the hydrated proniosomal dispersion.

3.2.6 Droplet size and distribution

The average droplet sizes calculated for the niosomal and hydrated proniosomal dispersions were 224.6 ± 8.1 d.nm and 698.3 ± 357.8 d.nm, respectively. Niosomes can range from 10 to 1000 nm depending on the different components in the dispersions (Navya et al., 2014). The polydispersity index (Pdl) for both the niosomal (0.453 ± 0.136) and hydrated proniosomal

dispersions (0.702 ± 0.219) were indicative that both were broadly polydispersed, considering the average Pdl values were higher than $\bar{D} > 0.4$ (Nobbman, 2014).

3.3 Membrane diffusion experiments

Both the niosomal and hydrated proniosomal dispersions released α -lipoic acid to a satisfactory degree. For each dispersion, the average flux value and standard deviation (calculated using the slope of the straight lines for amounts released per time), and the average cumulative amount area ($\mu\text{g}/\text{cm}^2$) of active released in 6 h. The niosomal dispersion released α -lipoic acid to a higher degree than the hydrated proniosomal dispersion, with average %release values of $3.123 \pm 0.30\%$ and $2.128 \pm 0.31\%$, respectively. The resulting average flux values for both dispersions were calculated as $467.49 \pm 51.82 \mu\text{g}/\text{cm}^2\cdot\text{h}$ for the niosomal and $332.01 \pm 49.04 \mu\text{g}/\text{cm}^2\cdot\text{h}$ for the hydrated proniosomal dispersion. It should be kept in mind that the dispersion with the higher release rate is not necessarily the dispersion obtaining better skin diffusion results.

3.4 Diffusion experiment

3.4.1 Transdermal diffusion study

The transdermal diffusion studies revealed that both the niosomal and hydrated proniosomal dispersions carried α -lipoic acid through the skin into the receptor compartments. The calculated average flux from the niosomal dispersions was $187.43 \pm 25.95 \mu\text{g}/\text{cm}^2\cdot\text{h}$ and from the hydrated proniosomal dispersion, $36.94 \pm 6.11 \mu\text{g}/\text{cm}^2\cdot\text{h}$. The lower diffusion of the hydrated proniosomes could possibly be due to the higher viscosity of this dispersion, adding to the overall resistance of the flow of the active ingredient through the dispersion (Cross et al., 2001). Furthermore, the placebo Franz cells did not have any detectable concentrations of active ingredient present in their receptor compartments either and may also be attributed to the rapid reduction of α -lipoic acid to its reduced state, dihydrolipoic acid (Perricone, 2000).

3.5 Tape stripping

The concentrations of α -lipoic acid were examined in each grouping of skin layers, i.e. stratum corneum-epidermis (strip 2-15) and epidermis-dermis (remainder of skin sample).

3.5.1 Stratum corneum-epidermis

HPLC analysis revealed both dispersions delivered α -lipoic acid to the stratum corneum-epidermis (as seen in Figure 3). Results also revealed that the niosomal dispersion obtained slightly higher diffused amounts of active ingredient after 12 h (2.411 ± 1.32 $\mu\text{g/ml}$), compared to the hydrated proniosomal dispersion (2.166 ± 1.47 $\mu\text{g/ml}$). α -Lipoic acid is considered to have higher affinity towards lipophilic regions compared to aqueous environments, which may be the reason that both dispersions delivered detectable concentrations of the active in the stratum corneum-epidermis (Podda et al., 1996). Similar results were obtained from a previous study conducted on the topical delivery of α -lipoic acid by Podda et al., (1996).

3.5.2 Epidermis-dermis

As mentioned before, the intended target site for the delivery of α -lipoic acid was the epidermal skin layer. Figure 3 illustrates the results obtained for both the stratum corneum-epidermis and the epidermis-dermis, which indicated that both the niosomal and hydrated proniosomal dispersions successfully delivered the active, but to a different extent. The niosomal dispersion obtained an average concentration of 5.077 ± 1.47 $\mu\text{g/ml}$, almost double the value from the hydrated proniosomal dispersion (2.854 ± 1.43 $\mu\text{g/ml}$). The difference in concentrations may be attributed to the higher viscosity of the hydrated proniosomes, therefore increasing the overall resistance to the flow of the α -lipoic acid through the dispersion itself (Cross et al., 2001). Furthermore, the results obtained from two individual studies were similar to the results from this study. The α -lipoic acid was found to rapidly penetrate the skin and accumulate in the dermal skin region, possibly due to the sebaceous gland and other lipid-rich components present in this layer (Beitner, 2003; Podda et al., 1996). Once again, no concentrations of the active ingredient were detected in the placebo Franz cells, which may be due to the reduced α -lipoic acid in the skin layers (Perricone, 2000).

Figure 3: The concentrations ($\mu\text{g/ml}$) of active ingredient detected in the (a) stratum corneum-epidermis and the (b) epidermis-dermis skin layers from niosome (N) and hydrated proniosome (PN) dispersions after 12 h. The indicated squares and lines represent the median and average concentrations respectively (n=10).

3.6 Statistical analysis

The correlation between the average flux values ($\mu\text{g}/\text{cm}^2\cdot\text{h}$), both released and diffused, were examined for each dispersion. The results indicated there were no statistically significant correlation between the flux values for both the niosomal ($p=0.2765$) or hydrated proniosomal (340 $p=0.6943$) dispersion. The diffusion data obtained from the stratum corneum-epidermis and epidermis-dermis were further analysed by performing two-way ANOVA tests. Results showed the two skin layers, conjointly, had a statistically significant difference in the average amounts of α -lipoic acid present for both the dispersions ($p=0.0445$) tested and of each skin layer ($p=0.0075$).

Each skin layer was then individually examined, to determine the targeting ability of both (345 dispersions. Both the niosomal and hydrated proniosomal dispersion delivered α -lipoic acid to the stratum corneum-epidermis, with no significant difference ($p=0.6995$). For the epidermis-dermis however, statistically significant differences were identified ($p=0.0404$). A T-test was performed and showed that the hydrated proniosomal dispersion had no statistically significant (350 higher affinity for the epidermis-dermis compared to the stratum corneum-epidermis ($p=0.3046$). The tests again proved that the niosomal dispersion had better targeting ability towards the epidermis-dermis compared to the stratum corneum-epidermis ($p=0.01492$).

It was further decided to perform a non-parametric test, i.e. Mann-Whitney U-test, on both dispersions to compare the p-values obtained from the T-tests. The non-parametric test (355 confirmed the findings from the T-test, showing that the hydrated proniosomal dispersion delivered the active to both skin layers relatively equally ($p=0.2413$), showing no particular affinity towards the epidermis-dermis. As for the niosomal dispersion, significant targeting to the epidermis-dermis was evident compared to the stratum corneum-epidermis ($p=0.0257$). Therefore, the niosomal dispersions' delivery to the target site, namely the dermal layer was (360 better when compared to the hydrated proniosomal dispersion.

4 Conclusion

The physical characterisation of both dispersions (niosomal and hydrated proniosomal dispersion) was performed. The results obtained from the TEM and pH measurements

indicated both dispersions formed clear unilamellar vesicles with pH values that fell within the required ranges for topical formulations. According to the zeta-potential averages values determined for the vesicle dispersions, both showed good and stable values higher than -30 mV. The droplet size and size distribution results pointed to the niosomal dispersion being the carrier of choice for α -lipoic acid, due to the narrower size distribution and smaller average vesicle sizes measured for this system.

α -Lipoic acid is a lipophilic compound, therefore the results determined for the aqueous solubility (0.33 ± 0.002 mg/ml) and log D value (-1.21) during this study are understandable. These properties however indicated that α -lipoic acid may have suboptimal skin penetration during the skin diffusion studies. Results obtained from the release studies showed satisfactory release of α -lipoic acid from both vesicle systems therefore, both the niosomal and hydrated proniosomal dispersions successfully released and delivered the active ingredient to the stratum corneum-epidermis for possible skin penetration. The niosome vesicles released the α -lipoic acid better compared to the hydrated proniosomal vesicles, which may be because the active ingredient has affinity to the added sorbitol in the dispersion, or due to the higher viscosity of the dispersion resulting in a decreased penetration rate through the dispersion itself (Cross et al., 2001).

Transdermal delivery from both dispersions was observed during the skin diffusion studies, resulting in α -lipoic acid concentrations detected in the receptor compartments from both dispersions. Again, the values obtained from the hydrated proniosomal dispersions were found to be smaller compared the concentrations transdermally diffused by the niosomal dispersion. It should be kept in mind that the smaller concentrations of α -lipoic acid that were initially released from the hydrated proniosomal vesicles may influence further diffused concentrations. Additionally, the higher viscosity of the hydrated proniosomal dispersions may also have attributed to the smaller concentrations initially released. The concentrations of α -lipoic acid detected in the receptor compartments (representing the bloodstream) are less than the recommended daily dose of α -lipoic acid for systemic use that ranges between 200-600 mg (Beitner, 2003). The transdermal diffusion of α -lipoic acid did not form part of the intended aim

of the study, but this is not considered a negative adverse effect because of the advantages of oral α -lipoic acid supplementation.

Statistical analysis revealed no significant differences ($p=0.6995$) between the concentrations of active detected in the stratum corneum-epidermis after tape-stripping, from the two dispersions. The accumulation of the α -lipoic acid in the stratum corneum-epidermis can be ascribed to the affinity of this compound for the lipophilic demeanour of this skin layer (Podda et al., 1996).

According to previous diffusion studies conducted on several topical formulations containing α -lipoic acid, similar results were obtained to those observed with the niosomal and hydrated proniosomal dispersions (Beitner, 2003; Podda et al., 1996).

Concentrations of α -lipoic acid were found in the target skin layer, namely the epidermis-dermis. Statistical analysis revealed the niosomal dispersion delivered the active significantly better compared to the hydrated proniosomal dispersion ($p=0.040$). This amelioration in the delivery ability of the niosomal dispersion, compared to the hydrated proniosomal dispersion, may be due to the higher concentrations of active ingredient released followed by the higher amount accumulated in the stratum corneum-epidermis and ultimately reaching the epidermis-dermis.

In conclusion, the targeted delivery of two vesicle systems were examined and compared to determine the most suitable carrier for the topical delivery of α -lipoic acid. The intended target site for α -lipoic acid was the epidermal-dermal layers, which are considered an extensive metabolic region where an antioxidant such as α -lipoic acid can act against reactive species. The niosomal dispersion was found to be the better suited option of vesicle delivery compared to the hydrated proniosomal dispersion.

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Disclaimer

Any opinion, findings and conclusions, or recommendations expressed in this material are those
425 of the authors and therefore the NRF does not accept any liability in regard thereto.

Conflict of Interest

All authors of this article declare there are no conflicts of interests.

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Table 1: List of all ingredients used during the preparation of a niosomal and hydrated proniosomal dispersion (10 ml sample)

Ingredient	Activity	Quantity
α -Lipoic acid	Active ingredient	500 mg
Span [®] 60	Emulsifier	500 mg
Cholesterol	Stabiliser	1000 mg
Chloroform	Organic solvent	10 ml
Sorbitol*	Humectant/carrier	4000 mg
dH ₂ O	Solvent	10 ml

*Carrier used for the proniosomes

Table 2: Chromatographic conditions used during HPLC analysis of samples containing α -lipoic acid

Column used	Venusil XBP C18 (2) 150x 4.6 mm
Mobile phase	45% Acetonitrile (ACN)/0.1% phosphoric acid (H ₃ PO ₄)
Flow rate	1 ml/min
Retention time	The analyte elutes at ± 7 min
Run time	± 9 min
Detection	Diode array detector at 210 nm
Solvent/receptor phase	PBS at pH 7.4

Figure legends

Figure 1: TEM micrograph of the niosomal vesicles with a size of 277.19 nm

Figure 2: TEM micrograph of the hydrated proniosomal vesicles with a size of 200.68 nm

Figure 3: The concentrations ($\mu\text{g/ml}$) of active ingredient detected in the (a) stratum corneum-epidermis and the (b) epidermis-dermis skin layers from niosome (N) and hydrated proniosome (PN) dispersions after 12 h. The indicated squares and lines represent the median and average concentrations respectively (n=10)

Figures:

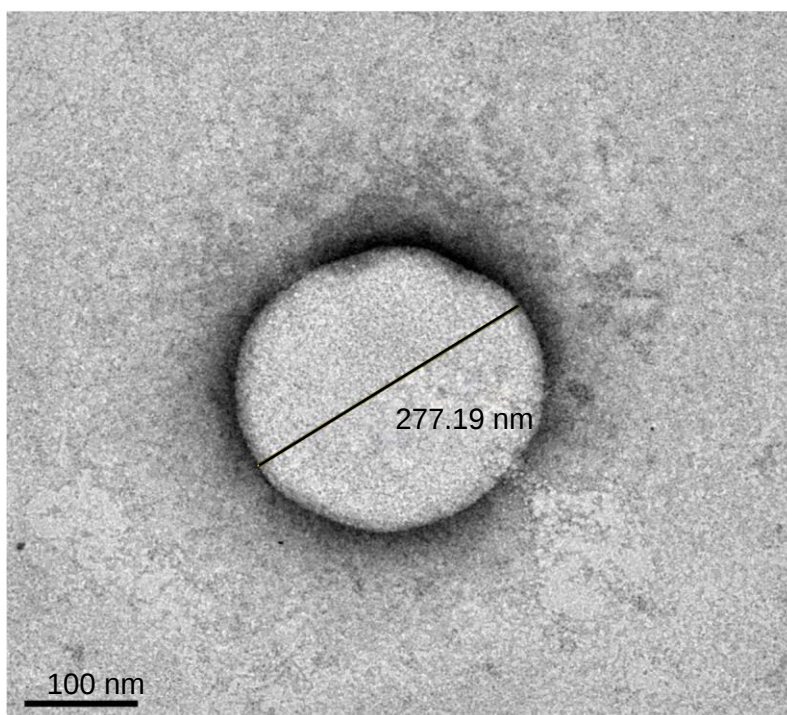


Figure 1: TEM micrograph of the niosomal vesicles with a size of 277.19 nm

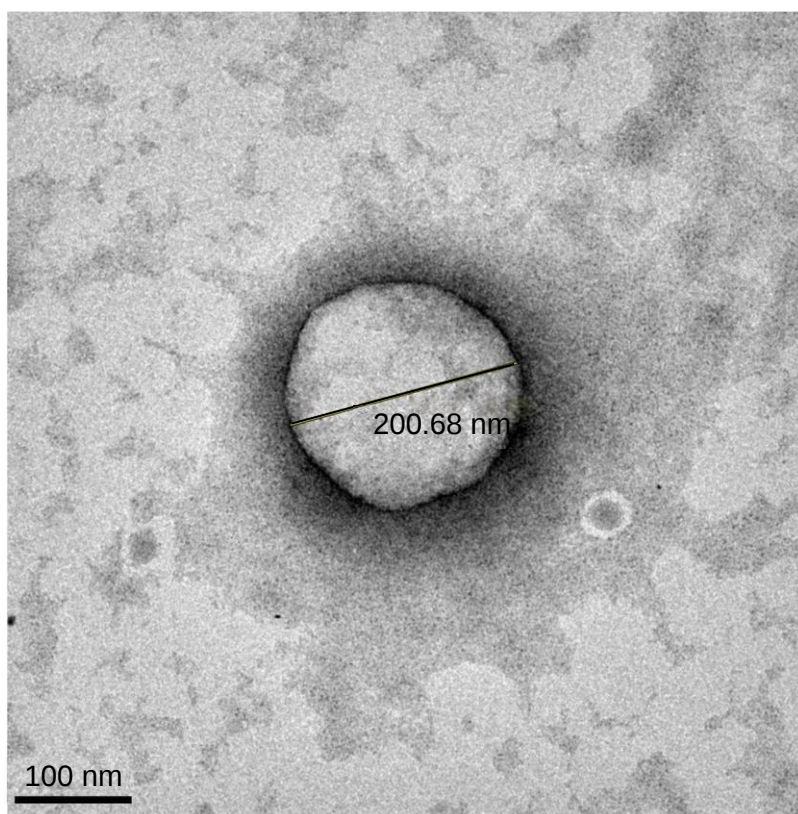


Figure 2: TEM micrograph of the hydrated proniosomal vesicles with a size of 200.68 nm

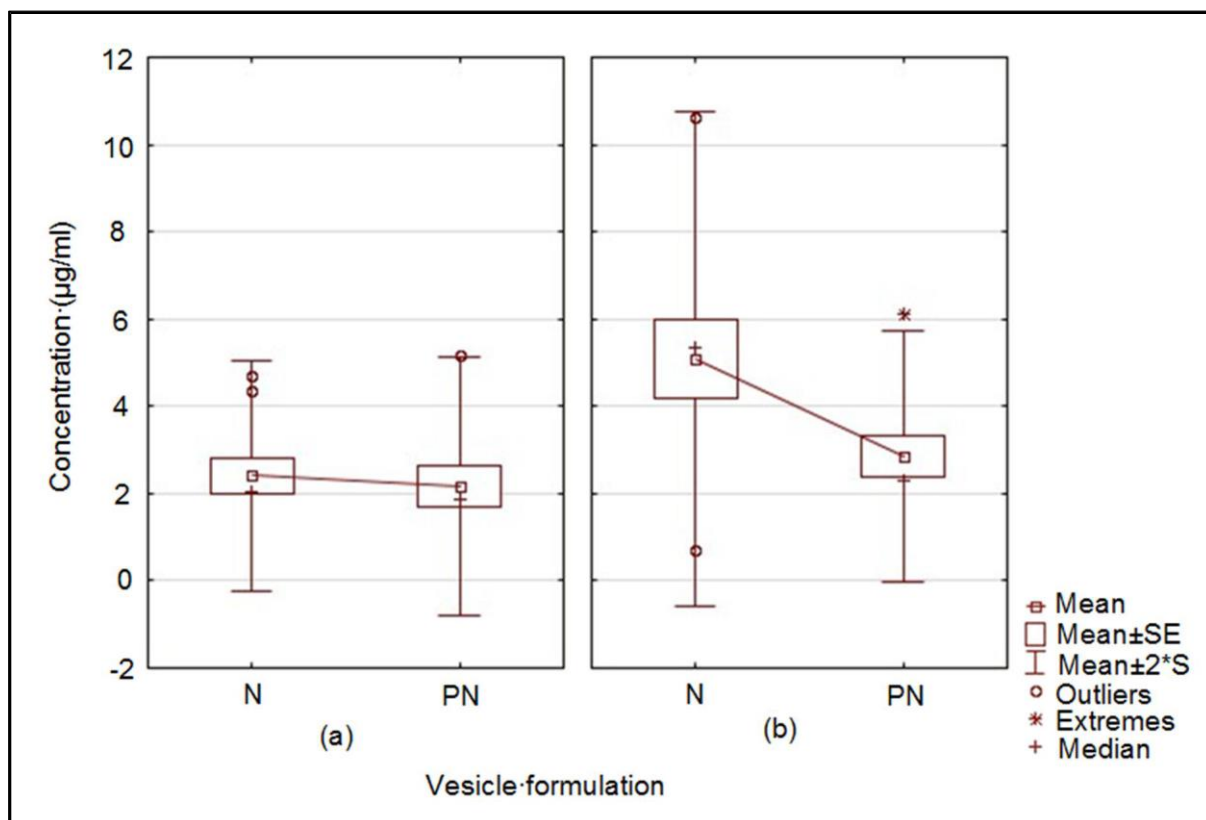


Figure 3: The concentrations (µg/ml) active ingredient detected in the (a) stratum corneum-epidermis and the (b) epidermis-dermis skin layers from niosome (N) and hydrated proniosome (PN) dispersions after 12 h. The indicated squares and lines represent the median and average concentrations respectively (n=10)

Chapter 4

ARTICLE FOR PUBLICATION IN

SKIN PHARMACOLOGY AND PHYSIOLOGY

Chapter 4 is written in article format for the purpose of publication in *Skin Pharmacology and Physiology*. Appendix F includes the complete guide for authors of this journal. No formatting was used, other than advised by the guide for authors, during the writing of this article. However, to ease reading and improve neatness, the paragraphs were justified.

Clinical efficacy of topical formulations containing α -lipoic acid in the treatment of skin ageing and inflammation

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Keywords: α -Lipoic acid, Skin aging, Inflammatory skin disease, Topical delivery, Niosomes

Abstract

Background: The octanoic acid derivative, α -lipoic acid, is an endogenous antioxidant famous for being effective in both lipid and aqueous environments. This unique ability enables the antioxidant to scavenge reactive oxygen species (ROS) in both the aqueous matrix and inside the cells itself. This universal antioxidant can also improve the functionalities of other endogenous antioxidants, such as vitamins E and C, resulting in overall ameliorated defences against oxidative stress. Additionally, α -lipoic acid attains the ability to inhibit certain transcription factors, such as NF- κ B involved in the body's inflammatory response, therefore deeming this compound an anti-inflammatory agent. **Aim of the study:** The aim of the clinical studies was to evaluate the efficacy of a topically applied formulation of niosomal vesicles containing α -lipoic acid for the possible treatment of premature signs of skin ageing and inflammatory skin diseases. **Methods:** Non-invasive measuring instruments were used to evaluate the changes, over time, in skin hydration, -topography, -viscoelasticity and erythema levels. Two separate clinical studies, i.e. anti-ageing (four week) and anti-inflammatory (8 days) studies were conducted. **Results and discussion:** Results obtained showed improvement of skin hydration, scaliness, elasticity parameters (Q0, Q1, Q2) and erythema levels, but no significance was observed between the effects of the active test formulation (ATF) and control groups.

1 Introduction

α -Lipoic acid is a unique and potent antioxidant produced within the body, and is sometimes referred to as the universal antioxidant due to its ability to accumulate in both lipid and aqueous environments [1]. Antioxidants protect cells from oxidative stress by inactivating free radicals before they can cause damage to nearby cells. Oxidative stress is a major contributor to the bodies ageing process, therefore to counterbalance these deleterious free radicals we must have adequate antioxidant defences. With increasing age, the production of ROS (main contributor to oxidative stress) also increases, but the opposite is true for the antioxidants. This imbalance results in the appearance of wrinkles, fine lines and age spots [2, 3, 4, 5]. Another sign of aged skin is the increased inelasticity of the dermal layers, resulting in the skin becoming less capable to respond to external stressors [6].

α -Lipoic acid not only scavenges free radicals, but also possesses the ability to regenerate other antioxidants that were inactivated by these free radicals. It is easily absorbed through skin layers and has significant benefits for skin cells by not only preventing, but also potentially reversing the ageing process [7].

Researchers found the beneficial value of either consuming or topically applying extra α -lipoic acid for increased overall antioxidant activities and the treatment of other medical conditions. The topical application of α -lipoic acid resulted in the improvement of facial lines, skin colour

and skin texture with no instances of peeling or skin irritation [1]. In addition, α -lipoic acid can inhibit transcription factors, such as NF- κ B, involved in the inflammatory systems and attains the ability to down-regulate certain inflammatory processes leading to the attenuation of further cytotoxic cytokine and free radical production [8, 9]. It was found that topical and oral α -lipoic acid may be efficacious for the treatment of inflammatory skin conditions such as psoriasis, atopic dermatitis and dermatitis [10].

To topically deliver and accumulate the α -lipoic acid into the dermal layers of the skin, a vesicle carrier was used. The vesicle system selected was based on its suitability for cosmetic products and its low irritation profile. Consequently, non-ionic surfactant vesicles, niosomes, were used during this study. The encapsulation of the α -lipoic acid into niosomal vesicles aimed to enhance the skin penetration, protect the active compound against degradation, to accumulate the α -lipoic acid in the target dermal layers and mask its unpleasant smell [11, 12].

This study consisted of two separate clinical efficacy studies, i.e. the anti-ageing study where skin hydration, topography and elasticity changes were evaluated over a treatment period of four weeks, and the anti-inflammatory study that examined the erythema levels of the skin after a five-day treatment period.

To conclude, an active test formulation (ATF) consisting of niosomal vesicles with encapsulated α -lipoic acid was assessed by evaluating the following properties:

- skin hydration levels, by evaluating the stratum corneums' water content;
- skin topography changes, by measuring the skins' roughness, scaliness, smoothness and wrinkling (SELS parameters);
- skin elasticity changes (Q-parameters);
- anti-inflammatory activity, by evaluating skins' erythema levels.

2 Materials and methods

2.1 Materials

The ATF contained niosomal vesicles encapsulating α -lipoic acid, which was compared to a placebo (control group) containing no active ingredient. The chloroform used during the preparation of the vesicle dispersions was evaporated using a Büchi® (Switzerland) rotary evaporator, equipped with an Interface I-100, Vacuum pump V-100 Heating bath B-100 and Rotavapor® R-100. The ATF and placebo consisted of all the components listed in Table E.1. For the irritation patch studies, commercially available 1% hydrocortisone acetate (w/w) cream (Biocort cream, Akacia™ HealthCare (Pty) Ltd) was utilised as positive control on the 1% sodium lauryl sulphate (SLS) (w/v) irritated skin areas.

Table 1: List of the ingredients used in the ATF and placebo with their respective functionalities in the preparations

2.2 Methods

The bioengineering, non-invasive instruments used during both clinical efficacy studies included the Corneometer® CM 825 [13] for skin hydration measurements, the Visioscan® VC 98 [14] for topographic changes in the skin surface, the Cutometer® MPA 580 [15] for elasticity assessment and the Mexameter® MX 18 [16] for evaluation of erythema levels. Prior to every study, the calibration status of all instruments was verified according to the calibration check procedures of the instrument manufacturer.

2.2.1 Non-invasive measurements

To measure the hydration level of the skin, the Corneometer® CM 825 [13] was used. The dielectrical constant (electrical capacitance value) of the skin was measured using fringing field capacitance sensors. Changes in the water content of the skin result in changes in the dielectric constant of skin, making it possible to measure accurately even small changes in the skin's hydration levels by means of the precision measuring capacitor. These changes in water content of the skin are converted into arbitrary units (AU) of hydration.

The changes in skin surface topography were evaluated using the Visioscan® VC 98 [14]. This instrument consists of a high resolution charge-coupled device (CCD) camera and two specially arranged halogenide light sources. The resulting ultraviolet light illuminates the skin surface uniformly. To avoid any reflective interference from deeper skin layers, the ultraviolet light's intensity, spectrum and arrangement are adjusted accordingly. The skin area (6 mm × 8 mm) is evaluated by capturing a clear and non-glossy image with the built-in CCD camera; the displayed high-resolution black and white images, clearly showing wrinkles and skin surface properties, are digitised and analysed by SELS® software (Courage & Khazaka electronic GmbH), utilising the Surface Analysis of Living Skin (SELS) method to calculate a number of SELS parameters which are used to assess the skin surface [17].

The measured changes in skin topography by the Visioscan® VC 98 [14] are characterised and quantified by wrinkles (SEw), smoothness (SEsm), roughness (SEr) and skin scaliness (SEsc), parameters. SEw is the proportion of horizontal and vertical wrinkles that recognise aging and wrinkles. SEsm reflects the mean width and depth of the wrinkles. In contrast to SEsm the roughness parameter (SEr) reflects the unevenness and asperity of the skin [18].

Scaliness is associated with the level of skin dryness or dehydration of the stratum corneum and is quantified by SEsc. A lower value indicates less scaliness with more skin hydration [19].

The viscoelastic properties of the skin were evaluated using the Cutometer® MPA 580 [15]. Negative pressure (400 mbar) is applied to the skin with the intended purpose of measuring the resistance (firmness) and response (elasticity) of the skin towards the deformation. After a pre-determined period, the suction is discontinued and the measurements are displayed as a curve, known as the deformation curve [15]. The set of parameters used in the study, known as the Q-parameters, was discovered to be more accurate yet relatively simple to determine when

compared to the conventionally used R-parameters. Therefore, the viscoelasticity properties of the skin were measured using the Q-parameters known as Q0, Q1, Q2 and Q3 [20,21].

To determine the anti-inflammatory activity of the topical formulation, the Mexameter® MX 18 [16] was used. This instrument measures the haemoglobin content in the upper skin layer. Blood is coloured red because haemoglobin absorbs green ($\lambda = 568$) light and reflects red ($\lambda = 660$ nm) light. The differences in light absorption by haemoglobin result in a certain intensity of reflected light which can be measured. The probe of the Mexameter® emits the specific light wavelengths and a receiver measures the light reflected by the skin. As the quantity of emitted light is defined, the quantity of light absorbed by the skin can be calculated. The redness value is expressed as arbitrary units ranging from 0 – 999. Erythema is an increase in skin redness caused by hyperaemia of superficial capillaries and is a result of skin irritation and inflammation. The redness of the skin is directly related to the concentration of haemoglobin in the skin, which increases with hyperaemia of the superficial capillaries. Measuring haemoglobin in the skin will therefore quantify skin redness to the level of erythema; the higher the haemoglobin levels in the skin, the higher the inflammatory action present [16].

2.2.2 Volunteers

In vivo studies were done to evaluate the anti-ageing and anti-inflammatory abilities of a topical α -lipoic acid formulation. Twenty-four human volunteers were enrolled for each clinical study and all were required to meet the pre-set inclusion and exclusion criteria. The criterion was designed to minimise inter-volunteer variations and covered topics related to age, gender, skin type, ethnicity and other health conditions. To reduce variation between skin anatomical differences, Caucasian (Skin Fitzpatrick I-III) female volunteers were enrolled in the studies [22,23]. Clear instructions were given to the volunteers on the washout period, which commenced seven days prior to each study. During this period, all volunteers were required only to use the supplied Dove® soap on the tested skin sites for the remainder of the studies. Normal cleansing routines were allowed however not on the specific tested skin sites, as this would influence the results. Due to the nature of both the anti-aging and anti-inflammation studies, no volunteer was allowed to topically apply or ingest products containing vitamin A (or derivatives thereof) and corticosteroids within three months prior to the commencement of the studies [24,25,26].

2.2.3 Treatment protocol: Anti-ageing and erythema study

Due to the variations in hydration levels on the different anatomical sites of the body, both clinical studies used the volunteers' volar forearms as the treatment areas [27]. The ATF was applied to the volunteers' dominant arm and the placebo (control) was applied on the non-dominant forearm. Initial baseline measurements were taken to determine each volunteer's natural moisture and skin properties to attain more valid results. To further limit variations, each volunteer received a marked stencil to ensure the exact same skin site was continuously treated

and tested. The precise amount of formulations that were applied per application, were pre-calculated to ensure the concentration of active was between 1-3 $\mu\text{l}/\text{cm}^2$ (mg/cm^2).

The four week anti-ageing study was conducted to evaluate the effects α -lipoic acid encapsulated in niosomal vesicles had on the hydration, topography and elasticity of the skin. Formulations were applied twice daily between 6:00 and 08:00 in the morning and 18:00 and 20:00 in the evening. Due to the small quantity needed for each application, the topical formulations were given in colour-coded, pre-filled syringes to remain anonymous to the volunteers.

The erythema study consisted of the evaluation of α -lipoic acids' anti-inflammatory action over a period of five days. The study also included a seven-day washout phase, followed by a two-day irritation period and five-day treatment period. Pre-irritated skin was required to measure percentage improvement of the erythema levels over time and therefore, 1% SLS was used to induce inflammation to the tested skin sites [28].

During the erythema study, initial measurements were taken on five specified areas on the non-dominant inner arm, which measured each volunteer's natural erythema levels (skins' redness). Thereafter, an occlusion patch containing 1% SLS in four Finn Chambers[®], with one empty chamber to serve as a control, was applied to the volunteers' arm. After 24 h, the patch was removed and inspected for any leakage, which may have occurred during the occlusion period. Volunteers were instructed to refrain from exercise or bringing the patch into contact with water, to ensure the irritant remained in contact with the skin. After removal, the skin was left for a further 24 h to enable it to fully develop its inflammatory response. Measurements were taken on day 3 (T0) and compared to each volunteers' initial measurement to ensure inflammation had been induced. Three pre-filled, color-coded syringes containing topical formulations were then given to each volunteer. Table 2 summarises the different skin sites, whether it was pre-irritated and which product was applied. For the purposes of the anti-inflammatory assessment, formulations were applied three times daily and measurements were taken on days 1 (T0), 2 (T1), 3 (T2) and 6 (T3).

Q-parameters were used to analyse the changes in the skin's viscoelasticity properties, i.e. maximum recovery area (Q0), elastic recovery (Q1), viscous recovery (Q2) and viscoelastic recovery (Q3).

Table 2: Tested skin sites and products applied during clinical efficacy studies

2.3 Statistical analysis

The raw data obtained for each parameter was evaluated by means of statistical analysis. The anti-ageing and erythema patch study was conducted to examine the properties of the ATF in comparison to the placebo (control group) at 95% probability, as a function of time for the duration of treatment ($p < 0.05$).

Statistical analyses were carried out using IBM SPSS Statistics Version 23. Linear mixed models were used to assess the influence of treatment and time on the various clinical markers, namely the hydration level, SELS parameters (surface analysis of living skin), elasticity (Q-parameters) and erythema levels. An unstructured or first-order autoregressive (AR(1)) covariance structure was used. Treatment and time were treated as Fixed effects and subjects as Random effects. p-Values were obtained from the Type III Test for Fixed effects. Bonferroni adjustment for multiple comparisons was made and

Cohen's d-value was used to evaluate the practical significance of the results. An effect size (d)≈0.20 is indicative of a small effect or practical non-significant difference, d≈0.50 points towards a medium effect or practical visible difference; whereas d≈0.80 signifies a large effect or practical significant difference [27].

The percentage change over time, relative to each parameters baseline values (T0), was calculated using the following equation:

$$\text{Percentage change (\%)} = ((T_x - T_0) / T_0) \times 100 \quad \text{Equation 1}$$

Where:

T0 = Baseline measured value for each parameter

Tx = Measured values at the different time intervals for each clinical study

3 Results and discussion

3.1 Anti-ageing study

3.1.1 Skin hydration level

Results from the statistical analysis revealed the ATF (p=0.008) and the placebo (p=0.001) significantly increased the hydration levels of the tested skin sites after a four week treatment period. After this period, the applied ATF increased the hydration levels by 13.00%; whereas the placebo increased the value by 23.75%. The percentage hydration changes over time are graphically depicted in Figure 1.

Figure 1: Percentage change in skin hydration levels over time for the ATF and placebo after four weeks treatment

3.1.2 Skin surface topography change

3.1.2.1 Roughness

Both the ATF and placebo increased the skin roughness over a treatment period of four weeks, but to no statistical significant degree according to the p-values calculated at each time point. Hence, results obtained revealed no statistical significant differences in the values for neither the ATF (p=0.469), nor the placebo (p=0.235) over the four week treatment period; furthermore, the skins' roughness increased by 4.92% after treatment with the ATF and by 11.68% with the placebo. Figure 2 illustrates the percentage changes over time for both the ATF and placebo.

Figure 2: Percentage change in skin roughness over time for the ATF and placebo after four weeks treatment

3.1.2.2 Scaliness

Results obtained for the skin scaliness changes revealed no statistical significant differences after treatment with neither the ATF ($p=0.07$), nor the placebo ($p=0.058$) over four weeks. Both formulations decreased the skin scaliness parameter measurements over the treatment period, indicating a positive effect on the scaliness of the skins' surface, however, no practical significant differences in the data were identified by the Cohen's test. The ATF treatment reduced skin scaliness with 9.98% and the placebo with 12.15% (as seen in Figure 3).

Figure 3: Percentage change in skin scaliness over time for the ATF and placebo after four weeks treatment

3.1.2.3 Smoothness

The skins' smoothness decreased after four weeks treatment with both the ATF and placebo. Statistical significant reduction was identified for the placebo ($p=0.039$), but not for the ATF ($p=0.193$) over the four week treatment period. After the treatment period the ATF resulted in a 3.62% reduction in overall skin smoothness, a smaller effect compared to the placebo, which caused the smoothness to decrease by 9.62% (as seen in Figure 4).

Figure 4: Percentage change in skin smoothness over time for the ATF and placebo after four weeks treatment

3.1.2.4 Wrinkling

Unfortunately, the wrinkle appearance of the skin increased after the four week treatment with both formulations. No statistical significant differences in values were identified for either the ATF ($p=0.549$) or the placebo ($p=0.071$). Figure 5 shows the percentage changes over time in the wrinkle appearance of the skin for both tested formulations. After four weeks, the treatment with ATF resulted in an increased wrinkle appearance of 10.21% compared to the baseline measurement. A smaller effect was observed by the placebo, which caused the value to increase by 0.10% after the treatment period.

Figure 5: Percentage change in wrinkle appearance over time for the ATF and placebo after four weeks treatment

3.1.3 Skin elasticity change

3.1.3.1 Q0 parameter

Following the treatment period of four weeks, there was a statistical significant difference in the measured values of parameters Q0 ($p=0.005$), but this was not found to be true for the other parameters assessed.

A decreasing value determined for this parameter represents an increase in skin firmness [15]. After the four weeks treatment, both the ATF and placebo ($p=0.085$) resulted in increased skin firmness; the ATF increased the skins' firmness to a statistical significant ($p=0.005$) degree, whereas the placebo ($p=0.085$) change was found not statistically significant. The percentage changes over time in the mean values are shown in Figure 6.

Figure 6: Percentage change in the Q0 parameter values over time for the ATF and placebo after four weeks treatment

Both the ATF and placebo delivered a skin firming action over the treated period, but to different extents. The application of ATF resulted in a 7.24% increase in skin firmness compared to the placebo's 4.35%.

3.1.3.2 Q1 parameter

The application of both the ATF and placebo resulted in the increase in this parameter. The elastic recovery of the skin increases with an increase in Q1 parameter values [15]. No statistical significance was identified between the measured values from the baseline measurement and after the four week treatment with the ATF ($p=0.182$) or the placebo ($p=0.636$).

Figure 7: Percentage change in the Q1 parameter values over time for the ATF and placebo after four weeks treatment

The ATF and placebo increased the skins elasticity as seen from Figure 7. After the treatment period, the application of ATF resulted in a 2.26% increase in the Q1 parameter and the placebo produced a 2.57% improvement.

3.1.3.3 Q2 parameter

The viscous recovery of skin increases with an increase in the Q2 [15]. Results of both tested formulations showed increases in the viscous recovery over the treatment time. No statistical significant differences were identified between the baseline measurements and the T4 measurements for the ATF ($p=0.096$) or the placebo ($p=0.602$). The changes in Q2 parameter values are graphically depicted over time in Figure 8.

Figure 8: Percentage change in the Q2 parameter values over time for the ATF and placebo after four weeks treatment

From Figure 8, it can be seen that the ATF initially revealed a delayed action, followed by a constant increase to T4. The placebo produced fluctuations in the Q2 parameter values, but ultimately resulted in a 3.23% increase in the viscous recovery of the skin. The ATF produced an increase in Q2 parameter values of 3.83% at the last measurement, leading the opponent placebo.

3.1.3.4 Q3 parameter

An increase in the Q3 parameter value means the overall viscoelasticity of the skin increased [15]. The ATF reduced the Q3 parameter measurements over the treatment time of four weeks, whereas the placebo increased the Q3 parameter value. None of the differences in measured values were identified as statistically significant for either the ATF ($p=0.438$) or the placebo ($p=0.851$) after the four week treatment period. The percentage changes over time are depicted in Figure 9.

Figure 9: Percentage change in the Q3 parameter values over time for the ATF and placebo after four weeks treatment

From Figure 9, it is evident the two tested formulations resulted in different effects on the Q3 parameter values. The ATF decreased the viscoelastic recovery by 1.49% over four weeks, whereas the placebo increased the value by 1.57%.

3.2 Erythema study

The erythema measurements, following initial skin irritation (T0), were compared to the erythema values after the five days treatment period. The skin areas treated with the 1% hydrocortisone cream (w/w), placebo, ATF and the negative control all showed statistical significant differences in the erythema levels between the measured T0 values and the values obtained after the five days of treatment (T3). The untreated skin did not show any statistically significant decrease ($p=0.147$) in the erythema levels measured after the five-day period, but did reduce the inflammation slightly.

As anticipated, the treatment with the positive control, namely the 1% hydrocortisone cream (w/w), caused a statistical significant decrease in the erythema values measured after the five-day treatment period. No effect was seen between T0 to T1 measurements for the positive control, which could be indicative of a possible delayed effect of the 1% hydrocortisone cream (w/w). This means the anti-inflammatory action of this particular cortisone cream only takes effect after 24 h.

The negative control, the area pre-irritated but not treated with any formulations, showed constant increased anti-inflammatory activity by producing statistically significant differences at T1 ($p=0.016$), T2 ($p=0.005$) and T3 ($p=0.001$).

Using Cohen's test for practical significant differences in values, the results revealed the ATF ($d=0.752$), positive control ($d=0.777$) and placebo ($d=0.673$) all illustrated visible significant differences in the baseline measured values compared to the post-treatment erythema values. The percentage improvement in erythema levels and mean measured values for each individual skin site are summarised in Figure 10.

Table 3: The mean Mexameter® values measured and percentage changes in erythema levels for each individual skin site

The positive control had the best anti-inflammatory action after five days treatment and decreased the erythema levels by 16.6%, the negative control decreased the erythema levels and reduced the inflammation by 9.4% and the ATF resulted in a 13.9% decrease in erythema levels (almost similar as for the placebo) attaining a slightly smaller outcome of 13.8%. The skin site without any irritant or treatment also decreased in erythema levels by 5%. The percentage changes in erythema levels over time for each tested skin site are graphically illustrated in Figure.10.

Figure 10: Percentage change in skin erythema levels for all tested skin sites during the erythema study

No difference in the anti-inflammatory action between the ATF and placebo were identified after five days treatment. Both formulations produced statistical and practical significant differences in the erythema levels after 48 h of treatment.

4 Conclusion

Results obtained revealed the ATF and placebo both improved the hydration levels, scaliness, erythema levels and some elasticity parameters (Q0, Q1 and Q2). Additionally, the skins' roughness, smoothness, wrinkling and the Q3 parameter did not show improvement after four weeks treatment with either tested formulation. A possible explanation for this is that the amount of active ingredient is not adequate for the desired anti-ageing and anti-inflammatory effects. The daily dosage of α -lipoic acid is 300-600 mg, which is extensively higher than the amount of active present in the ATF.

Measurements obtained using the Corneometer® revealed statistical significant increases in the hydration levels of the tested skin sites after treatment with the ATF as well as the placebo. Conclusively, the treatment with both the ATF and the placebo resulted in increased skin hydration levels therefore no distinguished hydration effect could be attributed to the active ingredient.

When the skins' condition improves, the Visioscan® clinical SELS parameters used during the topography evaluations should have decreased (roughness, scaliness and wrinkling) with only the smoothness parameter showing an increased value. The ATF decreased the scaliness of the skin, but also the smoothness parameter. Additionally, after treatment of the ATF the roughness and wrinkle appearance of the skin also increased, therefore, the application of ATF did not result in improved skin topographic properties.

The Q-parameters measured by the Cutometer® were used to evaluate the elasticity changes in the skin. For an overall improvement of elasticity, Q0 must decrease and parameters Q1, Q2 and Q3 must increase. Results revealed the ATF decreased the Q0 parameter significantly ($p=0.005$), therefore increasing the skin firmness. Q1 and Q2 parameters also showed improvement after the four week treatment with ATF with 2.26% and 3.83% increases,

respectively. Furthermore, the Q3 parameter decreased by 1.49% after the treatment with ATF, contradictive to the aforementioned results obtained; the control formulation caused this parameter to increase.

Following the results evaluated from the anti-inflammatory study, the application of ATF resulted in a 13.9% reduction in erythema levels of pre-irritated skin after five days' treatment. Although the ATF showed anti-inflammatory action, the placebo also caused the erythema levels to decrease by 13.8%, therefore, the anti-inflammatory action cannot be attributed to the presence of the active ingredient. As expected, the positive control delivered the best anti-inflammatory action by a 16.6% decrease in erythema levels.

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Disclaimer

Any opinion, findings and conclusions, or recommendations expressed in this material are those of the authors and therefore the NRF does not accept any liability in regard thereto.

Conflict of interests

The authors declare there is no conflict of interest.

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Tables:**Table 1:** List of the ingredients used in the ATF and placebo with their respective functionalities in the preparations

Ingredients	Quantity	Functionality
α -Lipoic acid*	5 g	Active ingredient
Span [®] 60	5 g	Emulsifier
Cholesterol	10 g	Stabiliser
Chloroform	100 ml	Organic solvent (evaporated)
Xanthan gum	2 g	Thickener/Stabiliser
dH ₂ O	100 ml	Organic solvent

Table 2: Tested skin sites and products applied during clinical efficacy studies

Skin area	Pre-treatment	Product applied
1	1% SLS pre-treatment	Positive control – 1 % cortisone cream
2	1% SLS pre-treatment	Placebo
3	1% SLS pre-treatment	ATF
4	1% SLS pre-treatment	Negative control – No product applied
5	No pre-treatment	No product applied

Table 3: The mean Mexameter® values measured and percentage changes in erythema levels for each individual skin site

Treatment	Time point	Baseline changes (Tx - T0)	
		Mexameter® MX 18 (mean ± SD)	%Change over time (%)*
Positive control: 1% hydrocortisone cream (w/w)	T0	341.30±78.13	-
	T1	338.69±65.65	-0.4%
	T2	322.12±64.36	-4.7%
	T3	280.56±54.65	-16.6%
Placebo	T0	306.21±66.82	-
	T1	297.53±62.85	-2.1%
	T2	283.40±55.90	-6.6%
	T3	261.22±50.98	-13.8%
ATF	T0	304.90±59.18	-
	T1	298.51±63.94	-1.5%
	T2	289.12±51.65	-4.6%
	T3	260.42±48.16	-13.9%
Negative control	T0	304.54±60.24	-
	T1	289.84±60.21	-4.4%
	T2	287.31±55.82	-5.1%
	T3	274.45±56.44	-9.4%
Untreated skin	T0	231.62±55.09	-
	T1	219.90±51.59	-3.9%
	T2	218.01±54.15	-5.0%
	T3	215.57±45.20	-5.4%

*Calculated using Equation 1

Figure legends:

Figure 1: Percentage changes in skin hydration levels over time for the ATF and placebo after four weeks treatment

Figure 2: Percentage changes in skin roughness over time for the ATF and placebo after four weeks treatment

Figure 3: Percentage changes in skin scaliness over time for the ATF and placebo after four weeks treatment

Figure 4: Percentage changes in skin smoothness over time for the ATF and placebo after four weeks treatment

Figure 5: Percentage changes in wrinkle appearance over time for the ATF and placebo after four weeks treatment

Figure 6: Percentage changes in the Q0 parameter values over time for the ATF and placebo after four weeks treatment

Figure 7: Percentage changes in the Q1 parameter values over time for the ATF and placebo after four weeks treatment

Figure 8: Percentage changes in the Q2 parameter values over time for the ATF and placebo after four weeks treatment

Figure 9: Percentage changes in the Q3 parameter values over time for the ATF and placebo after four weeks treatment

Figure 10: Percentage changes in skin erythema levels for the all tested skin sites during the erythema study

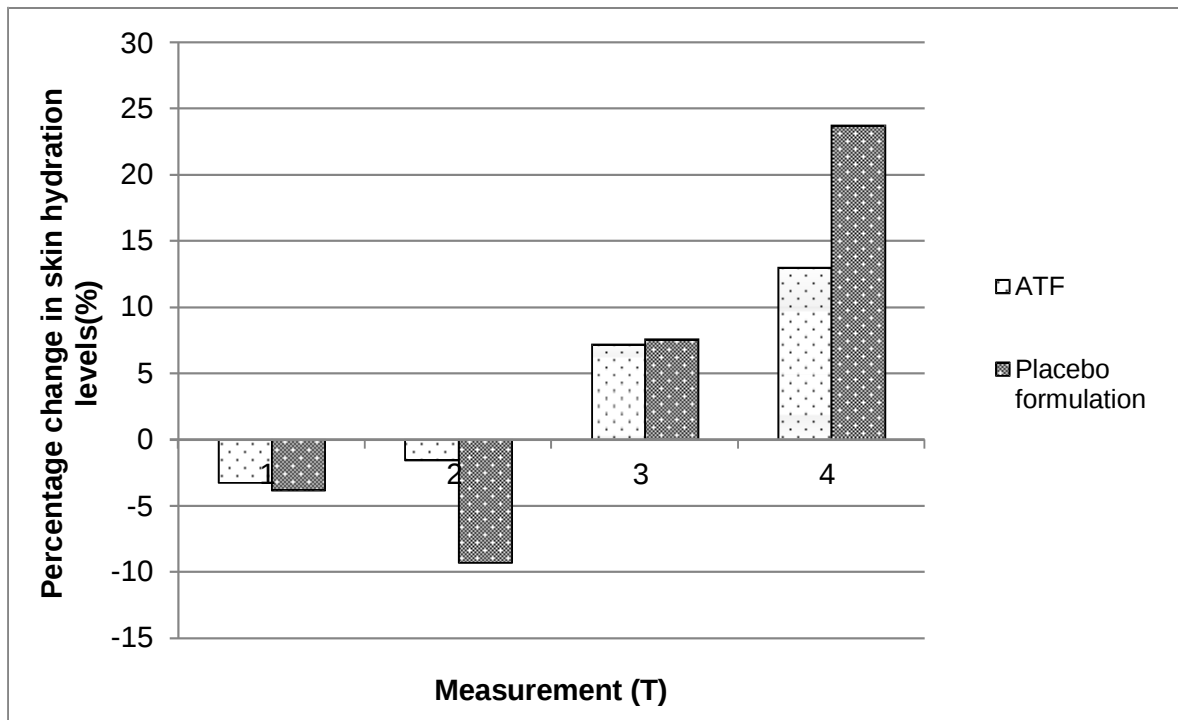


Figure 1: Percentage changes in skin hydration levels over time for the ATF and placebo after four weeks treatment

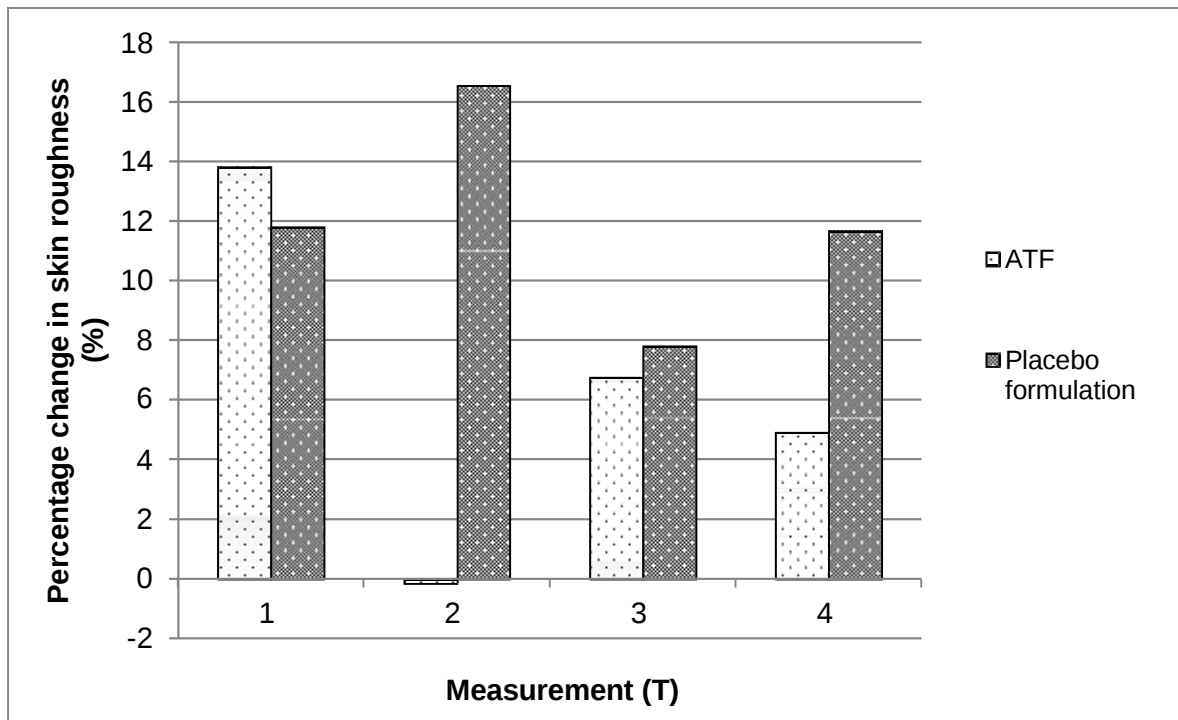


Figure 2: Percentage changes in skin roughness over time for the ATF and placebo after four weeks treatment

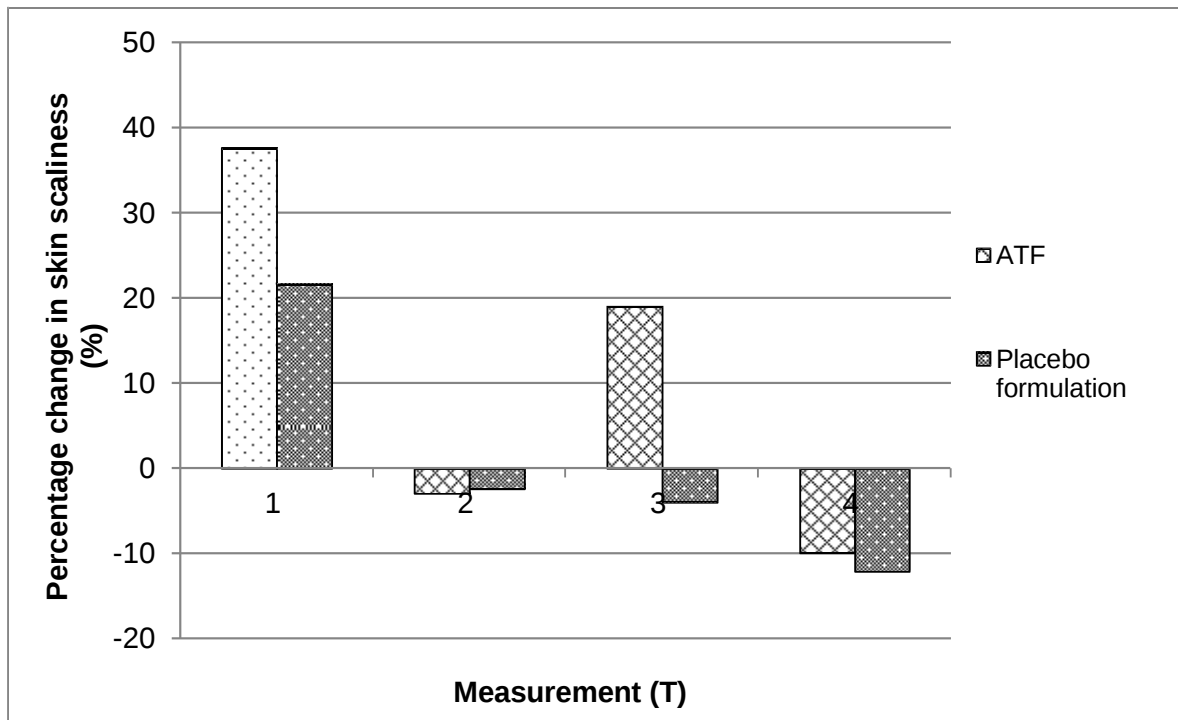


Figure 3: Percentage changes in skin scaliness over time for the ATF and placebo after four weeks treatment

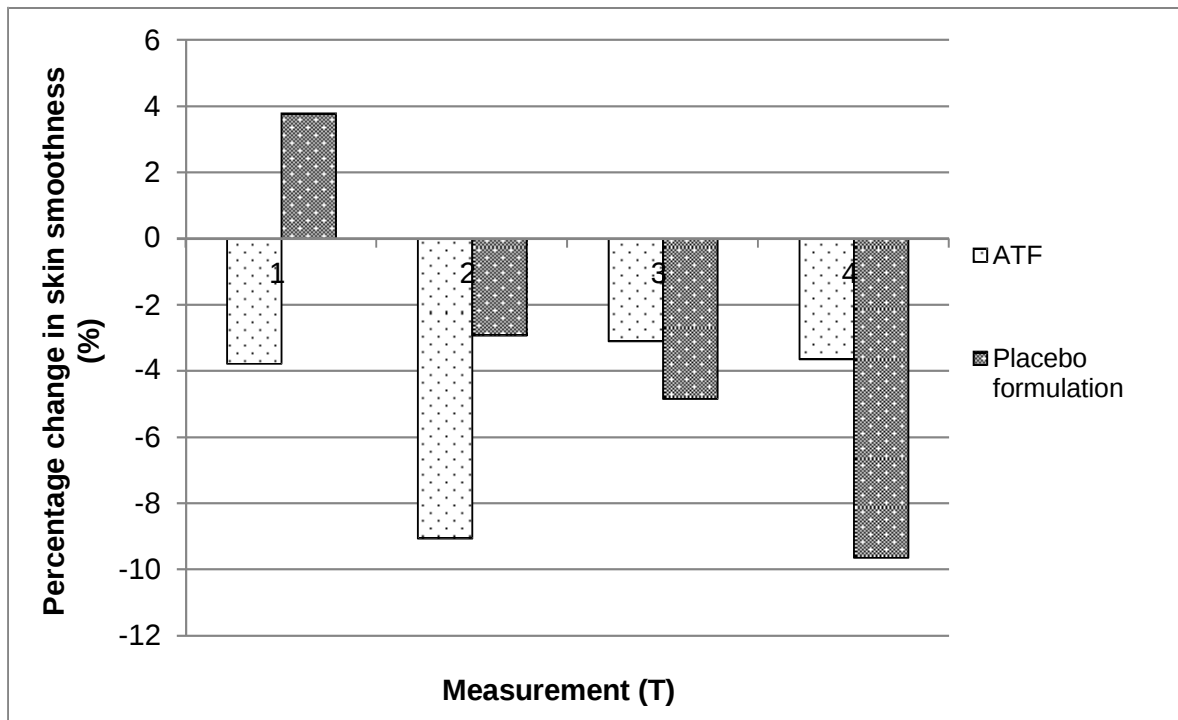


Figure 4: Percentage changes in skin smoothness over time for the ATF and placebo after four weeks treatment

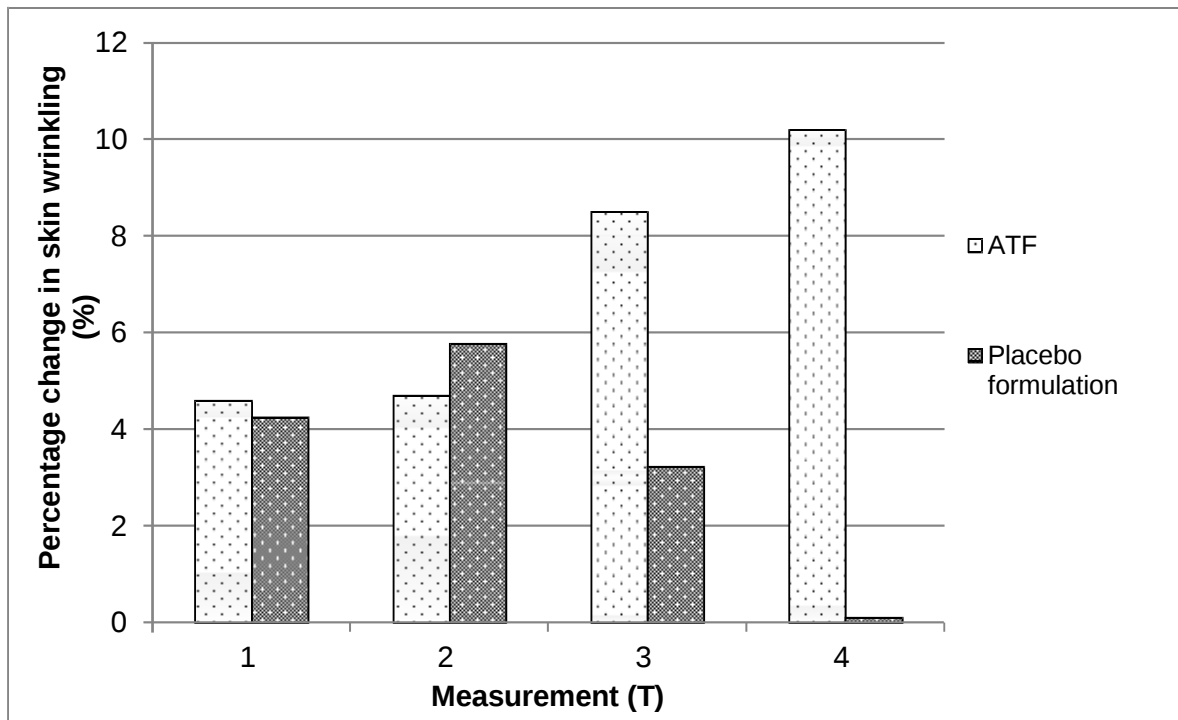


Figure 5: Percentage changes in wrinkle appearance over time for the ATF and placebo after four weeks treatment

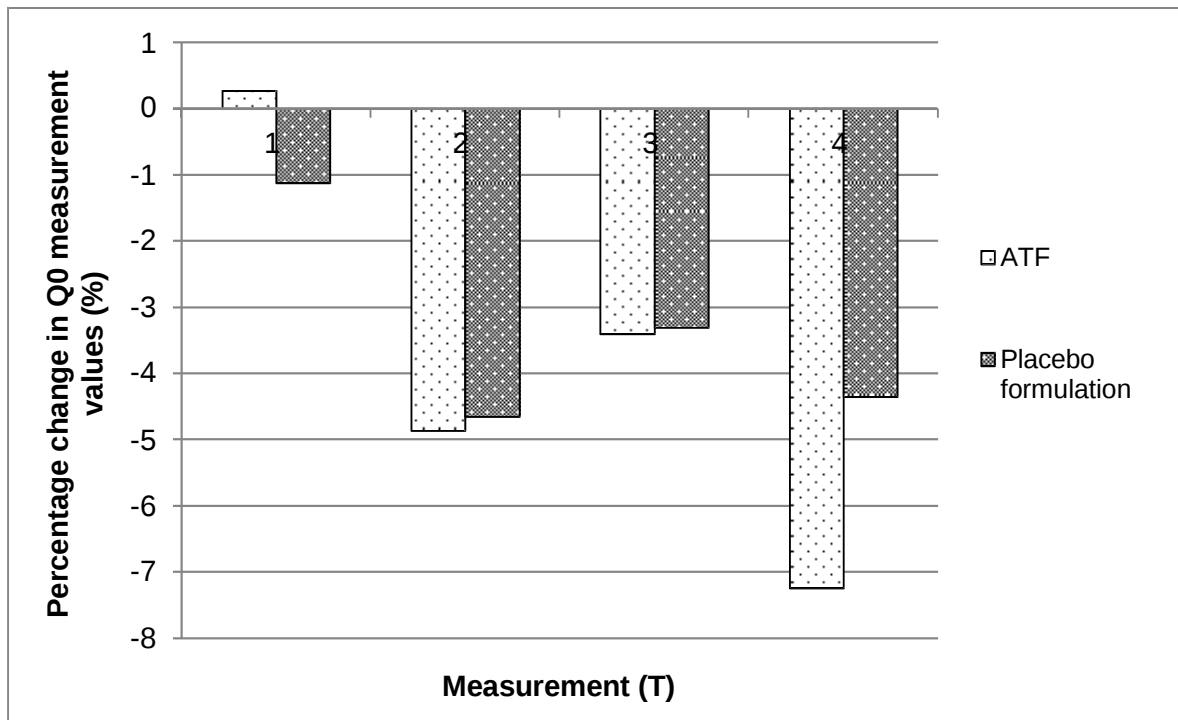


Figure 6: Percentage changes in the Q0 parameter values over time for the ATF and placebo after four weeks treatment

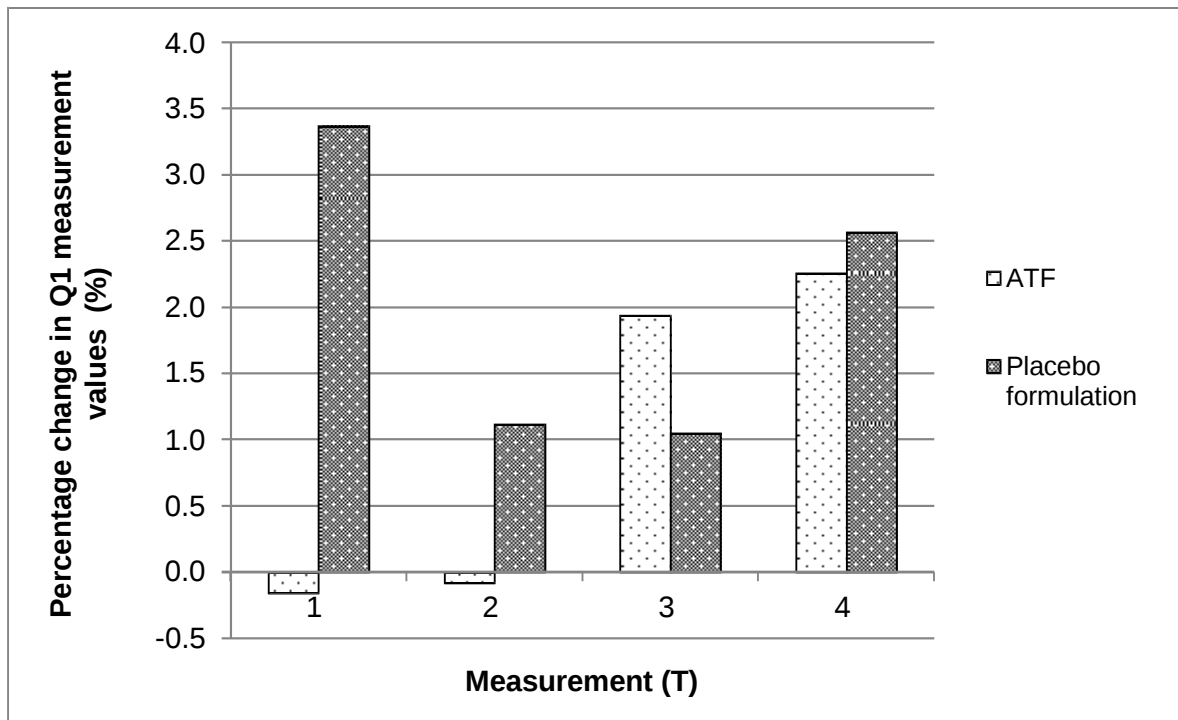


Figure 7: Percentage changes in the Q1 parameter values over time for the ATF and placebo after four weeks treatment

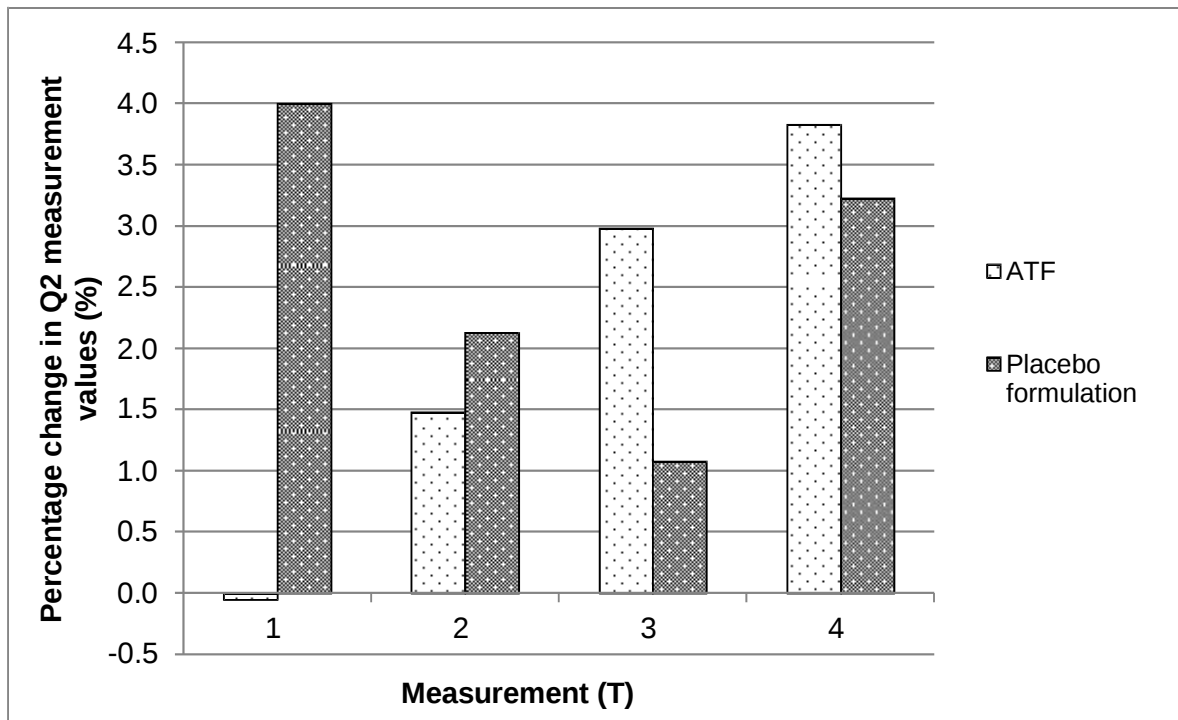


Figure 8: Percentage changes in the Q2 parameter values over time for the ATF and placebo after four weeks treatment

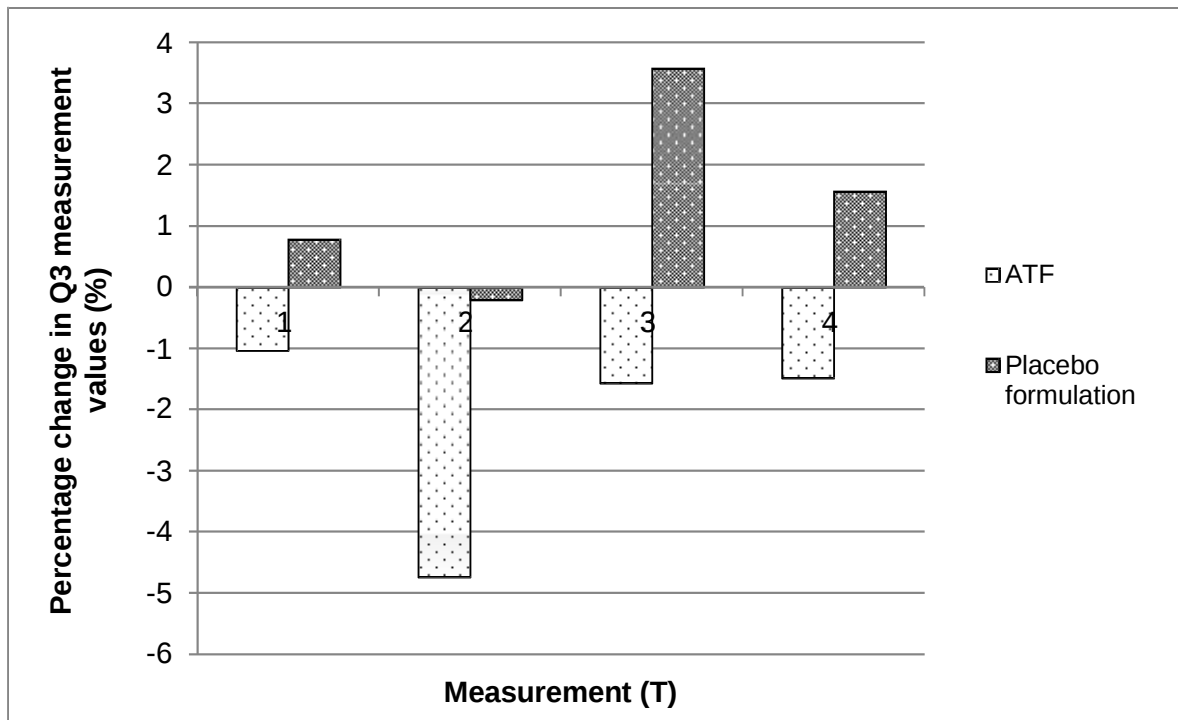


Figure 9: Percentage changes in the Q3 parameter values over time for the ATF and placebo after four weeks treatment

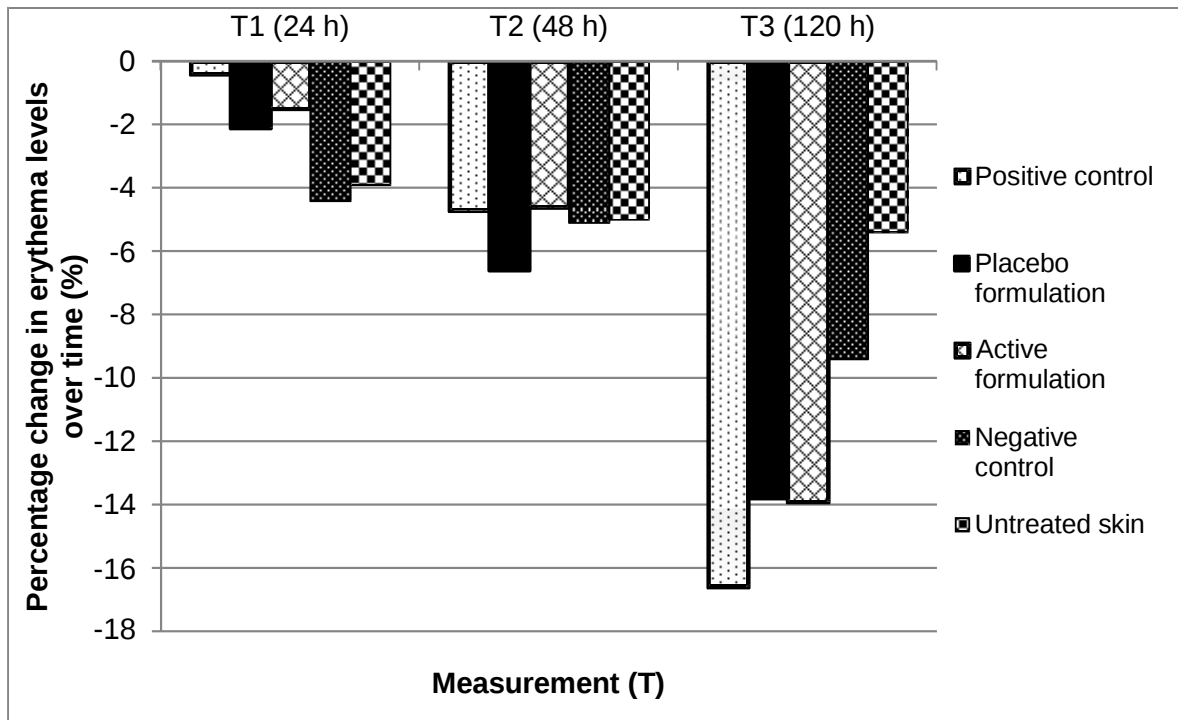


Figure 10: Percentage changes in skin erythema levels for the all tested skin sites during the erythema study

Chapter 5

FINAL CONCLUSION AND FUTURE PROSPECTS

The tenacious stratum corneum layer is deemed the restricting layer for any active ingredient that aims to permeate the skin. The complex and fairly unique composition of the components present in this layer sets the requirements for topical formulations at high standards. The transdermal delivery of active compounds provides advantages such as the circumvention of the first-pass metabolism, painless self-administration and convenient application directly to the target sites (Menon, 2002:S4; Prausnitz & Langer, 2008:1261; Savoji *et al.*, 2014:1).

The endogenous antioxidant used as the active ingredient during this study was selected based on its potent abilities to ameliorate the internal antioxidant defences and act as a topical anti-inflammatory agent. α -Lipoic acid can scavenge reactive species itself and has additional abilities to regenerate other antioxidants, ultimately increasing their concentrations. Unfortunately, this compound is susceptible to various degradation processes and is considered to be poorly soluble in water although known as an amphiphilic molecule (Biewenga *et al.*, 1997:315; Lee & Hughes, 2002:409; Li & Lim, 2016:253; Maczurek *et al.*, 2008:1465; Rochette *et al.*, 2013:116).

Therefore, the aim of this study was to formulate niosomal and hydrated proniosomal vesicles, which not only successfully encapsulated α -lipoic acid, resulting in the protection of the compound against degradation, but also enhanced its penetrations and accumulated the active inside the dermal layers where needed.

In order to achieve these aforementioned aims, the following objectives were set:

- The development and validation of a HPLC method to quantitatively determine the concentrations α -lipoic acid in both vesicle systems.
- The determination of α -lipoic acid's aqueous solubility and log D values.
- The formulation of niosomal and hydrated proniosomal dispersions for topical delivery containing α -lipoic acid. The characterisation of both the niosomal and hydrated proniosomal dispersions in terms of formation, droplet size, entrapment efficacy and zeta-potential.
- Conducting membrane release studies on both dispersions to determine the release of α -lipoic acid from each vesicle system.

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- The transdermal delivery of the active ingredient in terms of skin diffusion studies.
 - Performing the tape stripping method to determine the concentration of α -lipoic acid delivered to the dermal target layers by each dispersion.
 - Conducting clinical efficacy studies to evaluate the effectiveness and possible skin reactions to the best suited vesicle system containing the active ingredient compared to placebo formulations.

The new HPLC analytical method was developed and validated with the guidance and expertise of Prof Jan du Preez. The method proved reliable, accurate and reproducible, therefore it was suited for its intended purpose throughout this study. This method was used to analyse all samples during the study, including the samples obtained from the Franz cells during the diffusion studies.

The aqueous solubility of α -lipoic acid determined in this study was found to be 1.33 ± 0.002 mg/ml (32 °C) in phosphate buffer solution (PBS, pH 7.4). This value is close to the value 0.224 mg/ml stated in literature by Drugbank (2016) and by Toxnet (2011), reporting an aqueous solubility of 0.127 mg/ml (25 °C) for α -lipoic acid. Possible reasons for the differences in the values determined may be attributed to the temperature changes or different mediums used in the experiments. The skin permeation of α -lipoic acid was not considered ideal, according to Naik *et al.* (2000:319), because it did not meet the required aqueous solubility for ideal skin permeation of > 1 mg/ml.

The log D value determined also indicated that the penetration of α -lipoic acid might be suboptimal, considering it was found to be -0.21 ± 0.03 , which did not fall into the acceptable range of 1 to 3 for ideal skin permeation (Williams, 2003:36). The value determined in this study closely resembles the value obtained from a similar study on α -lipoic acid that was found to be -0.78 (Snyman, 2009:56).

Both the niosomal and hydrated proniosomal dispersions appeared as milky, off-white homogenous dispersions, with a creamy texture and a unique smell. The characterisation of both vesicle dispersions revealed good and acceptable pH results and formed well-defined unilamellar vesicles according to the classification described by Arora *et al.* (2012:97). The average zeta-potential values indicated both the niosomal (-34.7 ± 2.5 mV) and hydrated proniosomal (-55.9 ± 2.2 mV) dispersions were good and stable, as the values were greater than -30 mV (Honary & Zahir, 2013:270). The droplet size and polydispersity index (Pdl) measurements also indicated both dispersions were broadly polydispersed due to the obtained value of $\text{Đ} > 0.4$, and the distribution curves were fairly narrow and similar in shape (Nobbmann, 2014).

α -Lipoic acid was released from both vesicle dispersions and delivered to the stratum corneum-epidermis for the purpose of skin permeation. The average cumulative amount of α -lipoic acid per unit area was found to be higher for the niosomal dispersion, compared to the hydrated proniosomal dispersion. This may be attributed to the higher viscosity of the hydrated proniosomal dispersion. Therefore the active ingredients' penetration rate through the formulation limits the ultimate release through the vesicle.

After the conduction of skin diffusion studies, transdermal diffusion was observed from both dispersions. All Franz cells (excluding control groups) contained α -lipoic acid in their receptor compartments, indicating the possible delivery of an active ingredient into the bloodstream. Beitner (2003:847-848) stated the recommended daily dose of α -lipoic acid oral supplementation was 200 to 600 mg, which is higher than the concentrations reaching the receptor compartments. The transdermal delivery of this active component isn't necessarily considered an adverse effect, keeping in mind the various health benefits of oral α -lipoic acid supplementation.

The tape stripping method revealed no statistical significant differences between the concentrations of active ingredient delivered to the stratum corneum-epidermis from the two dispersions ($p = 0.6995$). Similar results were obtained during previous diffusion studies including α -lipoic acid formulations (Beitner, 2003:842; Podda *et al.*, 1996:631). Due to the many metabolic processes occurring in the dermal layers and the presence of collagen and elastin, this is the intended target area for the active ingredient. After analysis on the individual skin layers, it was found that significantly higher concentrations of α -lipoic acid were delivered by the niosomal dispersion compared to its opponent ($p = 0.040$), therefore this last mentioned vesicle system was selected for further clinical efficacy experiments.

Results obtained from the 28 day anti-ageing study, resulted in the skin hydration levels rising significantly ($p = 0.008$) and the overall appearance of skin scaliness decreased by 9.98%. Unfortunately, the placebo formulation acting as the control group, also caused these effects, but to different degrees. The conclusion made from the elasticity parameter changes, were that the topical treatment of an active test formulation (ATF) containing α -lipoic acid resulted in the improvement of the maximum recovery area, and the increase of both the elastic and viscous recovery of the skin tested. Again, some parameters measured retrograded during the treatment period, such as the roughness, smoothness and overall wrinkle appearance. The Q3 parameter for skin elastic evaluation illustrated the 28-day treatment of topical ATF decreased the viscoelastic parameter by 1.49%.

The anti-inflammatory effect of ATF was evaluated in terms of the seven-day erythema study. The conclusions that could be made after the results were analysed indicated the ATF did in fact improve the erythema levels by 13.9%, but this is similar to the 13.8% improvement of the placebo formulation. The positive control improved the skins' erythema levels by 16.6%, therefore validating the results obtained by the ATF. Furthermore, the untreated skin did not show any significant changes over the treatment period, also acting as a validation of the results collected.

To conclude, no statistical significant differences in the anti-inflammatory abilities were identified between the ATF and placebo formulation. This may be due to suboptimal concentrations of active ingredient delivered to the target dermal layers. According to the detected concentration of α -lipoic acid in the epidermis-dermis during the skin diffusion studies, $5.077 \pm 1.47 \mu\text{g/ml}$ of active was present in the target layers after 12 h. The conclusions made after clinical efficacy studies may indicate that this resulting amount of α -lipoic acid is not adequate for the desired anti-ageing and anti-inflammatory effects to present. Furthermore, the recommended daily dose of α -lipoic acid is 200 to 600 mg, which is a much larger concentration of α -lipoic acid than the concentrations delivered by the ATF.

Future prospects:

- Evaluating an increased concentration of α -lipoic acid in topical formulations to determine the concentration dependent effects of the active ingredient.
- Compare the transdermal and topical diffusion of α -lipoic acid encapsulated into other vesicle systems such as nano-emulsions or nano-emulgels.
- Optimise suitable formulations that result in an α -lipoic acid depot in the epidermis-dermis layer, therefore reducing the systemic absorption.
- Conduct clinical efficacy studies to compare the therapeutic effects of other vesicle systems encapsulating α -lipoic acid.

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Appendix A

VALIDATION OF THE HPLC ANALYTICAL METHOD FOR ASSAY ANALYSIS FOR α - LIPOIC ACID

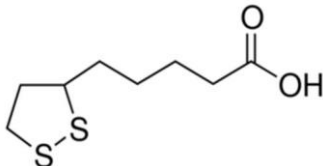
A.1 Purpose of validation

The validation process of the high performance liquid chromatography (HPLC) method was essential in ensuring a creditable, repeatable and sensitive analytical method to successfully detect α -lipoic acid concentrations in the samples tested throughout the entire study. All procedures were done in the Analytical Technology Laboratory of the North-West University (NWU), Potchefstroom, South Africa, under the guidance of Prof Jan du Preez.

A.2 Chromatographic conditions

The specific chromatographic conditions used during the development and validation of α -lipoic acid are seen in Table A.1.

Table A.1: Summary of the chromatographic conditions for the validation of α -lipoic acid

Active ingredient: α-lipoic acid	
Analytical instrument	The HPLC analysis of α -lipoic acid was done using an Agilent 1100 series (Agilent Technologies, Palo Alto, CA). It consists of a G1311A quaternary pump, equipped with a G1315A diode array detector, G1322A vacuum degasser, G1313A autosampler injection mechanism, solvent module and HP ChemStation Software.
Column	Venusil XBP C ₁₈ (2) 150 x 4.6 mm
Mobile phase	45% Acetonitrile (ACN)/0.1% phosphoric acid (H ₃ PO ₄)
Flow rate	1 ml/min
Retention time	The analyte elutes at approximately 7 min
Run time	Approximately 9 min
Detection	Diode array detector at 210 nm

A.3 Preparation of standard and samples

Two standard solutions were used during the development and validation of this analytical method, each with different concentration ranges. This was to ensure an accurate standard curve for each experiment depending on the peak area values. Both solutions were prepared following the same method, with the only difference being the initial amount of α -lipoic acid used.

Standard solutions and samples tested in each test were prepared by weighing α -lipoic acid (5 mg or 20 mg) and dissolving it in 50 ml methanol (MeOH). The solutions were then transferred to a 100 ml volumetric flask and made up to volume with phosphate buffer solution (PBS, pH 7.4), resulting in a 1:1 (MeOH:PBS) ratio. To ensure an accurate standard curve for each analysis, the standards were injected into HPLC by different injection volumes (2.5, 5.0, 10.0, 15.0 and 20.0 μ l) to yield a wider concentration range.

A.4 Preparation of phosphate buffer solution

Throughout this study, PBS (pH 7.4) was used for preparation and dilution purposes. The buffer solution was prepared by accurately weighing 1.463 g sodium hydroxide (NaOH) and adding it to 400 ml Milli-Q[®] water that was continuously stirred using a magnetic stirrer. A separate solution was then made by adding 6.525 g of monopotassium phosphate (KH_2PO_4) to 250 ml Milli-Q[®] water and this solution was also stirred and thereafter added to the NaOH solution. The concluding solution was again thoroughly mixed with the magnetic stirrer and adjustments were made to the pH of the buffer solution using H_3PO_4 to yield a final pH of 7.4.

A.5 Validation parameters

A.5.1 linearity and range

According to the USP (2012:880), an analytical method's linearity is defined as its capability, in a given range, to produce results that are directly proportional to the analytes' concentration in the tested sample. The linear regression analysis for α -lipoic acid was performed on the plot of the peak areas versus the concentration, $\mu\text{g/ml}$. The following equation was used to determine the linearity value of α -lipoic acid:

$$y = mx + c$$

Equation A.1

Where y refers to the peak area of active (α -lipoic acid), m is the slope of the line, x is the concentrations of active ($\mu\text{g/ml}$) and c is the y-intercept.

A mother solution was prepared following the same method as used for the standard preparation in Section A.3. Further dilutions were made for the linearity study as follows:

- The mother solution (5 ml) was diluted to 50 ml with PBS (pH 7.4) to obtain a 21.86 µg/ml solution.
- Subsequently a volume of 5 ml of the 21.86 µg/ml solution was further diluted to 50 ml with PBS (pH 7.4) to obtain a 2.186 µg/ml solution.
- 1 ml of each solution was transferred to HPLC vials and injected on the HPLC at different injection volumes (2.5, 5.0, 10.0, 15.0, 20.0 µl) to generate a linearity curve.

Table A.2: Linearity results of α-lipoic acid standard solutions

Concentration	Average peak area
0.11	0.00
0.27	1.91
0.55	3.65
1.09	9.63
1.64	6.34
2.19	8.70
5.47	45.70
10.93	100.61
16.40	150.80
21.86	199.06
54.65	514.48
109.30	1030.06
163.95	1550.32
218.60	2055.39
R²	0.9999
Slope	9.418

The accepted regression coefficient (R^2) determined by the linear regression analysis should be ≥ 0.99 . The regression value (R^2) obtained for α-lipoic acid was 0.9999, indicating good stability of the analysis system, a high degree of linearity and a direct correlation between active concentration and response.

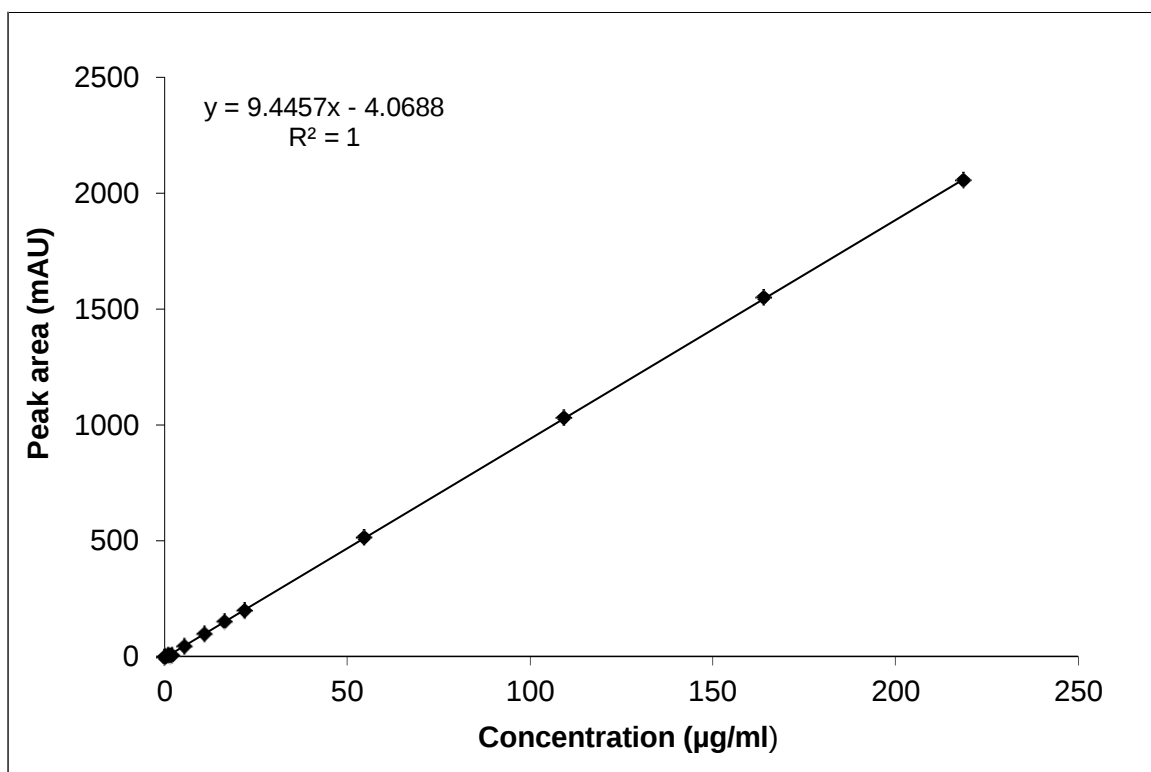


Figure A.1: Linear regression curve of α -lipoic acid standards

A.5.2 Accuracy

The accuracy of an analytical procedure, also known as the trueness, is described as the closeness of agreement between the measured value and the accepted reference value, or true value (USP, 2012:878). According to ICH (2005:10), a minimum of nine determinations over a minimum of three concentration levels covering the specified range is recommended to investigate the accuracy of an analytical method. The standard and three samples were prepared by dissolving α -lipoic acid in 50 ml MeOH and transferring 5 ml of the solution to a 50 ml volumetric flask. A second dilution was prepared by transferring 10 ml of the aforementioned dilution to a 50 ml volumetric flask. PBS (pH 7.4) was used for all dilution purposes.

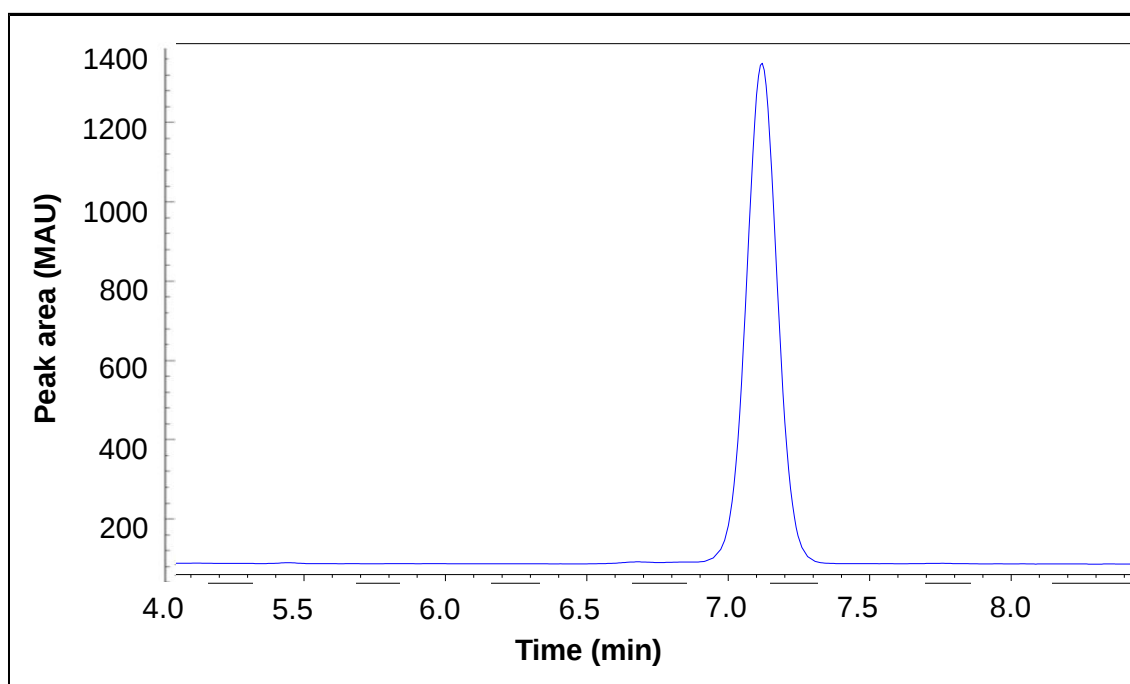
The amounts of α -lipoic acid weighed for each sample and the standard solution for the accuracy experiment was:

- Sample 1: 20.1 mg α -lipoic acid
- Sample 2: 20.2 mg α -lipoic acid
- Sample 3: 20.5 mg α -lipoic acid

All samples were injected onto the HPLC for analysis along with their two dilutions to give nine samples in total. Each sample concentration was injected into the chromatograph in duplicate.

Table A.3: Accuracy parameters of α -lipoic acid

Sample	Concentration spike ($\mu\text{g/ml}$)	Peak values			Recovery	
		1	2	Average	mg/100 ml	%
Dilution 3.2	4.10	34.1	34.0	34.05	4.03	98.40
Dilution 2.2	4.04	33.7	34.2	33.95	4.02	99.60
Dilution 1.2	4.02	32.6	32.8	32.70	3.89	96.80
Dilution 3.1	20.5	187.2	190.7	188.95	20.43	99.67
Dilution 2.1	20.2	189.2	188.5	188.85	20.42	101.10
Dilution 1.1	20.1	185.2	186.2	185.70	20.09	99.94
Stock solution 3	205.0	1997.0	2005.6	2001.30	212.30	103.56
Stock solution 2	202.0	1861.4	2002.3	1931.85	204.95	101.46
Stock solution 1	201.0	1949.0	1948.3	1948.65	206.73	102.85
					Mean	100.38
					SD	1.99
					%RSD	1.99

**Figure A.2:** HPLC chromatogram indicating the elution of α -lipoic acid standard solution

According to APVMA (2004:5), the mean percentage recovery of the analyte is a reflection of the accuracy of the analytical procedure and should be between 98 and 102%. The standard deviation (SD), as well as the percentage standard deviation (%RSD) were determined from the data collected from the accuracy tests and are shown in Table A.3. Over the concentration range of 4.10 to 201.00 $\mu\text{g/ml}$, the analytical method yielded an average recovery percentage of 100.38%, which is within the acceptance criteria.

A.5.3 Precision

The precision of an analytical method can be defined as the closeness of agreement amongst a series of measurements from multiple sampling of the same homogeneous substance under prescribed conditions (USP, 2013a:985). The precision of α -lipoic acid was examined in terms of the repeatability (intraday precision) and reproducibility (interday precision) of the analytical method.

A.5.3.1 Intraday precision (repeatability)

The repeatability of the analytical method was determined by conducting HPLC analysis on three concentrations of three different samples on the same day.

The three samples and standard (standard solution B) for the repeatability test were prepared according to Section A.3. The amounts α -lipoic acid weighed for each sample were:

- Sample 1: 20.1 mg α -lipoic acid
- Sample 2: 20.2 mg α -lipoic acid
- Sample 3: 20.5 mg α -lipoic acid

According to the United States Pharmacopoeia (USP), the criterion for intraday precision is a %RSD value of < 2% for acceptable method repeatability. All samples were analysed in duplicate by means of HPLC.

Table A.4: Intraday precision parameters of α -lipoic acid

Sample	Concentration spike ($\mu\text{g/ml}$)	Peak values			Recovery	
		1	2	Average	mg/100 ml	%
Dilution 3.2	4.10	34.1	34.0	34.05	4.03	98.40
Dilution 2.2	4.04	33.7	34.2	33.95	4.02	99.60
Dilution 1.2	4.02	32.6	32.8	32.70	3.89	96.80
Dilution 3.1	20.5	187.2	190.7	188.95	20.43	99.67
Dilution 2.1	20.2	189.2	188.5	188.85	20.42	101.10
Dilution 1.1	20.1	185.2	186.2	185.70	20.09	99.94
Stock solution 3	205.0	1997.0	2005.6	2001.30	212.30	103.56
Stock solution 2	202.0	1861.4	2002.3	1931.85	204.95	101.46
Stock solution 1	201.0	1949.0	1948.3	1948.65	206.73	102.85
					Mean	100.38
					SD	1.99
					%RSD	1.99

From Table A.4, it can be seen the %RSD of α -lipoic acid was 1.99% and this method is therefore acceptable according to the stated requirements of the USP.

A.5.3.2 Interday precision (reproducibility)

Three different samples of a known concentration were prepared on three different days and injected in triplicate to determine the reproducibility of the analytical method. According to APVMA (2004:5), the %RSD should be equal or less than 5% for a methods' reproducibility to be acceptable.

All standards and samples used in the reproducibility test were prepared in accordance with Section A.3.

Table A.5: Interday precision results obtained for α -lipoic acid

Days	%Recovery			Mean	SD	%RSD
Day 1	102.48	97.48	99.02	99.66	2.09	2.10
Day 2	102.25	102.23	102.12	102.20	0.06	0.06
Day 3	99.85	99.44	98.80	99.36	0.43	0.43
Mean between days				100.41	0.88	0.88

The %RSD value obtained for α -lipoic acid was 0.88% and therefore within the acceptable range for reproducibility (Du Preez, 2010b:7).

A.5.4 Limit of detection and limit of quantitation

An analytical procedure's LOD (limit of detection) value is described as the lowest amount of an analyte in a given sample, which can be successfully detected, but quantitation to a precise value is not possible under the specified experimental conditions (USP, 2012:880). The LOQ (limit of quantitation) value is defined as the lowest amount of an analyte in the given sample, which can be determined quantitatively with acceptable accuracy and precision (ICH, 2005:5).

The LOD and LOQ values of α -lipoic acid were determined by preparing a stock solution according to Section A.3. The initial amount of α -lipoic acid weighed for the stock solution was 5.10 mg, 1 ml of this solution was then transferred to a 10 ml volumetric flask and filled up to volume with PBS (pH 7.4) to obtain a 0.5 μ g/ml solution. 1 ml of the solution was then transferred to an HPLC vial for analysis by the HPLC. Four different injection volumes were used and each volume was injected seven times to give a total of 28 analysis values.

Table A.6: LOD and LOQ results obtained for α -lipoic acid

Injection volume	2.5	5.0	7.5	10.0
Concentration ($\mu\text{g/ml}$)	0.64	1.30	1.91	2.55
Peak area 1	6.63	10.17	19.61	26.31
Peak area 2	6.78	11.13	19.39	26.55
Peak area 3	6.25	12.96	19.57	26.55
Peak area 4	6.56	12.43	19.08	26.56
Peak area 5	6.39	14.27	19.29	26.71
Peak area 6	6.38	14.17	19.32	26.01
Peak area 7	6.97	13.87	19.10	26.37
Mean	6.6	12.7	19.3	26.4
SD	0.23	1.46	0.19	0.21
RSD%	3.52	11.51	0.99	0.81

From Table A.6 it can be seen the detection limit of α -lipoic acid has a %RSD value of 3.52. For acceptable precision and accuracy, the LOQ value to be determined should present with a %RSD less than 15%. The LOQ of α -lipoic acid was determined to be 1.30 $\mu\text{g/ml}$, with a %RSD of 11.51 %, which is within the acceptance criteria.

A.5.5 Robustness

According to the USP (2012:881), the robustness of a method is defined as the measurement of an analytical procedure's capacity to remain unaffected by deliberate variations of the method's parameters stated in the documentation regarding the specific procedure. The robustness shows the suitability and reliability of an analytical procedure during normal usage.

The following changes were made to the chromatographic method parameters and found to be acceptable:

- Wavelength: The wavelength could be altered by ± 5 nm without any ill effect.
- Flow rate: 0.8 – 1.2 ml/min
- Injection volume: 15 – 25 μl

The method was able to tolerate small changes in the chromatographic conditions and should therefore perform well under normal use.

A.5.6 Ruggedness

α -Lipoic acid was weighed and a stock solution was prepared in accordance with Section A.3. This solution was then transferred to two separate vials for system stability and repeatability determination.

A.5.6.1 System stability

One of the samples in Section A.5.6 was injected into the HPLC system to analyse the systems' stability. This was done for a period of 25 h to determine the degradation of the sample accurately throughout this period. By using the degradation percentage, it is possible to determine the time the sample is still usable. Samples are only viable for the period it takes to degrade with 2%.

Table A.7: Stability analysis of α -lipoic acid over 24 h

Time (h)	Peak area	Recovering (%)
0	644.50	100.00
1	644.90	100.06
2	644.30	99.97
3	645.70	100.19
4	646.80	100.36
5	644.20	99.95
6	642.00	99.61
7	643.60	99.86
8	643.70	99.88
9	668.90	103.79
10	668.70	103.75
11	658.50	102.17
12	657.40	102.00
13	655.80	101.75
14	648.90	100.68
15	652.10	101.18
16	658.80	102.22
17	663.40	102.93
18	659.60	102.34
19	654.10	101.49
20	657.70	102.05
21	657.90	102.08
22	656.80	101.91
23	659.50	102.33
24	658.50	102.17
Mean	653.45	101.39
SD	7.92	1.23
RSD%	1.21	1.21

From Table A.7, it can be seen that samples of α -lipoic acid deemed stable over a period of 24 h, with a recovering percentage of 101.39% and RSD% of 1.21%.

A.5.6.2 System repeatability

To determine the repeatability of the peak areas and retention times of α -lipoic acid, six consecutive injections of the same sample were injected under uniform conditions. The acceptable criterion for the system repeatability is a %RSD value of 2% or less (Du Preez, 2010a:5).

Table A.8: Repeatability data obtained for α -lipoic acid

Injection number	Peak area	Retention time (min)
1	558.70	4.73
2	556.40	4.73
3	556.80	4.74
4	556.20	4.74
5	555.90	4.73
6	556.60	4.73
7	557.30	4.73
8	558.00	4.73
Mean	556.99	4.73
SD	0.89	0.00
RSD%	0.16	0.06

From Table A.8 it can be seen the RSD% from the repeatability results was 0.16%, indicating that this analytical method is adequate and falls within the acceptable range.

A.5.7 Specificity

The definition of specificity is the assessment of the active ingredient or drug in the presence of other expected components without any interference from those particular components (ICH, 2005:4). The following method was used to determine the specificity:

A stock solution was used during this study and prepared by weighing 5 mg of α -lipoic acid and dissolving it into 100 ml MeOH; 1 ml of the stock solution was then transferred into five separate test tubes and prepared as follows:

- 1 ml stock solution
- 1 ml stock solution + 1 ml H₂O (amphoteric)

- 1 ml stock solution + 1 ml hydrogen chloride (HCl)
- 1 ml stock solution + 1 ml NaOH
- 1 ml stock solution + 1 ml hydrogen peroxide (H₂O₂)

These samples were injected into the HPLC for analysis in triplicate. According to McPolin (2009:84), for the specificity of a method to be acceptable, there should be no peaks interfering with the peak of the analyte in question.

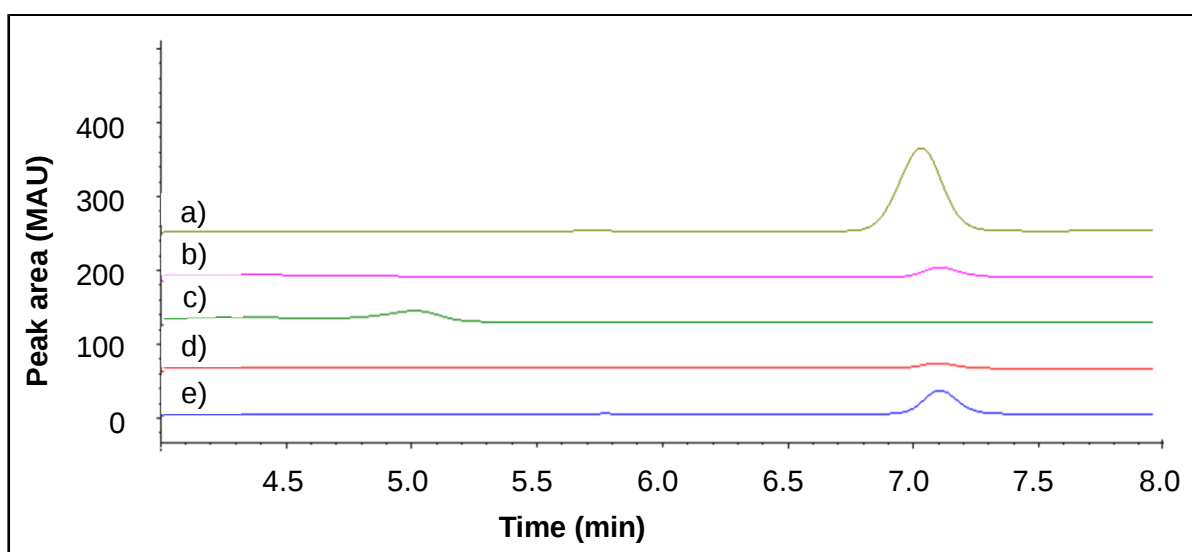


Figure A.3: Chromatographs of the results from the specificity determination of α -lipoic acid with a) additives, b) H₂O, c) HCl, d) NaOH and e) H₂O₂

From Figure A.3, it can be seen that no interference occurred with the peak detected for α -lipoic acid. The area of the peaks possibly varied due to the acidic nature of α -lipoic acid and the varied pH values of the added components. α -Lipoic acid is regarded as a weak acid (pKa 4.7), while NaOH is a widely used strong base hence, the resulting product will be of basic nature (Cichewics *et al.*, 2013:2; Ibrahim *et al.*, 2008:546; Ye *et al.*, 2015:1). According to LC/GC's Chromacademy (2016:7), the order of elution for acids and bases is as follows: weak bases > strong bases > weak acids > strong acids. This explains the decrease in retention time of α -lipoic acid.

Figure A.3 shows no interference of degradation components with the α -lipoic acid peak was indicated. Therefore the analytical method is acceptable and specific for α -lipoic acid detection.

A.6 Conclusion

The developed HPLC method for α -lipoic acid was found to be sensitive and reliable enough for the determination of α -lipoic acid concentrations in further niosomal and proniosomal studies. The method performed well and met *all* the validation parameters in this appendix, which shows it should be appropriate to analyse the α -lipoic acid in further tests.

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Appendix B

PREPARATION OF NIOSOMAL AND PRONIOSOMAL VESICULAR SYSTEMS TO ENCAPSULATE α -LIPOIC ACID

B.1 Introduction

The ingredients used and the method followed, when preparing vesicle systems, ultimately determines the structure and properties of that specific carrier. The physicochemical properties such as pH, droplet size and zeta-potential are largely dependent on the ingredients and ratio of the ingredients used during the preparation phase. Therefore, in this appendix, the ingredients used and the ratios in which they were used, along with the precise methods followed to prepare both the niosomal and proniosomal vesicles, are described. Once the formulation methods were finalised, it remained unchanged throughout the remainder of the study and was kept the same for all further experiments.

B.2 Pre-formulation

Pre-formulation studies are defined as research performed on the active ingredients and excipients' physicochemical properties prior to the onset of initial formulation. This ensures the rational development of safe, stable and efficacious dosage forms (Walters & Brain, 2009:491). The thin-film hydration method, described by Dua *et al.* (2012:15) and Kailash *et al.* (2013:1), was studied in respect of the usage of a rotary evaporator and hydration medium commonly stated in literature. For the preparation of proniosomes, the method known as the slow spray-coating method (Kakar *et al.*, 2010:230; Vasthist *et al.*, 2015:27) was studied and selected due to similar instruments available and ingredients used in literature.

B.3 Early formulation and problems encountered

During the early formulation phase, the method initially used was changed a multitude of times to finalise and correct it. A trial-and-error approach was followed where different dispersions were made and tested to determine the appropriate method for α -lipoic acid preparations.

The initial method used to formulate niosomes was derived from a dissertation by Candice Csongadi (Csongadi, 2015:148), which used a hotplate as a substitute for a rotary evaporator. The method was tested and found to be inappropriate, as the niosomal dispersions seemed

burnt after a short period on the hot plate, even at a low temperature. Since α -lipoic acid is heat sensitive and requires greater temperature control, the rotary evaporator was a more suitable choice to avoid further problems and ensure accurate temperature control. Further changes were also made to the ratio of components in the dispersions, from a ratio of 2:1:1 (Span® 60:active:cholesterol) to a ratio of 1:1:2. Dispersions ranging from 1 - 5% active ingredient were formulated to determine the most efficient concentration to be used in the study, depending on the amount successfully encapsulated by the niosomes and proniosomes. After eliminating two dispersions (2% and 4% α -lipoic acid) due to no significant increase in the values calculated, the remaining three concentrations (1%, 3% and 5% α -lipoic acid) were further tested, in duplicate, to select the most suited dispersion.

The entrapment efficacy (EE%) of both the niosome and hydrated proniosome dispersions were determined for the remaining three concentrations (1, 3 and 5%). According to Zabot *et al.* (2016:28), the EE% can be defined as the ratio between the active encapsulated inside the matrix and the total amount of active present. At first, a calibration curve was developed for all the tested vesicle systems at the specific pH of 7.4. Thereafter, the concentration of free active was determined by separating the formed vesicles from the un-encapsulated α -lipoic acid (Mahale *et al.*, 2012:53). This was done by placing the samples in suitable test tubes, then into a Beckman Coulter Optima L-100 XP ultracentrifuge, fitted with a 50.2Ti fixed rotor (Beckman Coulter, South Africa). The samples were centrifuged at 25 000 rpm for a period of 30 min and at a temperature of 4 °C. The supernatant was then injected into autosampler vials and analysed using the HPLC method described in Appendix A

Equation B.1 can be used to calculate the EE% of vesicle carrier systems and was used to determine the EE% of each of the respective concentrations of both vesicle systems (Abdellatif & Abou-Taleb, 2016:20; Kurakula *et al.*, 2012:37)

$$EE\% = (\text{Concentration}_t - \text{Concentration}_f) / \text{Concentration}_t \times 100 \quad \text{Equation B.1}$$

Where:

Concentration_t refers to the total concentration of active in the sample

Concentration_f refers to the concentration of free un-encapsulated active

Prior to the analysis of the samples, a standard curve was prepared in accordance with Section A.3. The calculated average EE% for both the niosome and hydrated proniosome dispersions, using the three different concentrations, are listed in Table B.1.

Table B.1: Results of the three dispersions of each vesicle system that was tested in duplicate to determine the most efficient concentration to pursue in the study

EE%		
%Active encapsulated	Niosomes	Proniosomes
1%	99.77	59.81
3%	99.94	69.74
5%	99.96	92.621

Results indicated that all the niosomal and hydrated proniosomal dispersions encapsulated the α -lipoic acid very well, but the ultimate deciding factor was the encapsulation of the different dispersions; the 5% concentration was chosen, since it had the highest proniosomal encapsulation also.

The 5% dispersion was selected for further examination. The required sonication period to yield a niosomal dispersion with an adequate stability profile was identified by means of zeta-potential and Pdl size testing.

Different proniosomal preparations were made to determine the correct amount of sorbitol needed to dry the entire dispersion. Initially 5 g was used, but it was found that 4 g was sufficient to dry the wet vesicle dispersion. To improve the drying process, a bigger round bottomed flask (200 ml) proved more adequate.

B.3.1 Preparation of vesicular systems

The initial methods used for the preparation of niosomes and proniosomes were the thin film hydration method (Dua *et al.*, 2012:15; Kailash *et al.*, 2013:1; Moghassemi & Hadjizadeh, 2014:28; New, 1990:36-38) and slow spray-coating method (Kakar *et al.*, 2010:230; Vasthist *et al.*, 2015:27), respectively. Both these methods were adapted to better suit the properties and limitations of α -lipoic acid, such as its heat instability (Ikuta *et al.*, 2013:212; Nishiura, 2013:1). All adaptations were examined to determine their influences and to establish if the vesicles did form successfully.

B.3.2 The general method used for the preparation of vesicular systems

The preparation method followed to prepare niosomes, consisted of dissolving the lipid mixture (cholesterol and lipophilic α -lipoic acid) in an organic solvent to assure a homogeneous mixture of lipids. According to Dua *et al.* (2012:15), the organic solvent generally used is chloroform or mixtures thereof, i.e. chloroform:MeOH. Once a clear lipid solution was obtained, the organic solvent was removed by evaporation until only a thin lipid film/cake on the walls of the round

bottomed flask remained. The heating bath of the rotary evaporator (Figure B.1) was controlled at a temperature (above the lipid's transition temperature) of 50 °C with an automatically regulated pressure of 332 bar (evaporation pressure of chloroform is at 50 °C).

The lipid film/cake was subsequently hydrated by adding a preheated aqueous medium to the flask. According to Dua *et al.* (2012:16), distilled water (dH₂O) is a suitable hydration medium and used because it is readily available. The dH₂O water was preheated to above the transition temperature (the same as the heating bath during evaporation). The mixture was agitated by using a magnetic stirrer to remove all possible lipid film from the walls.



Figure B.1: A Büchi® (Switzerland) rotary evaporator equipped with an Interface I-100, Vacuum pump V-100, Rotavapor® R-100 and Heating bath B-100 was used during the formulation process

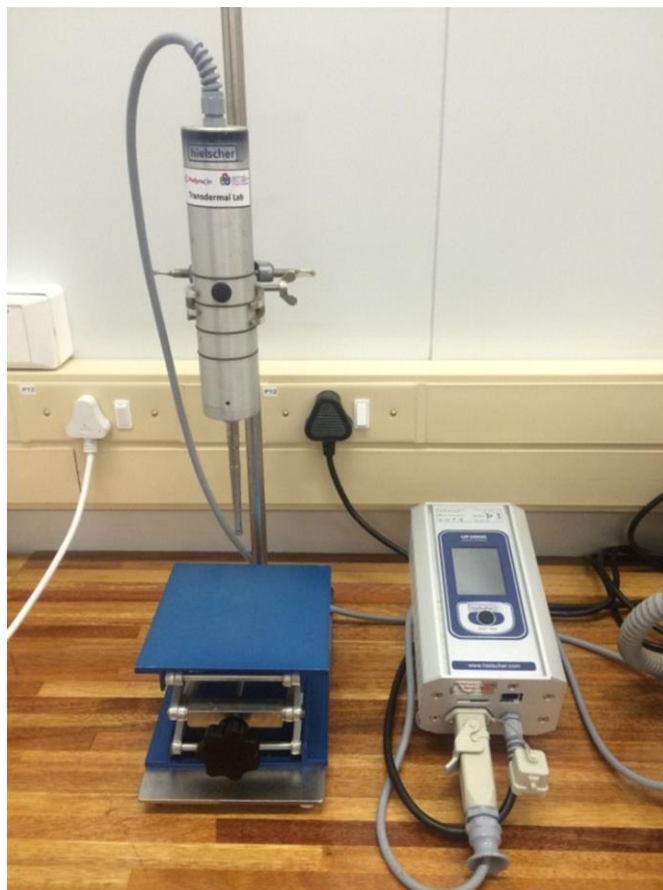


Figure B.2: A Hielscher Ultrasonic Processor UP200St was used to size the vesicles by means of sonic energy

Sonic energy was used by sonicating (Figure B.2) the hydrated vesicle dispersion to size the vesicles into smaller structures with fewer lamellae (Kalra & Bally, 2013:42). After sonication time of 45 sec, the dispersion was left at room temperature for optimal swelling of the vesicles (New, 1990:36-38).

B.3.3 The general method used for the preparation of provesicular systems

The slow spray-coating method involves the mixing of the surfactant in organic solvent and spraying it repeatedly on the desired carrier, while the organic solvent is evaporated under a specific pressure (Kakar *et al.*, 2010:230; Vasthist *et al.*, 2015:27). This is achieved by placing the carrier or coating material in a round bottomed flask and attaching it to a rotary evaporator (Figure B.1); the mixture is continuously dripped onto the carrier until all the organic solvent is completely evaporated. This method provides a thin surfactant coating on the carrier, which allows the formation of niosomes with a more uniform size distribution in relation to conventional preparation methods.

B.4 Ingredients used to formulate vesicular and pro-vesicular systems

B.4.1 α -Lipoic acid

α -Lipoic acid is the active ingredient that will be encapsulated into the niosome and proniosome vesicles in order to stabilise and protect it against breakdown, such as thermolysis and polymerisation. α -Lipoic acid is a derivative of octanoic acid with poor water solubility and bioavailability due to its unstable form (Li & Lim, 2016:253). It possesses the unique ability to scavenge ROS and recycles endogenous antioxidants such as vitamins C and E, making it an ideal investigational ingredient for topical delivery and possible treatment of skin ageing and inflammation.

B.4.2 Cholesterol

Cholesterol was added to the formula of the dispersions due to its ability to improve the rigidity and fluidity of vesicles, which in turn leads to less leakage of the vesicles (Karim *et al.*, 2010:378; Kumar & Rajeshwarrao, 2011:211; Varun *et al.*, 2012:633). Due to the insolubility of cholesterol in water, a synergic interaction between the hydrophobic alkyl chain of the surfactant and cholesterol forms bilayer structures in an aqueous medium (Ghosh *et al.*, 2015:2311). According to Mahale *et al.* (2012:49), the addition of cholesterol also has a modulating effect on the lipid bilayer's mechanical strength and cohesion.

B.4.3 Non-ionic surfactants

Non-ionic surfactants are preferred due to their ability to enhance solubility and ultimately increase the bioavailability of poorly soluble drugs (Mahale *et al.*, 2012:47) such as α -lipoic acid. Their compatibility, stability and their non-toxic nature is of importance for topical formulations. Amongst all the Tweens and Spans, Sorbitan-monostearate (Span[®] 60) produces the highest EE% and therefore was used in this study to form part of the vesicle's lipid layer (Kumar & Rajeshwarrao, 2011:210; Mali *et al.*, 2013:587; Ruckmani & Sankar, 2010:1124). Span[®] 60 also acts as a wetting agent, solubiliser and permeability enhancer when added to formulations (Kumar & Rajeshwarrao, 2011:209).

B.4.4 Organic solvents

Chloroform is a non-irritating, non-flammable (Peng *et al.*, 2016:264) organic solvent that is extremely suitable for the manufacturing and formulation of products intended for topical delivery. According to New (1990:33), chloroform is a popular solvent commonly used in the preparation of various types of vesicle systems and is convenient to handle.

B.4.5 Sorbitol

Sorbitol was used as carrier for the provesicular systems in this study. Singh *et al.* (2015:1674) and Sadiq *et al.* (2014:99) both stated that sorbitol is especially useful in the cases where the active ingredients were susceptible to hydrolysis. According to Patel and Packer (2008:241), acid hydrolysis, as well as enzymatic hydrolysis, influenced their values trying to quantify the baseline serum levels of α -lipoic acid. The lipid soluble component is dissolved in the organic solvent (chloroform) and carefully sprayed onto the sorbitol, thereafter the organic solvent is evaporated with a rotary evaporator to leave a dry provesicular system. During the hydration of the proniosomes, the sorbitol dissolves on addition of an aqueous medium and results in the formation of vesicles.

B.4.6 Purified water

All experiments throughout the study were done with Milli-Q[®] HPLC grade water (Merck-Millipore, Halfway House, South Africa).

B.5 Final dispersion formulas

Based on the data from the pre-formulation tests done initially, the final ratio of cholesterol:Span[®] 60: α -lipoic acid was 2:1:1 and remained consistent throughout all further experiments. After the formation of all vesicles, the formulated dispersion was sonicated for a period of 45 sec to produce the desired vesicle sizes.

Table B.2: Raw materials used during the formulation process of both niosomes and hydrated proniosomes dispersions, along with their respective concentrations and batch numbers

Ingredient	%m/m	Batch number
α -Lipoic acid	99.00%	117K0679
Span [®] 60	-	423065/141002
Cholesterol	92.50%	421293/122201
Chloroform	99.00%	1018441
Sorbitol	-	107758
dH ₂ O	-	Merck-Millipore

B.5.1 Formula for niosome preparation

Table B.3 shows the ingredients used during the formulation of niosomes during the study. Also listed is each ingredients' specific activity in the vesicle dispersion.

Table B.3: List of the ingredients used during the niosomal preparation process to formulate a 10 ml sample dispersion

Ingredient	Activity	Quantity
α -Lipoic acid	Active ingredient	500 mg
Span [®] 60	Emulsifier	500 mg
Cholesterol	Stabiliser	1000 mg
Chloroform	Organic solvent	10 ml
dH ₂ O	Solvent	10 ml

B.5.2 Preparation method for niosomes

The final method used to prepare all niosome dispersions for further experiments in this study are summarised and stated as follows:

- Preheat the heating bath of the rotary evaporator to a constant temperature of 50 °C.
- Accurately weigh the Span[®] 60, cholesterol and α -lipoic acid.
- Place all dry ingredients in a glass beaker and add 10 ml of chloroform to the dispersion.
- Pour the solution into a round bottomed flask and attach to a rotary evaporator.
- Rotate under pressure at 8 rpm at a temperature of 30 °C ($\pm$ 60 min) until a layer of mixture is deposited on the wall of the flask.
- Add 10 ml dH₂O to the dried surfactant film and place on a heater with a magnetic stirrer to agitate the film gently.
- Let it cool to room temperature before sonicating on ice for 45 sec.

B.5.3 Discussion

The final dispersions appeared homogenous with a creamy texture, an off-white to slight yellowish colour and a unique smell.

B.5.4 Formula of proniosomes

Table B.4 shows the ingredients used during the formulation of proniosomes throughout the study. Also listed is each ingredients' specific activity in the provesicle dispersion.

Table B.4: List of the ingredients used during the proniosomal formulation process to prepare a 10 ml sample dispersion

Ingredient	Activity	Quantity
α -Lipoic acid	Active ingredient	500 mg
Span [®] 60	Emulsifier	500 mg
Cholesterol	Stabiliser	1000 mg
Chloroform	Organic solvent	10 ml
Sorbitol	Humectant	4000 mg
dH ₂ O	Solvent	10 ml

B.5.5 Preparation method for proniosomes

The final method used to prepare all hydrated proniosome dispersions used for further experiments in this study is as follow:

- Preheat the heating bath of the rotary evaporator at a constant temperature of 50 °C.
- Accurately weigh the Span[®] 60, cholesterol, sorbitol and α -lipoic acid.
- Place Span[®] 60, cholesterol and α -lipoic acid in a glass beaker then add 10 ml chloroform to the dispersion and allow to dissolve completely.
- Pour the total amount of sorbitol into the round bottomed flask.
- Dropwise add small quantities of the solution onto the dry sorbitol and attach to rotary evaporator.
- Set the rotary evaporator at a pressure of 332 mpa and at 8 rpm.
- Evaporate the solvent from the dispersion until dry before dribbling more of the solution on the sorbitol (repeat until all the solution has been added and evaporated from the sorbitol).
- The process takes about 60 min and consists of continuous dripping and evaporating to give a dry dispersion.

Reconstitution of proniosomes:

- Add 10 ml dH₂O to the proniosomes and place on a heater with a magnetic stirrer to hydrate the dispersion evenly.
- Let it cool to room temperature before sonicating it on ice for 45 sec.

B.5.6 Discussion

The dry proniosomes appeared as a white free flowing granule dispersion with a unique smell. After reconstitution and sonication of the proniosomes, a milky off-white liquid with a creamy texture was produced and the unique smell was retained.

B.6 Conclusion

The niosomal and hydrated proniosomal dispersions appeared homogeneous, and manufactured in bulk for use in the characterisation, diffusion and clinical efficacy studies. For the release studies and skin diffusion studies, placebo dispersions were made following the same methods. These placebo dispersions contained no active ingredient and acted as negative standards to ensure accuracy and validity of the results obtained.

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Appendix C

PHYSICOCHEMICAL CHARACTERISATION OF VESICULAR SYSTEMS CONTAINING α -LIPOIC ACID

C.1 Introduction

In this appendix, the physicochemical properties of both the niosomal and proniosomal vesicles are discussed. The characterisation of the vesicles and their respective placebo dispersions were performed by testing specific parameters such as the morphology of the vesicles formed, viscosity and the EE% of the dispersion, to name a few. The methods and instruments used to measure each parameter are described and possible interpretations of the data collected provided. All samples used for testing and measurements were prepared in accordance with the final formulation method described in Sections B.5.3 and B.5.7.

C.2 Physicochemical characteristics determined for the vesicular and provesicular systems encapsulating α -lipoic acid

C.2.1 Transmission electron microscope (TEM)

To examine the morphology and formation of the vesicle systems, the transmission electron microscope was used. Only dispersions containing no active ingredient were viewed, due to the sensitivity of the TEM towards larger molecules such as α -lipoic acid and the possible precipitation that could damage the microscope. A FEI Tecnai G2 TEM (FEI, Holland) fitted with a Gatan bottom mount camera was operated by Dr A. Jordaan (Electron Microscopy Laboratory, NWU, Potchefstroom Campus) at 200 kV to capture the particles and their respective sizes.

To prepare the test samples, six drops of the respective dispersions were diluted with ± 10 ml of Milli-Q[®] water and thoroughly mixed. A drop of the hydrated vesicles was deposited onto a carbon-coated 300 mesh copper grid and left for 10 min to dry (Li *et al.*, 2016:43). The excess sample was carefully removed by means of filtration paper, where after osmium was added on top of the sample to oxidise the lipids (Bayinder & Yuksel, 2012:826) and ultimately stain the lipid films. According to Belazi *et al.* (2009:105), osmium is a stain used extensively in electron

microscopy of unsaturated lipids due to its oxidation abilities. Heavy metal salt solutions are used to incubate the samples to enhance their electron scattering properties (Barreto-Vieira & Barth, 2015:51; Reynolds, 1963:208).

Uranyl acetate was chosen as the negative stain, due to the increased stability when using this reagent instead of uranyl formate (Booth *et al.*, 2011:8). The samples were then washed with dH₂O and then coloured with lead citrate as a positive stain. Time was allowed for the samples to properly dry before analysis started with the microscope (120 kV).

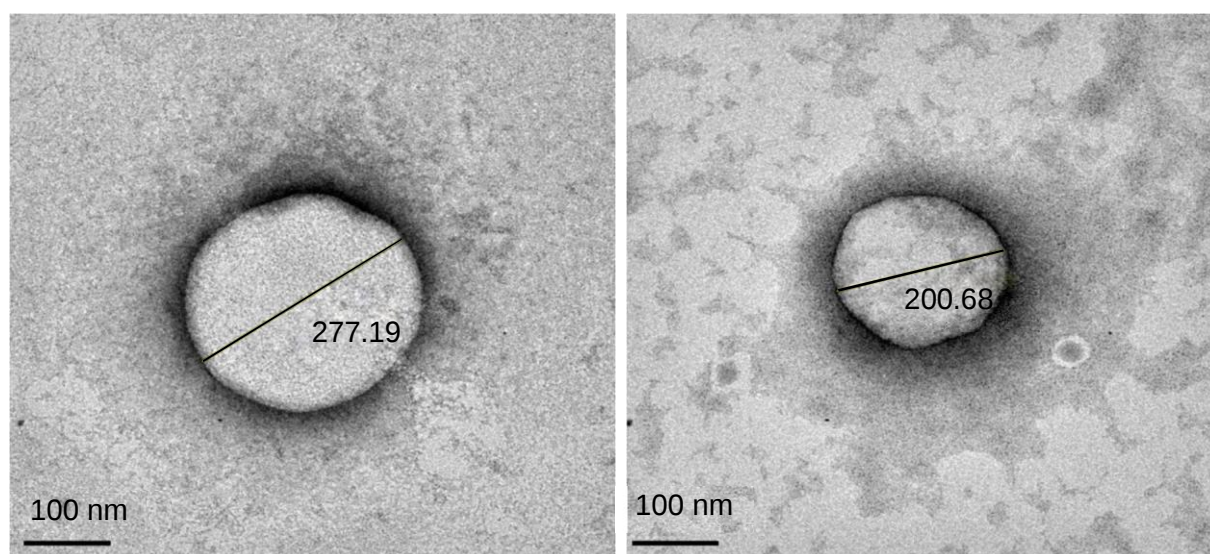


Figure C.1: TEM micrographs for the a) niosomes and b) proniosomes

The results obtained from the TEM (Figure C.1) for both niosomes and proniosomes shows that both dispersion formulas successfully formed vesicles. As reported by Navya *et al.* (2014:445), niosomes can differ in size between ranges of 10 – 1000 nm. Hence, both vesicle dispersions can be classified by their size as LUV (Arora *et al.*, 2012:97). The dark edges on both images illustrate the osmium that is fixed on the membranes and is an indication of the membranes strength. The niosomes (Figure C.1.a) had a more uniform membrane and appeared to be more spherical than the proniosome vesicles. On the proniosome (Figure C.1.b) image, there are additional molecules visible, which can possibly be the sorbitol used to dry the dispersion during the preparation process.

C.2.2 Viscosity

During the clinical efficacy studies (Appendix F), the dispersion of choice will have to be applied directly on the inner forearms of the volunteers. Therefore, the viscosity of both the niosomal and hydrated proniosomal dispersions, with their respective placebo dispersions, were measured to determine whether a thickening agent would be needed to ease the application process. A Brookfield® Viscometer model DV III Ultra (Middleboro, Massachusetts, United

States) was used to measure the viscosity of the different dispersions at pre-set shear rates. Approximately an hour before the measurement was taken, the heating bath was set on a controlled temperature of 25.5 °C and all four dispersions (niosome and hydrated proniosome dispersions with their individual placebo dispersions) were placed inside to heat to the desired temperature.

During each measurement, ± 10 ml of the tested dispersion was transferred to a cylindrical sample chamber enclosed in an adapter and flow jacket. The viscosity, or the dispersion's resistance to flow, was measured using either the smaller T-bar spindle 34 (SC4-34), or the bigger spindle 18 (SC4-18), rotating inside the sample chamber. The spindle was selected depending on the viscosity of the different samples tested. Multipoint measurements were taken every minute for a period of 6 min for each of the four dispersions. The averages of the individual samples were then calculated.



Figure C.2: Brookfield® Viscometer model DV III Ultra used during viscosity measurements

The results in Table C.1 show both placebo dispersions had a lower viscosity compared to the dispersions encapsulating α -lipoic acid in the vesicles. The proniosomes had an overall higher viscosity in comparison to the niosomes, probably due to the added sorbitol in the formulations. Cross *et al.* (2001:150) stated that a high viscosity causes resistance to the diffusion of active through the formulation and results of reduced permeation through the skin. For comparison purposes, the viscosity of water is known to be 1 cP and that of peanut butter, 250 000 cP,

therefore none of the four tested dispersions are proficient for topical application, and additional thickening agents are required to promote topical application (Hydramotion, 2016).

Table C.1: Average viscosity measurements in cP of both dispersions with their respective placebo dispersions

Vesicle dispersion		Viscosity (cP)	Torgue	Speed (rpm)	Spindle
Niosomes	Active encapsulated	11.31 ± 0.09	75.42	200	18
	Placebo	3.33 ± 0.06	22.25	200	18
Proniosomes	Active encapsulated	109.12 ± 19.39	35.18	200	34
	Placebo	12.10 ± 0.06	80.77	200	18

The slightly higher viscosity of the proniosomes encapsulating α -lipoic acid may affect the diffusion of this dispersion and decreased flux values during the diffusion studies can possibly be expected.

C.2.3 Potential of hydrogen (pH) determination

A Mettler Toledo SevenMulti™ pH/conductivity meter (Schwerzenbach, Switzerland), fitted with a glass Mettler Toledo InLab® 410 electrode (Schwerzenbach, Switzerland) was used to measure the pH values of the four different dispersions. Before pH measurements were taken, the electrode was calibrated using Mettler Toledo pH buffer solutions at various pH values (4, 7 and 10). During each measurement, the electrode probe was submerged into the sample dispersions and measurements were taken in triplicate.



Figure C.3: The Mettler Toledo SevenMulti™ pH meter was used during the pH measurements, with a glass Mettler Toledo InLab® 410 electrode

Healthy human skin's surface can be characterised by a pH value oscillating from 4.0 to 6.0 (Boer *et al.*, 2016:2). The ideal pH of topical formulations varies somewhat in different publications, resulting in different ranges being mentioned in literature for acceptable pH values that can be applied to the skin without damaging the cells. According to Kharat and Bathe (2016:149), the ideal pH of topical formulations falls in the range of 4.2 to 5.6; Mandal *et al.* (2016:3) states that the desired pH is from 5 to 9. Furthermore, it is a certainty that human skin is very sensitive to changes in pH, but the outermost stratum corneum is extremely resistant to changes in pH value and can tolerate a pH range of 3 to 9. This explains the varied pH ranges that are acceptable and clarifies the controversy of the ideal pH.

Table C.2: Results of pH measurements of both dispersions with their respective placebos

Vesicle dispersion		pH average
Niosomes	Active encapsulated	4.681 ± 0.01
	Placebo	6.264 ± 0.08
Proniosomes	Active encapsulated	4.736 ± 0.07
	Placebo	6.916 ± 0.10

The niosomal and proniosomal vesicles encapsulating α -lipoic acid had a lower pH value than the placebo dispersions, indicating the acidity of α -lipoic acid decreased the pH of the dispersion. The niosomes and proniosomes that encapsulated α -lipoic acid had pH values of 4.681 ± 0.01 and 4.736 ± 0.07, respectively, and both were considered acceptable and fell within the desired range.

C.2.4 Entrapment efficiency percentage

Zabot *et al.* (2016:28) describes the EE% as the ratio between the drug/active inside the encapsulating matrix and the total drug/active present. The EE% of each vesicle system was calculated using Equation C.1. The equation is based on the percentage α -lipoic acid successfully encapsulated by the vesicle system, relative to the total amount of α -lipoic acid. A calibration curve was initially developed at the precise dispersion pH (pH 7.4) for all of the vesicles systems. The concentration of the un-encapsulated α -lipoic acid was determined using a Beckman Coulter Optima L-100 XP ultracentrifuge (Beckman Coulter, South Africa) equipped with a 50.2Ti fixed rotor to separate the formed vesicles and free α -lipoic acid (Mahale *et al.*, 2012:53). The samples were placed in suitable tubes and centrifuged for 30 min at 25 000 rpm at a pre-set temperature of 4 °C. The resulting supernatant was then analysed by means of HPLC in accordance with Section A.2. The equation of the linear standard curve of α -lipoic acid was used to calculate the concentration of active in each of the individual vesicles systems. According to Kurakula *et al.* (2012:37) and Abdellatif and Abou-Taleb (2016:20), Equation B.1

can be used to successfully determine the EE% of each of the respective vesicle systems that will be formulated to carry α -lipoic acid into the skin layers. To calculate the EE%, a standard curve was first produced for each new sample in accordance with Section A.3.

Results obtained from the EE% studies showed high degrees of entrapment for both vesicle dispersions. The EE% measured for the niosome dispersion was determined as $99.96 \pm 0.02\%$. This value is in close agreement to the statement made by Souto and Müller (2007:222), explaining that EE% values for lipophilic active ingredients are typically between 90% and 98%. New (1990:37) also explained that lipid soluble actives (such as α -lipoic acid) could have high EE% of up to 100%, provided the active has not been overloaded. The proniosomes obtained lower entrapment levels than the niosomes, but were in accordance with the values obtained from a similar study (Ruckmani & Sankar, 2010:1124).

Table C.3: Entrapment efficiency results obtained for the 5% niosome and proniosomes

Vesicle dispersion	Entrapment efficiency
Niosomes	99.96 ± 0.02
Proniosomes	92.621 ± 2.76

When comparing these results to the EE% of α -lipoic acid encapsulated in other carriers such as cubosomes ($69.45 \pm 14.3\%$) and hydrogels ($52.65 \pm 11.7\%$), depending on the composition and preparation method, both the niosomes and proniosomes encapsulated α -lipoic acid to a desirable level. Keeping the results obtained in mind, it is evident the niosome dispersion encapsulated α -lipoic acid better than the proniosome dispersion.

C.2.5 Zeta-potential

The surface charges of the various vesicle systems were determined by means of zeta-potential to examine the stability of the dispersions (Kumar & Rajeshwarrao, 2011:213; Kurakula *et al.*, 2012:36). A higher zeta-potential value indicates a greater electrostatic repulsion force between individual particles, meaning the dispersion is more stable and devoid of agglomeration (Honary & Zahir, 2013:270; Li *et al.*, 2016:45; Singh *et al.*, 2016:1478).

To determine the zeta-potential of the colloidal dispersions, a Malvern Zetasizer Nano ZS (Malvern Instruments, Worcestershire, United Kingdom) was used. One drop of the sample dispersion was diluted with 20 ml Milli-Q[®] water and thoroughly mixed before being injected into the cuvette. The measurements were taken on the same day as the preparation of the tested samples. All measurements were taken in triplicate.



Figure C.4: The Malvern Zetasizer Nano ZS used to measure the zeta-potential, Pdl and size of the dispersions

The zeta-potential results of all four dispersions are summarised in Table C.4. The niosome dispersion obtained the lowest zeta-potential, but all tested dispersions resulted in good stability results. Honary and Zahir (2013:270) specified that a higher zeta-potential value (negative or positive), typically more than 30 mV, will maintain a stable system. If the zeta-potential is below a certain level, the droplets or colloids will ultimately aggregate due to the attractive forces. The determination of this stability parameter is essential, as no stability testing will be done on the final product, only efficacy experiments will be conducted on the most suitable dispersion.

Table C.4: Summary of the results obtained with the Malvern Zetasizer Nano ZS

Vesicle dispersion		Zeta-potential average (mV)
Niosomes	Active encapsulated	-34.7 ± 2.5
	Placebo	-43.6 ± 0.3
Proniosomes	Active encapsulated	-43.2 ± 1.3
	Placebo	-55.9 ± 2.2

From Table C.4 it is evident that both placebo dispersions were more stable than their corresponding dispersions containing α -lipoic acid, which can possibly be due to the interactions between the components and added acidic active ingredient. By interpreting these zeta-potential results, both the niosome and proniosome dispersions are considered stable with an

average value for all dispersions of -44.35 ± 21.2 , indicating only a small change of aggregation of particles (Manconi *et al.*, 2011:40).

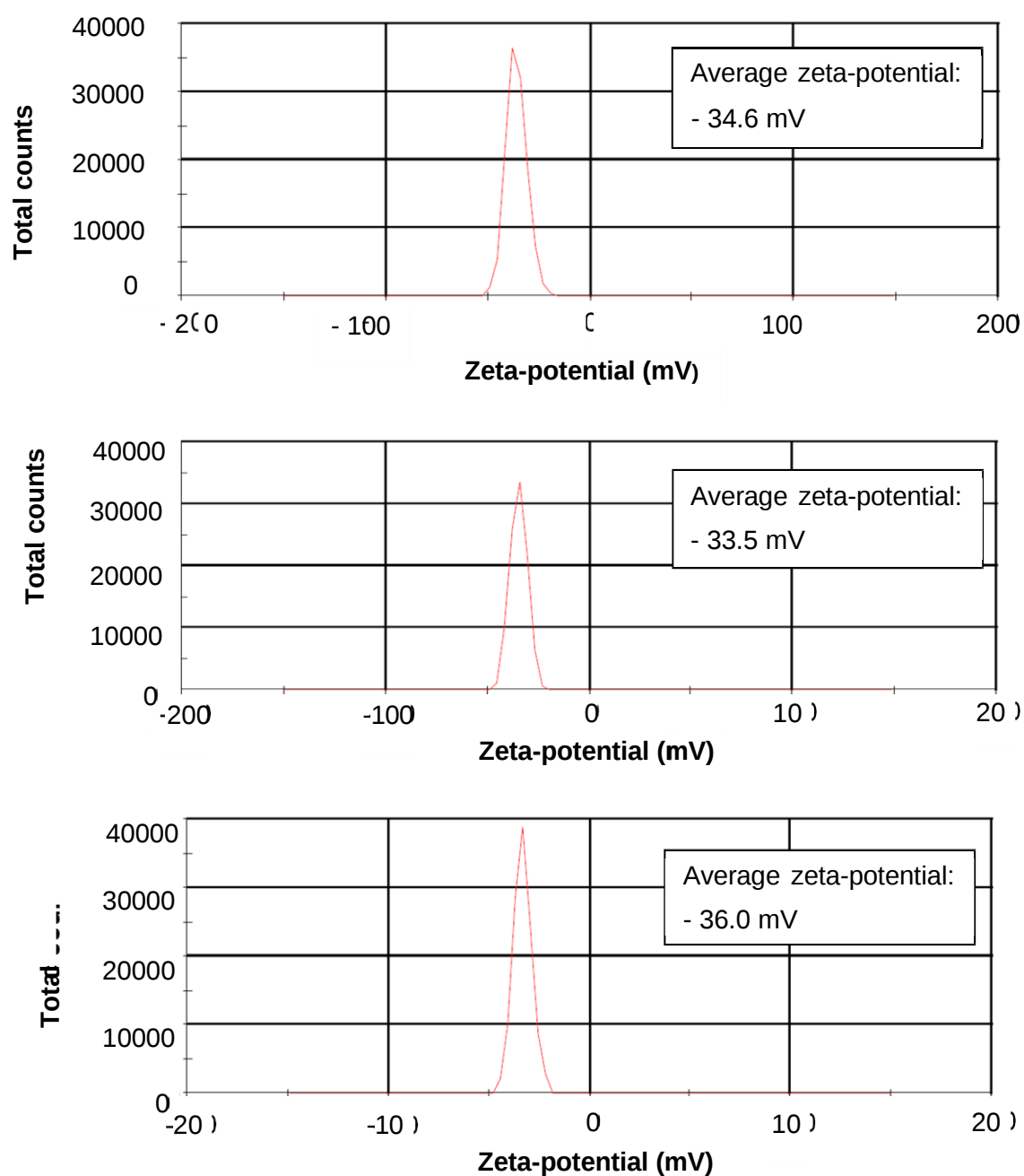


Figure C.5: Zeta-potential (mV) curves determined for 5% niosome dispersions measured in triplicate showing well-defined curves

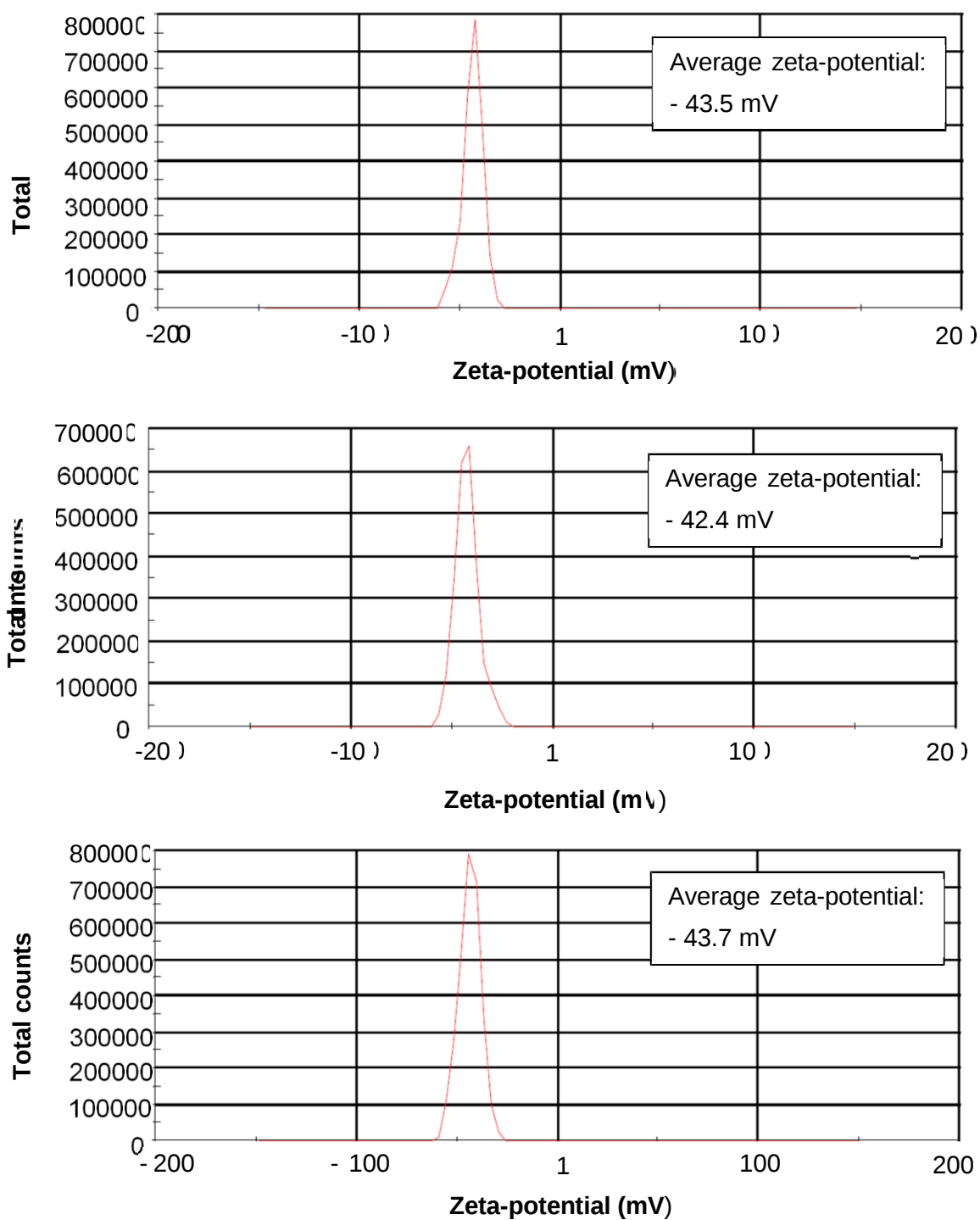


Figure C.6: Zeta-potential (mV) curves determined for 5% proniosome dispersions measured in triplicate

It can be seen from Figures C.5 and C.6 that the curves obtained during zeta-potential measurements were well defined and uniform, indicating good and stable dispersions for both the niosomes and proniosomes.

C.2.6 Droplet size and distribution

According to Shaji and Bhatia (2013:154), droplet size and distribution are some of the most important parameters for measuring a vesicle system. The size and distribution of each vesicle dispersion was measured using the Malvern Zetasizer Nano ZS, same as for the zeta-potential measurements. Measurements were taken in triplicate and the average values calculated. For each measurement, the samples were diluted by adding one drop of the dispersion to 25 ml of Milli-Q[®] water. According to Kumar and Rajeshwarrao (2011:213), the stability and EE% of vesicle systems are affected by the size of the droplets in the dispersions. Dispersions are considered more stable when the vesicles are small and their particles size distribution are narrow. For a uniform sample a Pdl of $\bar{D} = 0.0$ is required (Nobbman, 2014).

As mentioned before, the size range of niosomes are 10 – 1000 nm and can differ due to the various components in the formula used (Navya *et al.*, 2014:445). The average droplet size of the niosome dispersion containing α -lipoic acid (224.6 ± 8.1 d.nm) was found to be smaller when α -lipoic acid was incorporated into the formula, compared to the placebo niosome dispersion (817.2 ± 49 d.nm). In contradiction, the proniosome dispersion encapsulating the active ingredient obtained a bigger size measurement (698.33 ± 357.8 d.nm) compared to its placebo dispersion (312.23 ± 21.8). This variance in the results, obtained from both dispersions and their respective placebos, complicates the conclusion of why the average droplet sizes vary so much. A possible explanation is the addition of the sorbitol in the proniosome dispersion, which could influence the size measurements, or the affinity of α -lipoic acid to the components in the formula resulting in smaller vesicle sizes.

Table C.5: Droplet size and distribution results for niosome and proniosome dispersions

Vesicle dispersion		Average size (d.nm)	Average Pdl
Niosomes	Active encapsulated	224.60 ± 8.10	0.45 ± 0.13
	Placebo	817.20 ± 49.02	0.64 ± 0.05
Proniosomes	Active encapsulated	698.33 ± 357.80	0.70 ± 0.21
	Placebo	312.23 ± 21.88	0.54 ± 0.07

According to Nobbman (2014), all four dispersions are categorised as broadly polydispersed, considering the average Pdl measurements were higher than $\bar{D} > 0.4$. The niosome dispersion encapsulating α -lipoic acid had the best Pdl value (0.453 ± 0.136), compared to the proniosome dispersion with α -lipoic acid (0.702 ± 0.219).

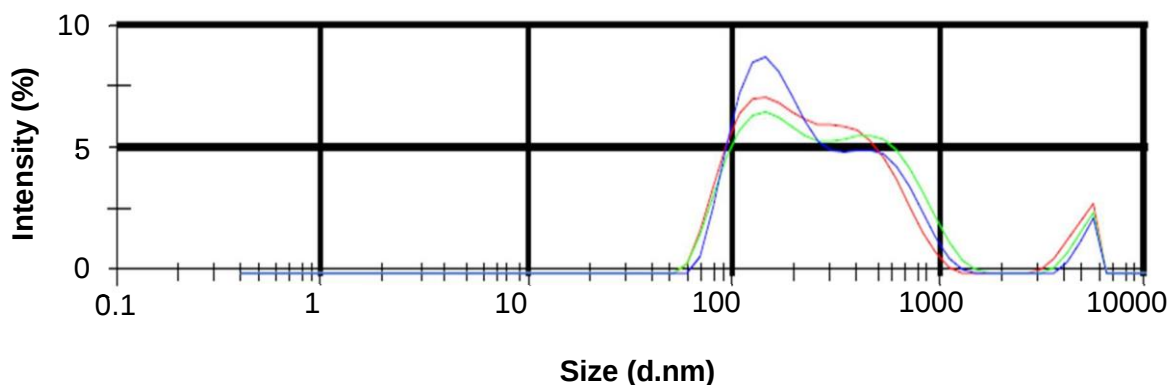


Figure C.7: Droplet size distribution curves determined for the 5% niosome dispersion

According to Ammar *et al.* (2011:148), the tail of the size distribution curve, as seen in Figure C.7, can be viewed as aggregations of some of the nanoparticles that formed and resulted in larger particles.

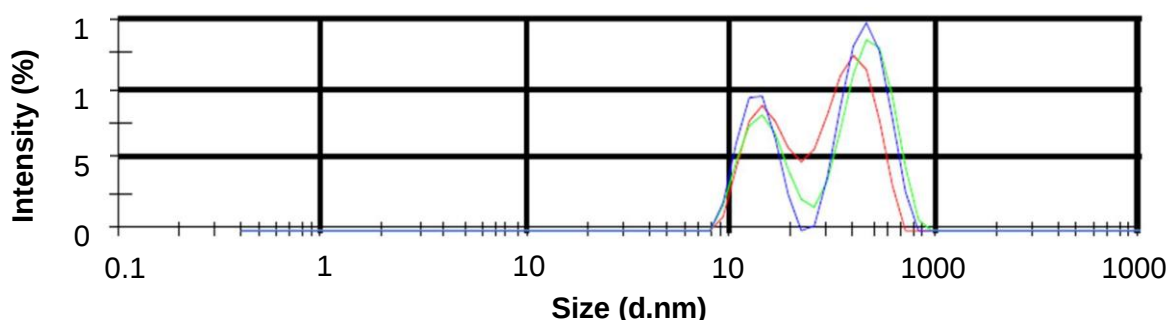


Figure C.8: Droplet size distribution curves determined for the 5% proniosome dispersion

From Figures C.7 and C.8, it can be seen that the size distribution curves collected for the niosome and proniosome dispersions with active ingredient were fairly narrow and similar in shape. The different sizes in each sample can be the result of fusion between the vesicles and caused by surface interactions that generate different sized vesicles in a formulation (Bayindir & Yuksel, 2010:2055).

C.3 Conclusion

To select the most suitable vesicle system to deliver α -lipoic acid successfully to the target dermal layers, the characterisation of both dispersions needed to be determined. It should be kept in mind that a vesicle system's characterisation properties affect their topical delivery potential and can indicate the stability of vesicle dispersions. No stability tests were done in this study, therefore the zeta-potential and size distribution measurements were of importance and an indication of the stability of the dispersions.

Both dispersions obtained good results from the TEM and pH meter, which indicated that both the niosome and proniosome dispersions formed clear unilamellar vesicles that were within the desired pH ranges for topical formulations. The zeta-potential averages for both dispersions were also good and indicated stable dispersions with values higher than – 30 mV.

The niosome dispersion showed better encapsulation of α -lipoic acid and overall better stability due to droplet size and size distribution, considering the values obtained from the Malvern Zetasizer Nano ZS. Although the proniosomes were more viscous than the niosomes, both dispersions will need an additional thickening agent before any clinical efficiency studies can take place. It should be kept in mind that membrane release studies, as well as skin diffusion studies still have to be performed on both dispersions, but considering the characterisation parameters determined the niosome dispersion seems to be the best-suited vesicle dispersion for α -lipoic acid.

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Appendix D

MEMBRANE RELEASE AND SKIN DIFFUSION STUDIES

D.1 Introduction

The Organisation for Economic Co-operation and Development (OECD) guidelines on dermal absorption, No. 156, states, "*in vitro* methods are designed to measure the penetration of chemicals into the skin and their subsequent permeation across the skin into a fluid reservoir, as well as to determine the partition into the different skin layers and possible deposition therein."

Isaac *et al.* (2015:1) also stated that for an active substance to be available through human skin, two consecutive steps are of importance, namely, the release from the vesicular system followed by its subsequent permeation through the skin layers. Therefore, this appendix discusses the release and permeation studies of the two topical dispersions containing α -lipoic acid, as well as their respective placebo dispersions for validation purposes.

The aim of this study is to deliver α -lipoic acid successfully to the dermal layers of the skin, where melanocytes and collagen are present, in the hope of potentially including the active in the treatment regimens for signs of skin ageing and inflammatory skin diseases, such as psoriasis. This will be done by encapsulating the α -lipoic acid within both niosomal and hydrated proniosomal dispersions, and establishing the most efficient vesicle system for further clinical efficacy studies.

Initially, the release studies were done on both vesicle dispersions to determine and quantify the amount α -lipoic acid that was successfully released from both vesicle systems. Thereafter, skin diffusion studies were done to determine the permeation behaviour and percentage α -lipoic acid that diffused by using excised human skin from abdominoplasty. The tape stripping method was used to quantify the amount of α -lipoic acid present in each specific skin layer to determine whether α -lipoic acid reached its target site, namely the epidermis-dermis layer.

D.2 Materials and methods

D.2.1 α -Lipoic acid dispersions

Both niosomal and hydrated proniosomal dispersions were prepared according to Appendix B. Placebo dispersions were made to act as controls throughout each study.

D.2.2 Sample analysis of α -lipoic acid by HPLC

All samples collected from the diffusion studies were analysed by means of HPLC in the Analytical Technology Laboratory (ATL) of the North-West University (NWU), Potchefstroom Campus, with the guidance of Prof J. du Preez. In Appendix A, the development and validation of the method used during this study is thoroughly discussed and deemed suitable for analysis of α -lipoic acid.

An Agilent 1100 Series (Agilent Technologies, Palo Alto, CA), equipped with a G1311A quaternary pump, a G1315A diode array detector, a G1322A vacuum degasser and G1313A autosampler injection mechanism was used. The software used for the analysis was the HP ChemStation software. The conditions and parameters remained the same throughout the membrane release and skin diffusion studies.

Table D.1: Chromatographic conditions

Column used	Venusil XBP C ₁₈ (2) 150 x 4.6 mm
Mobile phase	45% ACN/0.1% H ₃ PO ₄
Flow rate	1 ml/min
Retention time	The analyte elutes at approximately 7 min
Run time	Approximately 9 min
Detection	Diode array detector at 210 nm
Solvent/receptor phase	PBS at pH 7.4

D.2.3 Standard preparation for HPLC analysis

Two standards were prepared with different concentrations and injected into the HPLC by various volumes (5, 10, 15 and 20 μ l). Amounts of α -Lipoic acid were weighed (5.0 mg and 2.5 mg) and transferred to a 100 ml volumetric flask. MeOH (50 ml) was added to each solution and made up to volume with PBS (pH 7.4), respectively. After analysing the quantity released and diffused during the membrane release studies, it was decided that a higher concentration standard would be more accurate, therefore 20 mg and 50 mg standards solutions were used in the release and diffusion studies.

D.2.4 Aqueous solubility of α -lipoic acid

The aqueous solubility of α -lipoic acid was determined in PBS (pH 7.4), as it was used as the receptor solution during diffusion studies. During the solubility test, three test tubes were filled with 3 ml PBS (pH 7.4) each, where after an excess amount of α -lipoic acid was added to supersaturate the solutions. The tubes were placed into a Stuart Shaking water bath for 24 h at

a controlled temperature of 32 °C, mimicking the temperature of skin during diffusion studies (Williams, 2003:63). The test tubes were constantly inspected to ensure an excess of α -lipoic acid was still visible. All samples were then filtered through 0.45 μ m filters and injected into HPLC vials for analysis. A standard solution was prepared according to Section A.3 to obtain a standard curve and ensure validity.

D.2.5 *n*-Octanol-buffer partition coefficient of α -lipoic acid (log D)

The lipophilicity of an active is defined by Andrés *et al.* (2015:181) as the affinity of that specific active for a lipid environment at a specific pH. In the topical and transdermal research field, the log D value is an essential parameter to determine, since topically applied formulations should acquire lipophilic properties to permeate the skin successfully.

The log D was measured by examining the distribution profile of α -lipoic acid between the organic solvent (*n*-octanol) and an aqueous phase (PBS, pH 7.4). The concentrations of α -lipoic acid in the organic and aqueous phases were used to calculate the distribution coefficient (log D), as seen in Equation D.1. (Andrés *et al.*, 2015:182).

$$\text{Log D} = \text{Concentration}_a / \text{Concentration}_b \quad \text{Equation D.1}$$

Where:

Concentration_a - Concentration of α -lipoic acid in *n*-octanol

Concentration_b - Concentration of α -lipoic acid in PBS (pH 7.4)

The experiment was performed by equilibrating an equal amount of PBS (pH 7.4) and *n*-octanol over a period of 24 h to ensure co-saturation of both phases. The mixture was added to a separating funnel to separate the two liquids for 24 h; both volumes were tapped off into separate beakers the next day. The α -Lipoic acid was then added to 3 ml of the pre-saturated *n*-octanol in three separate tubes; these mixtures were placed in a heating bath and rotated for 10 min at 32 °C, thereafter, equal volumes of the pre-saturated PBS (pH 7.4) were added to three test tubes and allowed to rotate for a further 45 min. The samples were centrifuged at 4000 rpm for a period of 20 min for the two phases to separate completely. The two liquid phases were carefully separated into different beakers to be filtered through 0.45 μ m hydrophilic polyvinylidene fluoride membrane (PVDF) filters and injected into vials for analysis. A standard solution, according to Section A.3, was prepared to produce a standard curve for the HPLC analysis.

D.2.6 Preparation of phosphate buffer solution

The PBS (pH 7.4) used during the release and skin diffusion studies were prepared according to Section A.4. The pH of the PBS used in the receptor phase resembles the pH of human blood and therefore had a pH value of 7.4 (Rogers & McCutcheon, 2015:46).

D.2.7 Preparation of receptor and donor phase solutions

According to Collier and Bronaugh (1991:47), it is recommended that the receptor fluid for relatively lipophilic compounds should be specifically chosen to enable the compounds to partition freely within the receptor fluid. Keeping this in mind, the receptor phases for both the release and skin diffusion studies consisted of PBS (pH 7.4). The PBS was kept at a constant temperature of 37 °C prior to and throughout both studies to mimic the temperature of human blood.

The donor phases consisted of niosomal and hydrated proniosomal dispersions prepared according to Annexure B. Both dispersions and their placebo dispersions were kept at 32 °C (skin's surface temperature) prior to loading the Franz cells. A magnetic stirrer was inserted into the dispersions to ensure the dispersion was heated uniformly and remained homogeneous.

D.2.8 Preparation of human skin for diffusion studies



Figure D.1: The Zimmer™ electric dermatome with 2.5 cm width plate was used. Each donor's skin was dermatomed with a new sterile blade and placed on Whatman® filter paper

The procurement and exploitation of human skin during this study was approved, under reference number NWU-00114-11-A5, by the Research Ethics Committee of the North-West

University. Female Caucasian abdominal skin was collected after cosmetic surgery and frozen, within 24 h after removal, at - 20 °C (Brain *et al.*, 1998:167). Each donor signed an informed consent form prior to the collection of the skin and all patient information was kept confidential. The skin samples were prepared using a Zimmer™ electric dermatome (model 8821) fitted with a 2.5 cm width plate to dermatome the skin, and the samples were placed upon Whatman® filter paper (as seen in Figure D.1).

D.2.9 Permeation experiments

During the membrane release and skin diffusion studies, the same preparation method was followed with the only differences being either membrane filters or skin samples used, respectively, and the extraction times for each.

The vertical Franz diffusion cells (Figure D.2.a) used consisted of a top (donor) and bottom (receptor) compartment with a diffusional area of 1.13 cm². In both the membrane release and skin diffusion studies, 12 Franz cells were used, of which two contained placebo formulations to serve as a control.



Figure D.2: Photographs illustrating a) the donor and receptor compartments of a vertical Franz cell, b) a horseshoe clamp used to, c) securely assemble the Franz cell and d) Dow Corning® high vacuum grease to prevent leakage

PVDF membrane filters, with a pore size of 0.45 μm (Pall® Life Sciences, Michigan, United States), were used during the membrane release studies and dermatomed skin, with the stratum corneum facing upwards, for the skin diffusion studies. The membrane filters, or skin samples, were placed on the surface area of the receptor compartment, covered with the donor compartment and sealed with Dow Corning® (Sigma-Aldrich, Germany) high vacuum grease to prevent any leakages occurring (Figure D.2.d). The two compartments were securely mounted and clamped together with a horseshoe clamp (Figure D.2.b) to ensure no movement or leakage would take place, as seen in Figure D.2.c.

PBS (pH 7.4; 2 ml) was injected into each receptor compartment along with a magnetic stirrer that continuously stirred the receptor fluid throughout the studies. Care was taken to avoid any air bubbles forming whilst injecting the PBS (pH 7.4), as this could influence the data obtained.

Each donor compartment was filled with 1 ml of the specific vesicle dispersion and covered with Parafilm® to avoid any spillage or loss of dispersion during extraction times. The Franz cells were then placed on a submersible Variomag® magnetic stirrer plate (Variomag, United States) and placed in a Grant water bath (Grant Instruments, United Kingdom) at 37 °C (Figure D.3).



Figure D.3: Franz cells arranged on a Variomag® magnetic stirrer plate and submerged in a Grant water bath set at a controlled temperature of 37 °C

The receptor compartments remained submerged throughout the study leaving the donor compartment above water, while retaining their 32 °C temperature to mimic skin's surface temperature (Williams, 2003:62).

During the membrane release studies, the receptor phases were carefully extracted every hour using specifically allocated syringes with tubes to ease the extraction process. All receptor compartments were refilled with PBS (pH 7.4, 37 °C) for the total duration of the study (6 h). For the skin diffusion studies, the receptor phases were extracted every 2 h for a period of 12 h. The receptor compartments were also refilled using PBS (pH 7.4, 37 °C) as in the membrane studies. The withdrawn samples were then injected into vials and analysed according to Appendix A by means of HPLC. The concentrations of α -lipoic acid released and permeated through the PVDF filters and skin, respectively, was then determined.

D.2.9.1 Membrane release studies

Prior to skin diffusion studies, the release of α -lipoic acid from the two vesicles dispersions was evaluated. If no release of α -lipoic acid was found during the release studies, skin diffusion studies would be deemed useless and no need for further clinical studies would be necessary.

The aim of the membrane release studies was to determine whether, and to what extent, α -lipoic acid was released and diffused from the vesicle dispersions and membrane filters, respectively. Niosomes and proniosomes encapsulating α -lipoic acid were formulated according to Appendix B and additional dispersions were prepared following the same methods, but containing no active to serve as controls during all studies. Standard solutions were freshly prepared for each study to ensure accuracy of values and comparison on the HPLC system.

D.2.9.2 Skin diffusion studies

The skin diffusion studies followed the membrane release studies after adequate release of α -lipoic acid was found. The aim of these studies was to determine the concentrations of α -lipoic acid that diffused through the skin layers by means of HPLC analysis, in conjunction with quantifying the amount of α -lipoic acid deposited in the distinct layers of the skin by conducting the tape stripping method.

D.2.9.3 Tape stripping

The aim of performing the tape stripping method was to remove the stratum corneum in order to calculate the penetration behaviour of α -lipoic acid. According to Nagelreiter *et al.* (2015:1), this method has widely been used in dermo-pharmacokinetic experiments and studies involving the *in vivo* bioavailability and bioequivalence of topical applied substances.

Lademann *et al.* (2008:2) stated that between five tape strips, the amount of stratum corneum removed might differ by 100%, which can essentially influence the interpretation of dermo-pharmacokinetics studies. This is due to several factors, such as the size of the corneocytes,

donor's age, skin type and race, therefore, to limit the amount of variables in this study, experiments were performed on skin of one donor and care was taken when applying and removing the strips to ensure uniformity, as Jacobi *et al.* (2005:94) stated this can profoundly influence the amount stratum corneum removed from each strip.

After completing the skin diffusion studies, all Franz cells were dismantled and skin samples were placed on Parafilm®, securely pinned to a solid surface. Using clean dry tissue paper, the skin was carefully dabbed to remove any remaining donor phase. 3M Scotch® Magic™ tape was cut to a length that covered the size of the diffusion area ($\approx 11.7 \text{ cm}^2$), which was visible due to the indent made by the Franz cells. The first strip was discarded as it formed part of the cleaning procedure to ensure all donor phase was removed. The next 15 strips used to remove the stratum corneum-epidermis (SCE) were placed in a polytop containing 5 ml PBS (pH 7.4) and left for extraction of α -lipoic acid to take place. The remaining epidermis-dermis (ED) was then cut into smaller pieces to increase the surface area and placed into a polytop containing 5 ml PBS (pH 7.4). All polytops were stored in the fridge ($2 - 5^\circ\text{C}$) overnight, where after the samples were filtered and HPLC analysis was performed the next day to determine the quantities of α -lipoic acid in each skin layer.

D.2.9.4 Data analysis

The data obtained from the membrane release studies were used to calculate the average percentage of active ingredient released (%) from each vesicle, together with the average concentration ($\mu\text{g}/\text{cm}^2$) of the active ingredient that successfully permeated the skin over a period of 6 h. For the skin diffusion studies, the average percentage diffused (%) and the average concentration ($\mu\text{g}/\text{cm}^2$) of active ingredient that penetrated through the skin layers were determined. The average flux values ($\mu\text{g}/\text{cm}^2\cdot\text{h}$) calculated from the release and diffusion studies were compared to examine any correlation between these two values. For the tape stripping studies, the concentration ($\mu\text{g}/\text{ml}$) that accumulated in the individual skin layers (SCE and ED) was determined.

Descriptive statistical tests were done on the release studies, SCE, ED and on the amounts of active ingredient found in the receptor compartments. Two-way analysis of variance (ANOVA) tests were performed with the dispersion (niosomes or proniosomes) and skin layer, as factors to determine whether the interaction between dispersions and skin layers was statistically significant. The p-values for these interactions were in some instances smaller than 0.05 ($p < 0.05$, i.e. statistically significant on a 5% level), giving rise to the comparison of skin layers for each dispersion separately. Therefore, T-tests were performed on each dispersion to compare a) the SCE and ED, b) the ED and skin diffusion and c) the SCE and skin diffusion; to

determine the targeting ability of both dispersions. These results were then validated by performing the non-parametric Mann-Whitney U-test on each of the two dispersions.

D.3 Results and discussion

D.3.1 Aqueous solubility

The ideal aqueous solubility of a substance to permeate the skin is higher than 1 mg/ml (Hadgraft, 1996:165; Naik *et al.*, 2000:319; Williams, 2003:37). The active compound, α -lipoic acid, is a poorly soluble compound with a low bioavailability (Li & Lim, 2016:1; Teichert & Preiss, 2008:288). According to Drugbank (2016), the predicted aqueous solubility of α -lipoic acid in water is 0.224 mg/ml, while Toxnet (2011) stated the experimental aqueous solubility of α -lipoic acid in water is 0.127 mg/ml (25 °C). In this study, the aqueous solubility of α -lipoic acid, determined in PBS (pH 7.4), was found to be 0.33 mg/ml (32 °C). The slight increase in value may possibly be due to the fact that at a pH of 7.4 α -lipoic acid will be fully ionised; thus increasing the solubility (pKa 4.52, Drugbank), and the different temperatures and mediums used during the experiments may have increased the active compounds' solubility. To conclude, the solubility of α -lipoic acid in PBS (pH 7.4) is not ideal for skin permeation.

D.3.2 *n*-Octanol-buffer distribution coefficient

According to Cichewics (2013:1), the predicted log D value of α -lipoic acid is 2.3, but very little supporting literature confirms this. An experimental log D value in PBS (pH 7.4), determined by Snyman (2009:56), was found to be - 0.78 at a temperature of 32 °C. In this study, the log D determined for α -lipoic acid was - 0.21, which is closely related to the value obtained by Snyman (2009:56), but it is an unfavourable value for penetration through the skin. Williams (2003:36) stated a log D value of 1 – 3 is required for optimal penetration and therefore, α -lipoic acid does not meet this standard and penetration may not be optimal with this determined value.

D.3.3 Membrane release studies

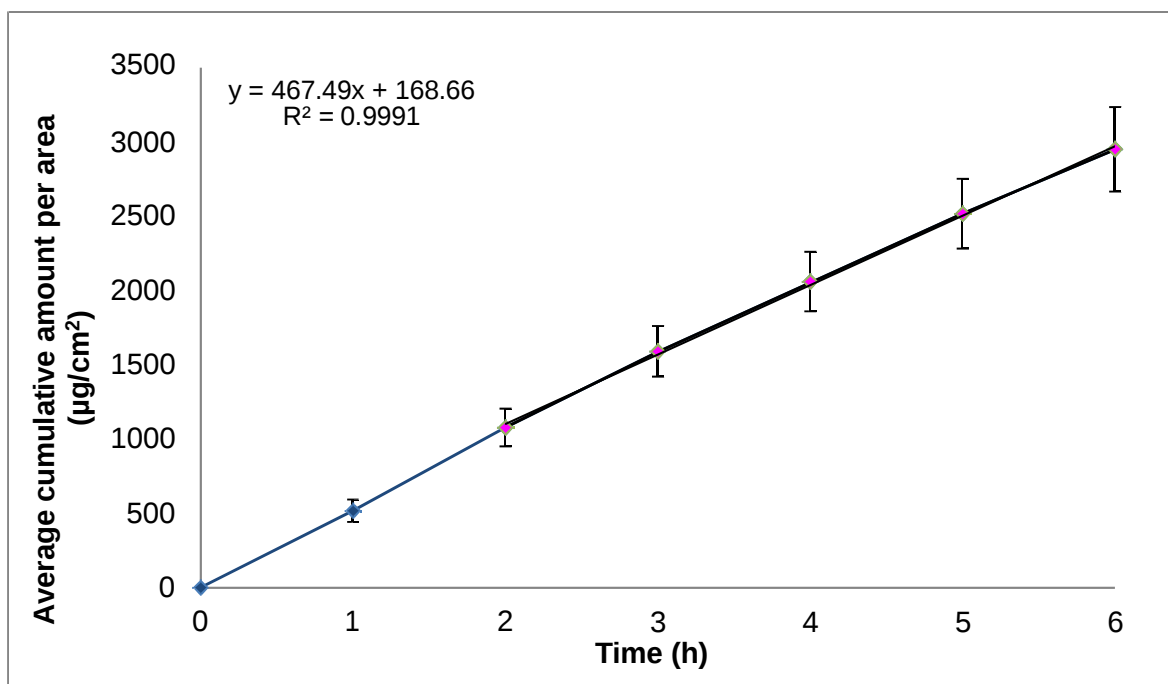


Figure D.4: Average cumulative amount per surface area ($\mu\text{g}/\text{cm}^2$) of α -lipoic acid that released from the niosomal dispersion, as a function of time to illustrate the average flux from the linear part of the graph (2 – 6 h; $n = 10$)

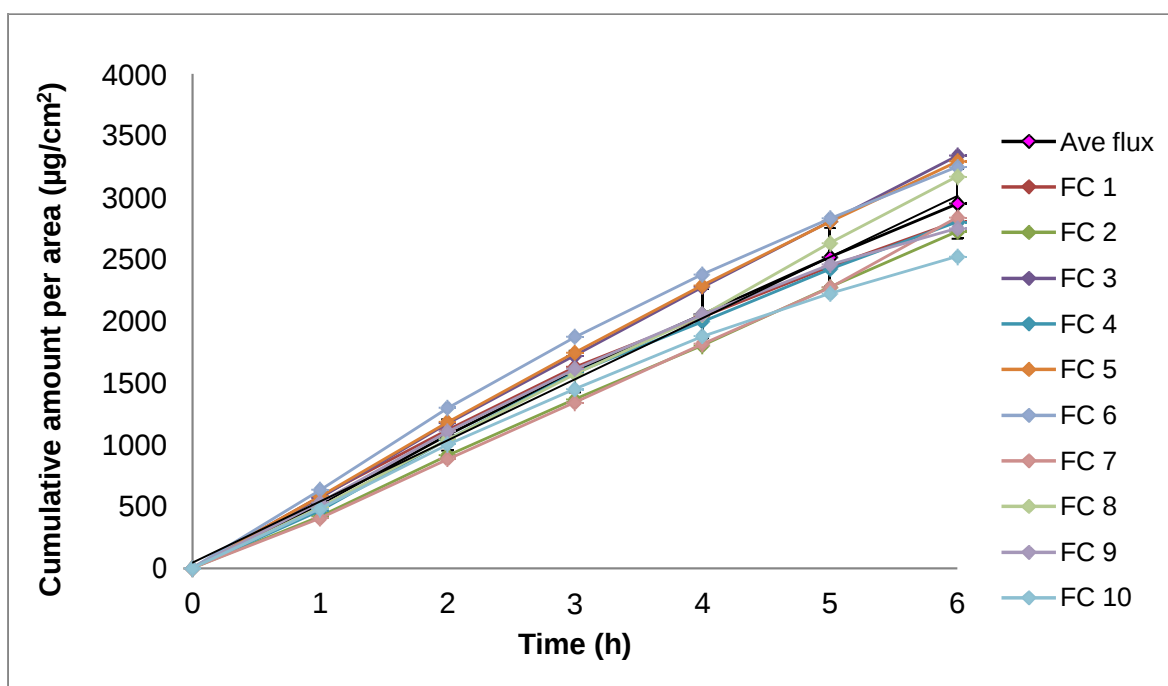


Figure D.5: Cumulative amount per surface area ($\mu\text{g}/\text{cm}^2$) of α -lipoic acid that released from the niosomal dispersion, as a function of time to illustrate the individual Franz cells during the 6 h membrane release study ($n = 10$)

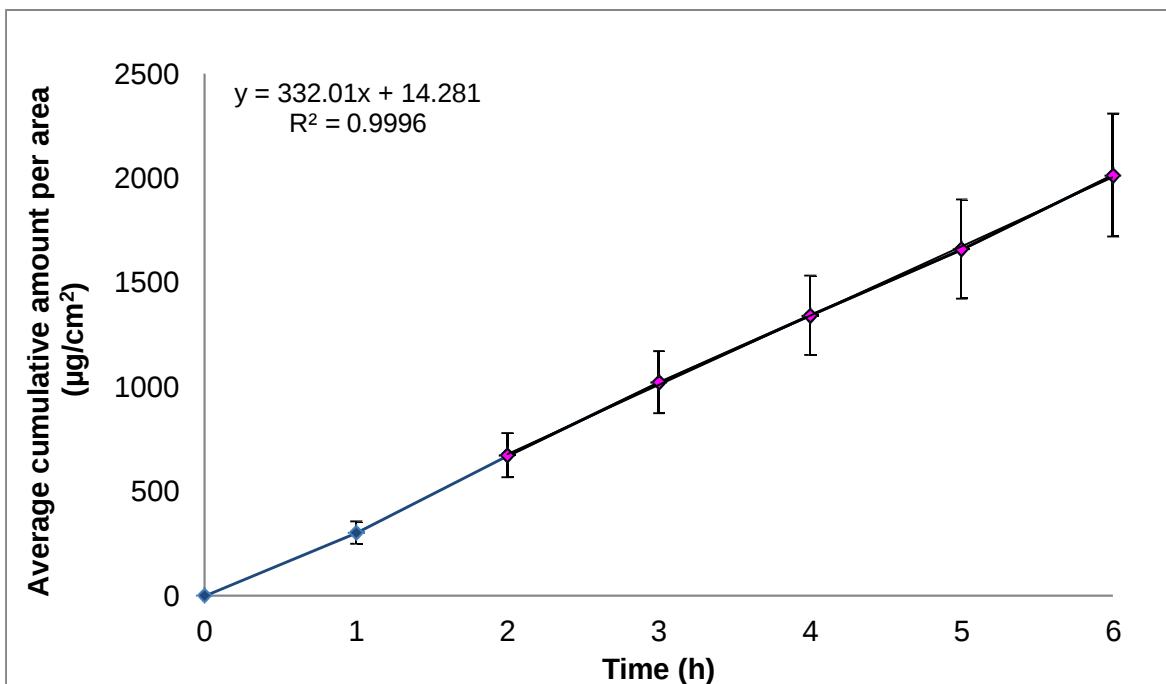


Figure D.6: Average cumulative amount per surface area ($\mu\text{g}/\text{cm}^2$) of α -lipoic acid that released from the proniosomal dispersion, as a function of time to illustrate the average flux from the linear part of the graph (2 – 6 h; $n = 10$)

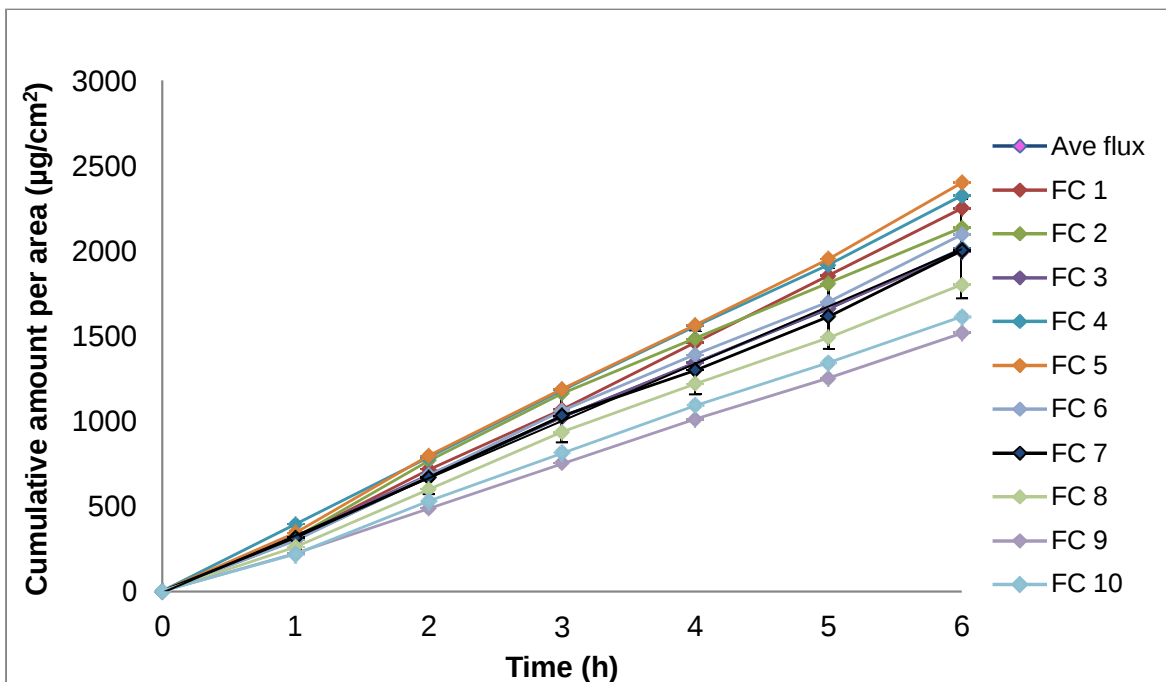


Figure D.7: Cumulative amount per surface area ($\mu\text{g}/\text{cm}^2$) of α -lipoic acid that released from the niosomal dispersion, as a function of time to illustrate the individual Franz cells during the 6 h membrane release study ($n = 10$)

As mentioned before, the aim of the release studies was to determine if the dispersions released α -lipoic acid to a desirable degree and to compare the average flux values for these dispersions. The average flux value, calculated from the slope of the straight line in Figures D.4 and D.6, illustrates the amount of α -lipoic acid that diffused through the PVDF filters during the 6 h release studies from both dispersions. Figures D.5 and D.7 graphically illustrate the cumulative amount of active ingredient detected per surface area ($\mu\text{g}/\text{cm}^2$) for all 10 individual Franz cells during the 6 h release studies. The outcomes of the membrane release studies for each dispersion can be seen in Table D.2. From the data obtained, it is evident that both the niosomal and hydrated proniosomal dispersion showed satisfactory release of the α -lipoic acid during the membrane studies.

Both the niosomal and proniosomal dispersions showed satisfactory release through the PVDF membrane filters, therefore further skin diffusion studies were conducted to determine the most efficient carrier for α -lipoic acid. It should be kept in mind that the vesicle system obtaining the best release results is not necessarily the vesicle system that best targets and delivers the active ingredient to the dermal layers of the skin.

Table D.2: Comparative summary of the results obtained from the membrane release studies over a period of 6 h (n = 10)

Dispersion	Average %released after 6 h	Average flux ($\mu\text{g}/\text{cm}^2\cdot\text{h}$)
Niosomes	3.123 ± 0.30	467.49 ± 51.82
Hydrated Proniosomes	2.128 ± 0.31	332.01 ± 49.04

Table D.2 indicates the average flux value calculated for the niosomal dispersion ($467.49 \pm 51.82 \mu\text{g}/\text{cm}^2\cdot\text{h}$) was higher than that of the proniosomal dispersion ($332.01 \pm 49.04 \mu\text{g}/\text{cm}^2\cdot\text{h}$). It is also evident that although the results for the niosomal dispersion dominated its opponents', both carriers obtained desirable values from the release studies. From Figure D.8 it can be seen that the niosomal dispersion attained the higher flux value released after the 6 h study.

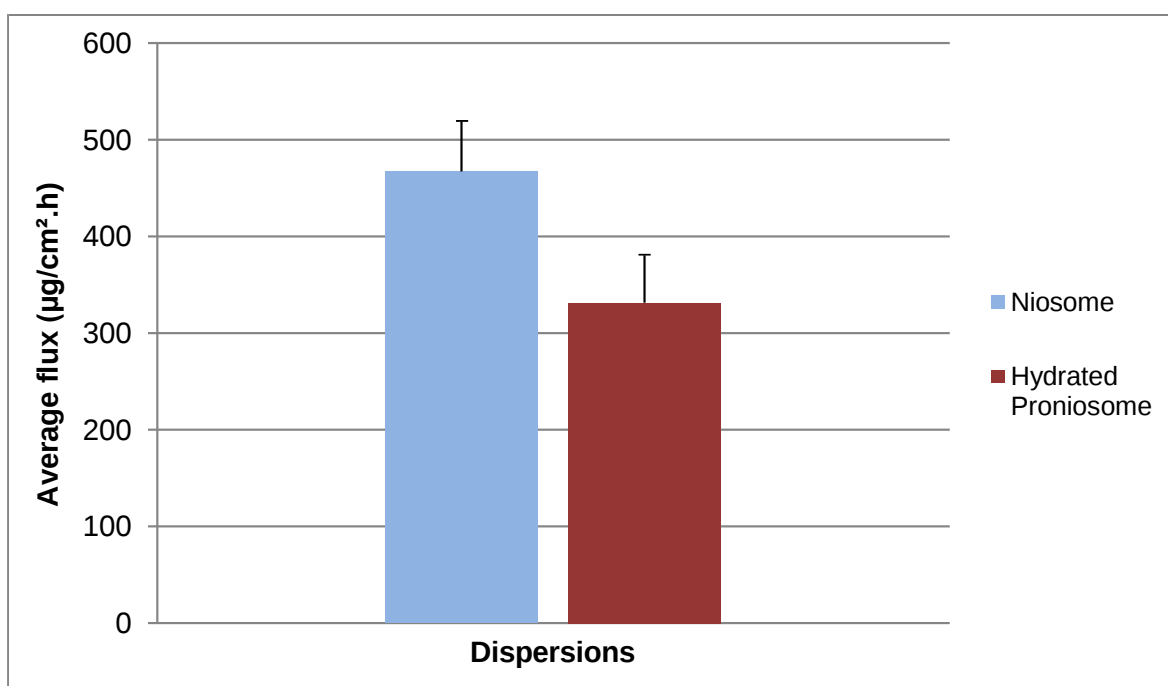


Figure D.8: Comparison between the average flux values (µg/cm².h) for the niosome and proniosome dispersions that released through PVDF filters over 6 h (n = 10)

D.3.4 Skin diffusion studies

To determine the percentage active ingredient that diffused through the skin, the receptor phases were analysed every 2 h up to 12 h by means of HPLC (Section A.2). The remaining skin samples were also analysed to determine the concentration of active in each individual layer (SCE and ED) of skin.

D.3.4.1 Transdermal diffusion

In both the niosomal and proniosomal diffusion studies, all 10 Franz cells had concentrations of α-lipoic acid present in their receptor compartments, indicating both dispersions delivered α-lipoic acid into the PBS (pH 7.4), representing the systemic circulation. Although α-lipoic acid is an endogenous compound to the skin, it was not detected in the control groups, therefore attaining no active ingredient in the receptor phase. This may possibly be explained by the rapid reduction that takes place due to the transformation of α-lipoic acid to its reduced state, i.e. dihydrolipoic acid, in the soma cells (Perricone, 2000:219).

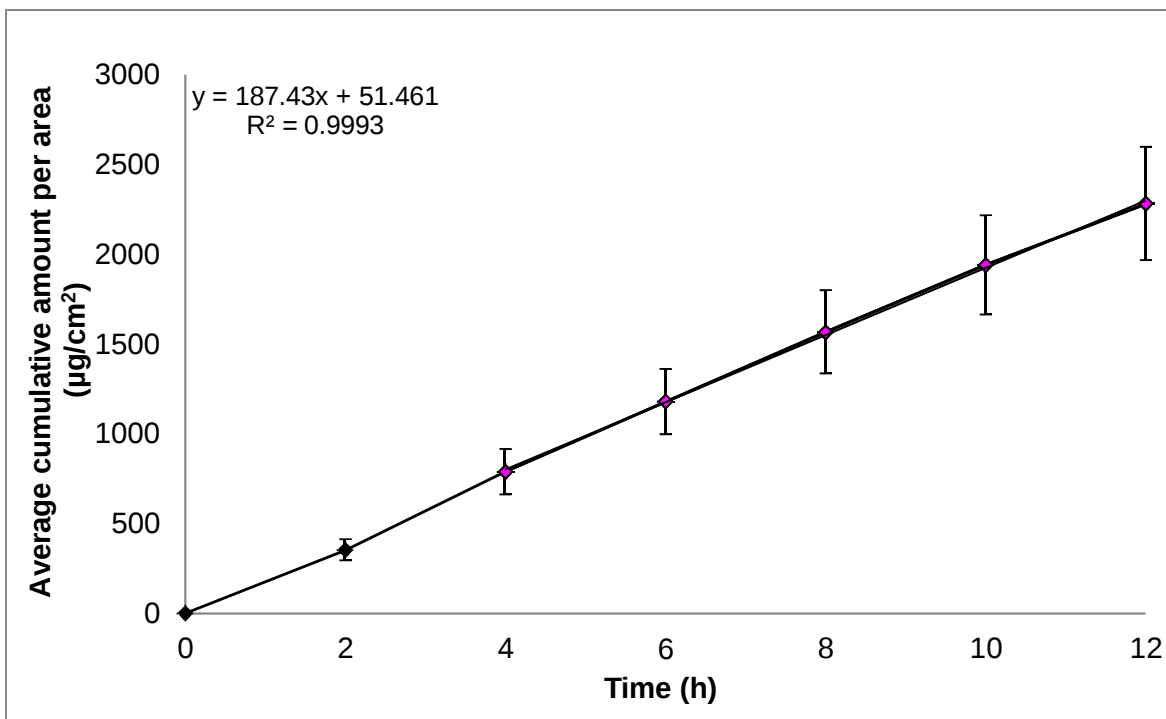


Figure D.9: Average cumulative amount per surface area ($\mu\text{g}/\text{cm}^2$) of α -lipoic acid that diffused from the niosomal dispersion, as a function of time to illustrate the average flux from the linear part of the graph (4 – 12 h; $n = 10$)

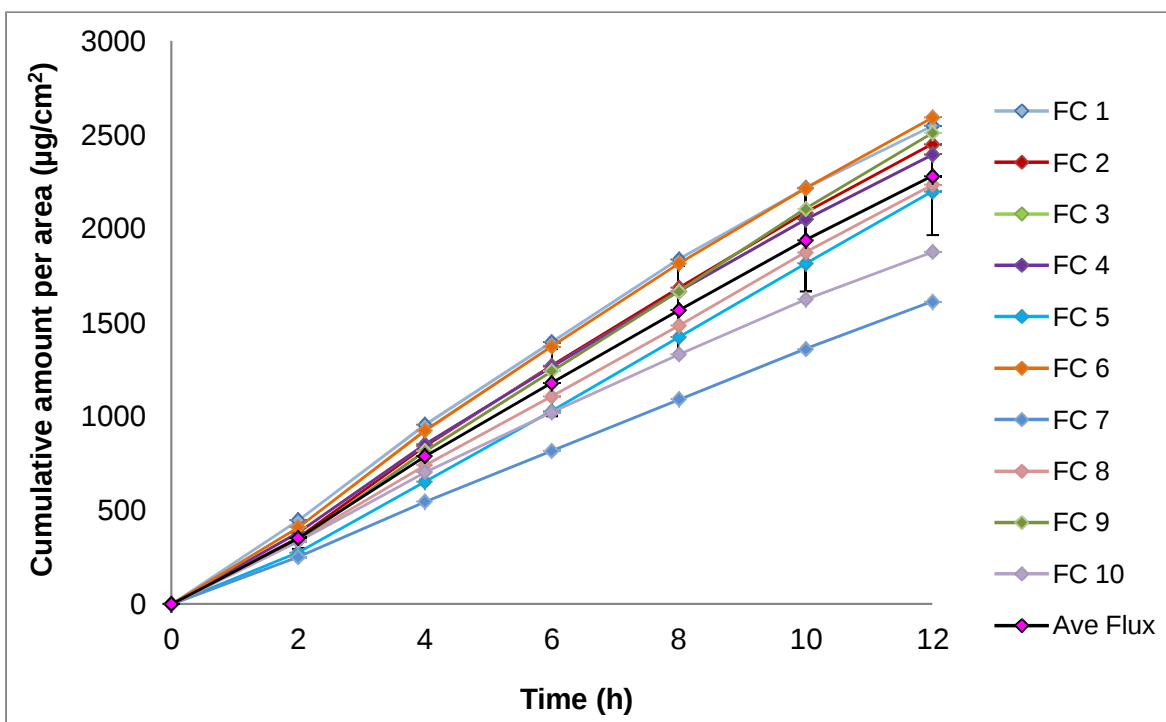


Figure D.10: Cumulative amount per surface area ($\mu\text{g}/\text{cm}^2$) of α -lipoic acid that diffused from the niosomal dispersion, as a function of time to illustrate the individual Franz cells during the 12 h skin diffusion study ($n = 10$)

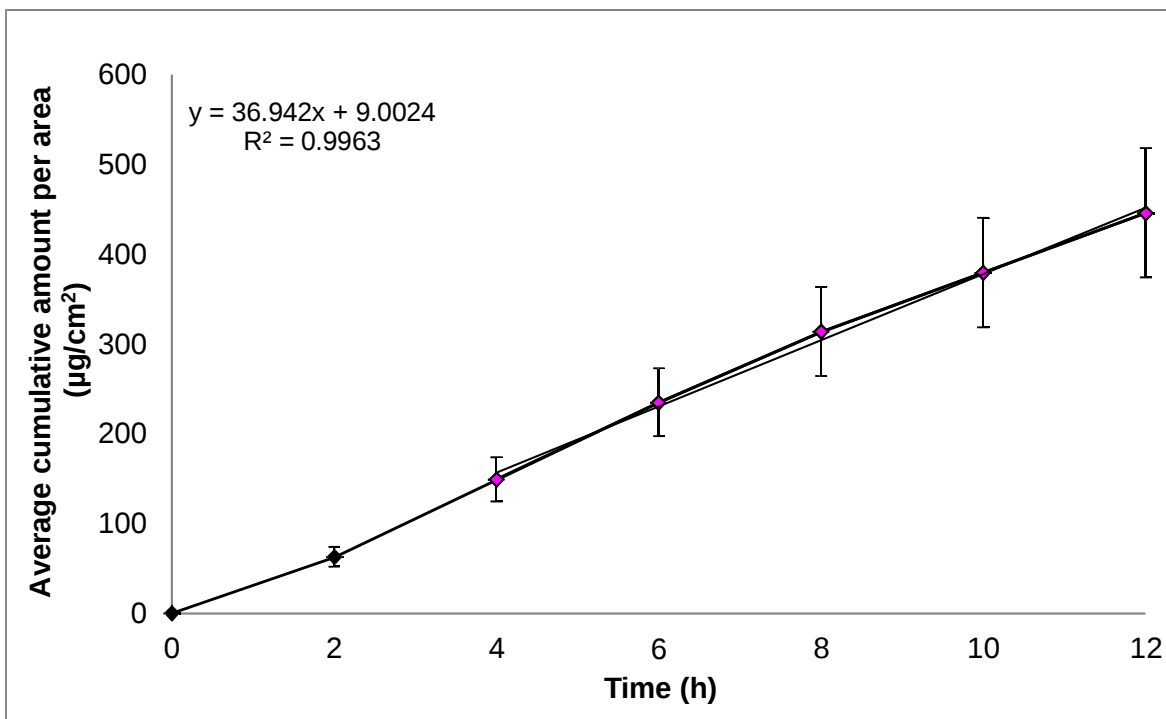


Figure D.11: Average cumulative amount per surface area ($\mu\text{g}/\text{cm}^2$) of α -lipoic acid that diffused from the hydrated proniosomal dispersion, as a function of time to illustrate the average flux from the linear part of the graph (4 – 12 h; $n = 10$)

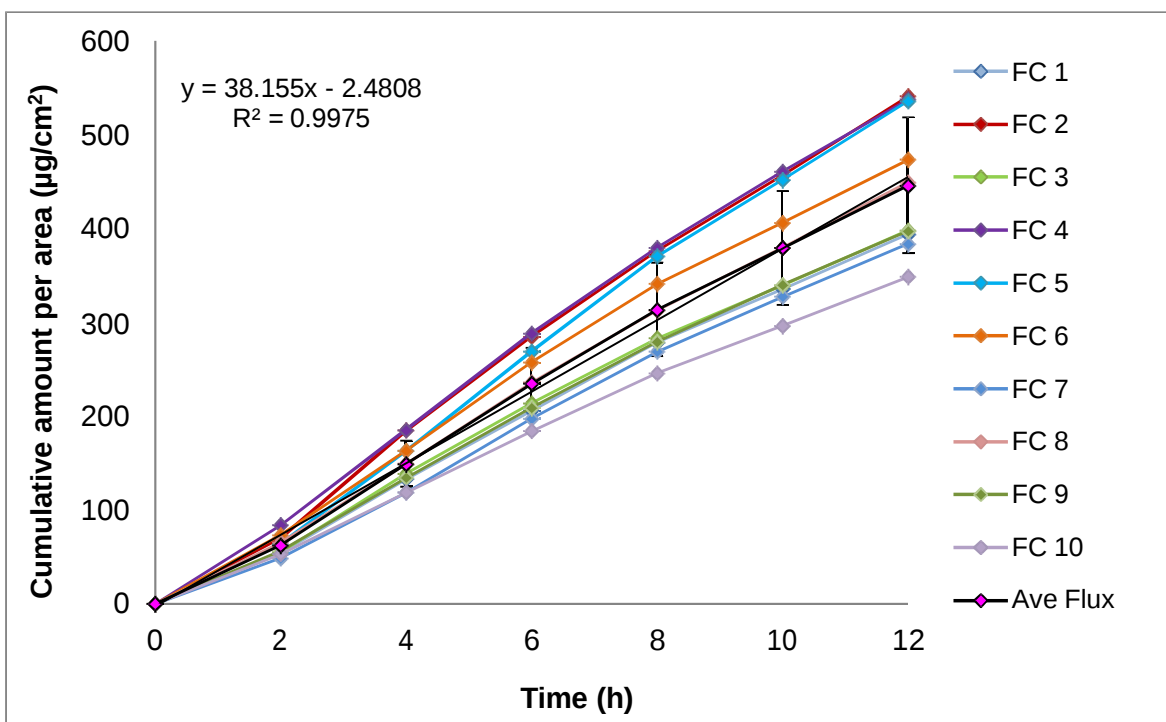


Figure D.12: Cumulative amount per surface area ($\mu\text{g}/\text{cm}^2$) of α -lipoic acid that diffused from the hydrated proniosomal dispersion, as a function of time to illustrate the individual Franz cells during the 12 h skin diffusion study ($n = 10$)

Table D.3: Comparative summary of the results obtained from the skin diffusion studies over a period of 12 h (n = 10)

Dispersion	Average %diffused after 6 h	Average flux ($\mu\text{g}/\text{cm}^2\cdot\text{h}$)
Niosomes	2.418 ± 0.33	187.43 ± 25.95
Hydrated Proniosomes	0.471 ± 0.08	36.94 ± 6.11

The niosomal dispersion ($187.43 \pm 25.95 \mu\text{g}/\text{cm}^2\cdot\text{h}$) had an overall better diffusion profile in comparison to the proniosomal dispersion ($36.94 \pm 6.11 \mu\text{g}/\text{cm}^2\cdot\text{h}$). This could be due to the viscosity differences between the two tested dispersions. The proniosomal dispersion was found to have a higher viscosity than the niosomal dispersion, possibly due to the addition of sorbitol. According to Cross *et al.* (2001:105), a higher viscosity of formulations may lead to a reduction in the permeation of an active ingredient.

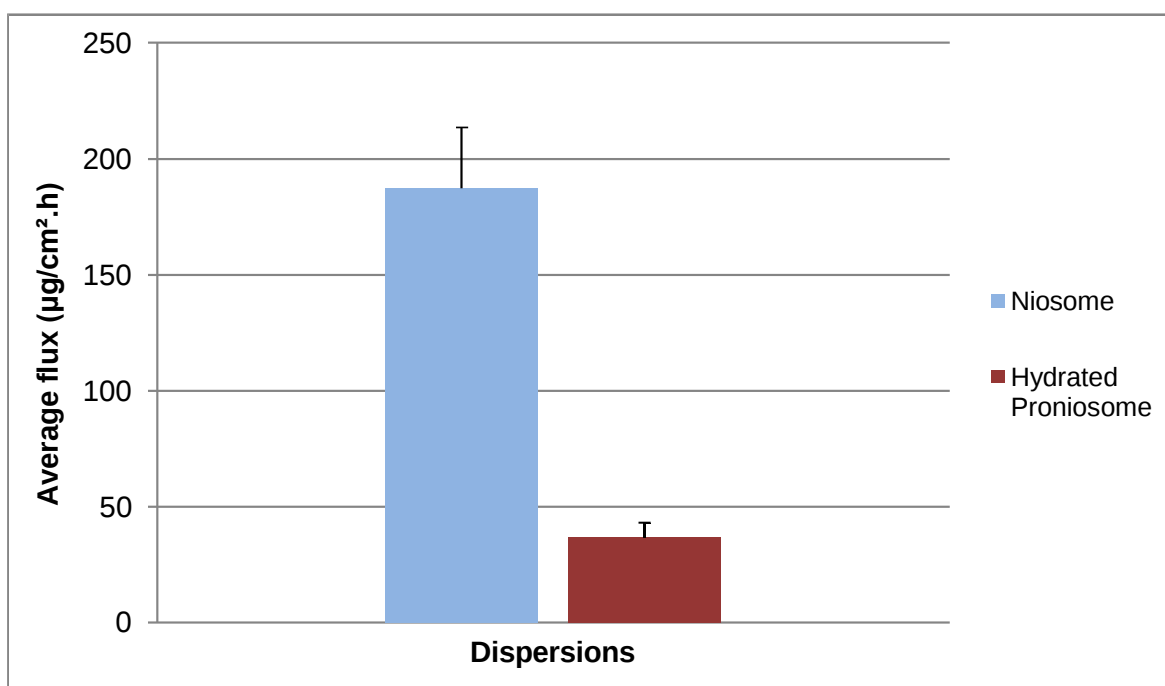


Figure D.13: Comparison between the average flux values ($\mu\text{g}/\text{cm}^2\cdot\text{h}$) for the niosome and proniosome dispersions that diffused through the skin over 12 h (n = 10)

D.3.4.2 Tape stripping

This method was performed to evaluate the concentration active ingredient in each skin layer (SCE and ED) after the 12 h diffusion study. For α -lipoic acid to be effective against signs of ageing and act against inflammatory skin conditions, accumulation in the ED is necessary.

D.3.4.2.1 Stratum corneum-epidermis

After HPLC analysis, concentrations of α -lipoic acid were found in the SCE delivered from both dispersions. All 10 Franz cells showed amounts of active present in the SCE, although the amounts were not that significant. It should also be mentioned that none of the control groups detected any concentrations of the active ingredient in the SCE. As mentioned before, the rapid reduction of α -lipoic acid to its reduced state, dihydrolipoic acid, may be the explanation for this phenomenon (Perricone, 2000:219). The average concentration of α -lipoic acid delivered from the niosomal dispersion ($2.411 \pm 1.32 \mu\text{g/ml}$) was slightly higher than that of the proniosomal dispersion ($2.166 \pm 1.47 \mu\text{g/ml}$) in the SCE. From Figure D.14.a, it is evident that the difference between the two dispersions is not significant and both vesicle systems delivered α -lipoic acid to the SCE. Furthermore, α -Lipoic acid is more lipophilic in nature and therefore has affinity for lipophilic regions, which could explain the concentrations detected in this skin layer (Podda *et al.*, 1996:632). Results obtained correlate with a similar study previously performed by Podda *et al.* (1996:632), which also observed the presence of α -lipoic acid in the stratum corneum skin layer after the topical application of a formulation containing α -lipoic acid.

D.3.4.2.2 Epidermis-dermis

The concentrations of the active ingredient found in the ED indicated which vesicle carrier was more efficient in delivering α -lipoic acid to the desired skin layer. The detected concentration of α -lipoic acid present in the ED was $2.854 \pm 1.43 \mu\text{g/ml}$ after delivery from the hydrated proniosomal dispersion and $5.077 \pm 1.47 \mu\text{g/ml}$ for the niosomal dispersion. The reason once again for no α -lipoic acid concentrations detected in the ED of the placebo formulations can be attributed to the reduction of α -lipoic acid to its reduced state (Perricone, 2000:219). The intended target site for α -lipoic acid encapsulated in both vesicle carriers were the ED and the obtained results showed both dispersions successfully delivered the active to this layer. According to two similar studies conducted on topical α -lipoic acid formulations, this active rapidly penetrates the skin layers and accumulates into this skin region possibly due to the presence of sebaceous gland and other lipid-rich components (Beitner, 2003:842; Podda *et al.*, 1996:632). The concentrations detected for the niosomal dispersion was almost twice as high as that of the proniosomal dispersion, obtaining concentrations similar to the amounts observed in the SCE.

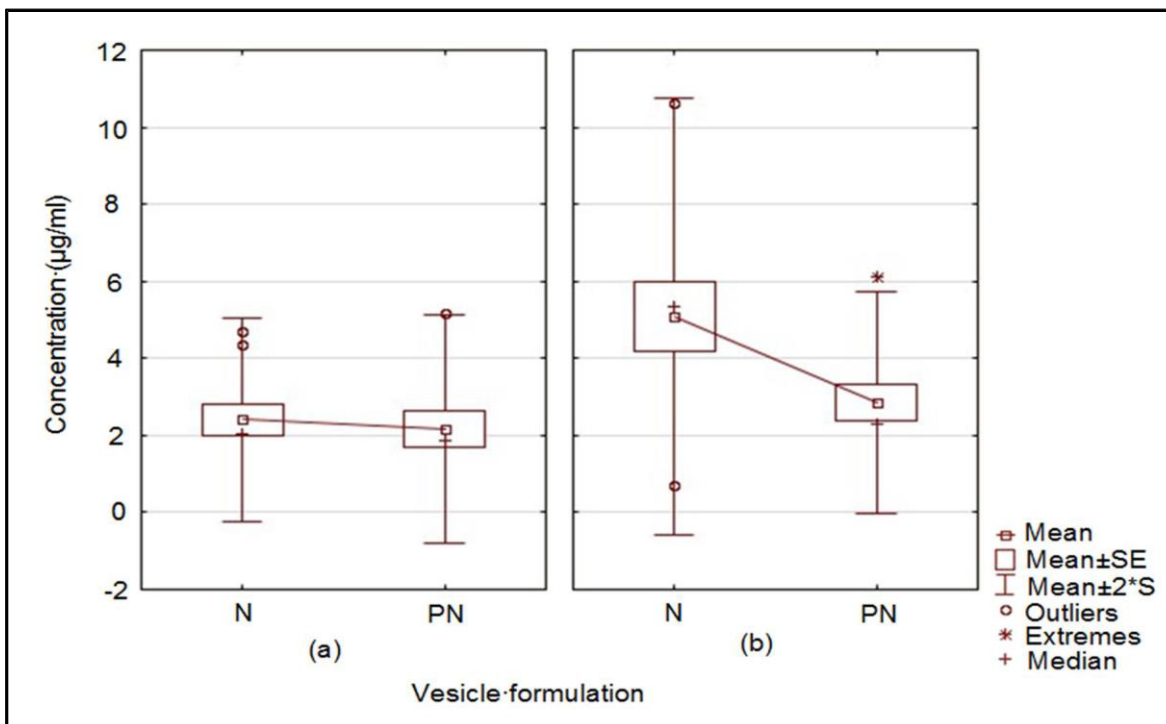


Figure D.14: Boxplots illustrating the concentration (µg/ml) of α-lipoic acid present in the a) SCE and b) ED from niosome (N) and proniosome (PN) dispersions. The median and average concentrations are indicated by the squares and lines, respectively.

D.3.5 Statistical data analysis of diffusion studies

The occlusion test was used to examine any correlation between the average flux (µg/cm².h) value released and diffused for each individual dispersion. Results showed no statistically significant correlation between the flux values for either the niosomes ($p = 0.2765$) or the proniosomes ($p = 0.6943$), which can be due to the small sample size of $n = 10$. The two-way ANOVA test that was performed on the data obtained from the SCE and ED, resulted in no significant interaction effect ($p = 0.1041$). Conjointly, the two skin layers showed a significant difference in the average amount of active ingredient present between the dispersion tested ($p = 0.0445$) and between each skin layer ($p = 0.0075$). Further analysis on each skin layer individually, showed both the niosomes and proniosomes successfully delivered the active ingredient to the SCE, but with no statistical significant difference ($p = 0.6995$) between the dispersions. The T-test for the ED exclusively indicated a better delivery from the niosomes with a $p = 0.0404$, proving the niosome dispersion produced a better targeted delivery than the proniosome.

The T-test was also performed on each dispersion to examine their respective targeting abilities and thus to validate the results obtained from the ANOVA tests conducted as aforementioned. Results for the proniosome dispersion indicated that between the two skin layers, no significant

difference ($p = 0.3046$) was seen and the proniosomes delivered the active ingredient to the same extent to both layers. That was not the case for the niosome dispersion, which provided a value of $p = 0.01492$, indicating a scientific difference between the average amounts of active ingredient in the ED and the SCE when delivered by a niosomal dispersion. To ensure the validity of these above-mentioned results, it was decided to perform a non-parametric Mann-Whitney U-test on both dispersions and compare the resulting p-values with that calculated using the T-tests from earlier. Results from the Mann-Whitney U-tests confirmed that the proniosomes targeted both skin layers equally ($p = 0.2413$), with no particular affinity for the SCE layer. Again, this was not the case for the niosomal dispersion, which showed a significant targeting difference between the SCE and the ED ($p = 0.0257$) meaning it would probably deliver α -lipoic acid more efficiently to the targeted dermal layers than the hydrated proniosome dispersion.

D.4 Conclusion

The determined aqueous solubility (0.33 mg/ml) and log D value (- 1.21) of α -lipoic acid was found to be unfavourable for skin permeation, indicating the transdermal delivery of α -lipoic acid may be suboptimal (Naik *et al.*, 2000:319). It should be kept in mind that α -lipoic acid is considered a lipophilic compound, thus supporting the results obtained from both the solubility and log D experiments.

The membrane release studies confirmed both the niosomal and hydrated proniosomal vesicles successfully encapsulated and released the active ingredient. The average flux for the niosomal dispersion ($467.49 \pm 51.82 \mu\text{g}/\text{cm}^2.\text{h}$) was higher when compared to that of the hydrated proniosomal dispersion ($332.01 \pm 49.04 \mu\text{g}/\text{cm}^2.\text{h}$) during the membrane release studies. A possible reason is the higher affinity of the α -lipoic acid for the additional component in the provesicular dispersion (sorbitol). Furthermore, the non-ionic surfactant (Span[®] 60) used in both dispersions is also considered a gelling agent, meaning it attains the ability to increase the viscosity of the dispersions over time (6 h studies). The sorbitol used as carrier in the hydrated proniosomal dispersions also contributed to the total mass and viscosity of these hydrated dispersions, ultimately limiting the total release through the thick gel that formed over the 6 h study period. The high release of the active ingredient out of the vesicles resulted in a high concentration possibly being available for skin diffusion.

The presence of α -lipoic acid in the receptor compartments indicated both the niosomal and hydrated proniosomal dispersions delivered the active transdermally (into the bloodstream). The average percentage α -lipoic acid, which diffused through the skin after 12 h for the niosomes ($2.418 \pm 0.33\%$), was higher than that of the proniosomes ($0.471 \pm 0.08\%$); this

means that for the 5% formulation, the average flux was $187.43 \pm 25.95 \mu\text{g}/\text{cm}^2\cdot\text{h}$ for the niosomal dispersion and $36.94 \pm 6.11 \mu\text{g}/\text{cm}^2\cdot\text{h}$ for the hydrated proniosomal dispersion. According to Beitner (2003:847-848), the daily supplementary amount of α -lipoic acid ranges between 200 – 600 mg. Considering the average %diffused through the skin from both 5% dispersions, the concentrations diffused after 12 h was calculated as 120.9 mg ($2.418 \pm 0.33\%$), delivered from the niosomal dispersion and 23.55 mg ($0.471 \pm 0.08\%$); from the hydrated proniosomal dispersion. Therefore, this obtained amount of α -lipoic acid is far less than the recommended dose for systemic use. The percentages diffused transdermally for both vesicle systems were small, but still above the LOQ of α -lipoic acid ($1.30 \mu\text{g}/\text{ml}$) determined in Appendix A.

The target site, for the delivery of α -lipoic acid, is the ED of the skin where most metabolic processes occur. Results from tape stripping indicated both vesicle systems delivered the α -lipoic acid into the SCE with no significant differences between the two systems ($p = 0.6995$). Furthermore, the results obtained from this study correspond to other skin diffusion studies conducted on topical α -lipoic acid formulations (Beitner, 2003:842; Podda *et al.*, 1996:631). The niosomal dispersion had significantly better delivery ($p = 0.040$) of the active ingredient into the ED, with a concentration of $5.077 \pm 1.47 \mu\text{g}/\text{ml}$ compared to the $2.854 \pm 1.43 \mu\text{g}/\text{ml}$ delivered from the hydrated proniosome dispersion. Although both vesicle systems showed good release and permeation abilities, it is evident from the results obtained that the niosomal dispersion was dominant.

Both the niosomes and proniosomes released high concentrations of active in the SCE, possibly due to the actives affinity for the lipophilic environment of this outer layer (Podda *et al.*, 1996:632). Furthermore, the targeting abilities of the niosomes were ameliorated with the higher concentrations of α -lipoic acid accumulating in the ED, where it was needed. The transdermal diffusion of α -lipoic acid from both systems was not desired, but also not considered a negative aspect due to the advantage of α -lipoic acid supplementation.

To conclude, the aim of this study was to achieve targeted delivery of the active to the ED. This was met by both vesicle systems, although the niosomes obtained better release and targeting results, therefore, it was decided to use this vesicle system for all further experimental work. The clinical efficacy of the niosomal dispersion containing α -lipoic acid will be determined for the possible treatment of premature signs of ageing and inflammatory skin diseases.

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Appendix E

CLINICAL EFFICACY OF TOPICAL FORMULATIONS CONTAINING α -LIPOIC ACID

E.1 Introduction

The natural occurring antioxidant, α -lipoic acid, was isolated for the first time in 1951, by Dr L.J. Reed and his fellow colleagues. It was only after they successfully isolated and purified the compound that its true potential as a potent antioxidant was discovered. Upon administration, α -lipoic acid is reduced to dihydrolipoic acid in the cells, which is considered an even more effective antioxidant and can scavenge the hydroxyl and nitric oxide radicals, as well as the superoxide radical (Perricone, 2000:219).

α -Lipoic acid can penetrate the skin rapidly and accumulates in the layers where it is most needed for antioxidant activity. It provides antioxidant protection when present in the skin and acts as an effective inhibitor of NF-kB, therefore is deemed a powerful anti-inflammatory agent. Inflammation of the skin can induce or promote skin conditions such as psoriasis and premature skin ageing, therefore the external application of antioxidants may be potentially useful in the treatment of these skin conditions (Perricone, 2000:219).

Skin ageing promotes molecular and structural degradation of the skin. The clinical implications of this degradation process include the formation of wrinkles, changes in skin colour and elasticity. The skins' dermal layer becomes inelastic and therefore less capable of responding to external stressors (Diridollou *et al.*, 2001:354). Skin diseases, such as psoriasis and eczema, result in elevated levels of erythema due to the inflammatory processes continuously present.

Therefore, to determine the clinical effects of α -lipoic acid encapsulated in niosomal vesicle carriers, the following properties were assessed:

- Skin hydration levels, by measuring the water content in the stratum corneum.
- Skin topography changes, by evaluating the scaliness, roughness, wrinkles and smoothness.
- Skin elasticity changes.
- Anti-inflammatory activity, by measuring the changes of erythema levels.

E.2 Materials and methods

E.2.1 Test formulations

Niosomal vesicle systems, prepared as discussed in Appendix B.5.2, were modified by adding appropriate viscosity enhancers until the formulation was suitable for use during the clinical efficacy experiments. The ATF, containing α -lipoic acid encapsulated in a vesicle system, was compared to a placebo formulation containing no active ingredient. The chloroform used during the preparation method was evaporated using a Büchi® (Switzerland) rotary evaporator fitted with an Interface I-100, heating bath B-100, vacuum pump V-100 and Rotavapor® R-100; all components present in the ATF and placebo formulation are listed in Table E.1. For the irritation patch studies, commercially available 1% hydrocortisone acetate (w/w) cream (Biocort cream, Akacia™ HealthCare (Pty) Ltd), was utilised as the positive control on the 1% sodium lauryl sulphate (SLS) (w/v) irritated skin areas.

Table E.1: Ingredients and their individual functionalities included in the preparation of 100 ml ATF

Ingredients	Quantity	Functionality
α -Lipoic acid*	5 g	Active ingredient
Span® 60	5 g	Emulsifier
Cholesterol	10 g	Stabiliser
Chloroform	-	Organic Solvent (evaporated)
Xanthan gum	2 g	Thickening agent/gelling agent
Milli-Q® water	100 ml	Organic solvent

*No active ingredient present in the placebo formulation

Table E.2: Average viscosity and pH measurements from both the ATF and placebo formulation prior clinical efficacy studies commenced

Formulation	Average viscosity (cP)	Average pH
ATF	7 909.4 $\pm$ 476.64	4.67 $\pm$ 0.01
Placebo formulation	10 776.0 $\pm$ 294.58	5.54 $\pm$ 0.00

Both test formulations were evaluated by means of viscosity (30 rpm, 25° C) and pH measurements. The averages of five measurements are listed in Table E.2. The measurements were taken to ensure the newly prepared formulations were sufficiently viscous to be applied topically and did not have a pH value that was not topically acceptable. The ideal pH for topically applied formulations are characterised by Kharat and Bathe (2016:149) as a pH

in the range of 4.2 to 5.6. Therefore, both the ATF and placebo formulations fell within the acceptable ranges for topical application and had adequate viscosity to be easily applied to the skin without the formulation running off the skin site.

E.2.2 Non-invasive measurements

All instruments used during the clinical efficacy experiments were non-invasive bioengineering instruments and included the Corneometer® CM 825 (Courage & Khazaka electronic GmbH, 2016a), the Visioscan® VC 98 (Courage & Khazaka electronic GmbH, 2016b), the Cutometer® MPA 580 (Courage & Khazaka electronic GmbH, 2016c) and the Mexameter® MX 18 (Courage & Khazaka electronic GmbH, 2016d). The instruments were checked and calibrated prior to every study to avoid any variations in the measurements.

E.2.2.1 Skin hydration level measurements



Figure E.1: Measurement with the Corneometer® CM 825 (Adapted from Courage & Khazaka electronic GmbH, 2015a).

The Corneometer® CM 825 (Courage & Khazaka electronic GmbH, 2016a) measures the hydration level of the skin surface. This is done by evaluating the skin's electrical capacitance value (di-electric constant) using fringing field capacitance sensors designed to measure small changes in the capacitance values. To ensure only the water content of the stratum corneum is calculated, the depth of the measurements only range between 10 and 20 μm . It is known that when the moisture in the skin increases, the capacitance and conduction values of the skin increase, subsequently these calculated changes are converted to arbitrary units (AU). Measurement times are kept short (1 sec) to minimise the occlusion effects, as the accumulated water that cannot evaporate under the glass probe influences the measurements (Berardesca

et al., 1997:127; Courage & Khazaka electronic GmbH, 2016a; Darleski & Fluhr, 2011:128; Li *et al.*, 2001:24).

The probe was placed vertically onto the desired area during measurements (as seen in Figure E.1). A spring provided a constant pressure on the skin area and a beep sound signalled when the measurement was successfully completed. The calculated measurement was then displayed on the computer software.

E.2.2.2 Skin surface topography measurements

The Visioscan® VC 98 (Courage & Khazaka electronic GmbH, 2016b) can analyse changes in skin topography, such as the living skin (SELS) parameters, using two special halogenide lights that are arranged on opposite sides of the probe to illuminate the skin uniformly. The ultraviolet lights' arrangement, intensity and spectrum are monitored to prevent any reflections from the deeper layers of the skin. A sharp, non-glossy image of the skin area (6 x 8 mm) is taken with a built in CCD (charge-coupled device) camera, where the wrinkles and skin surface properties can clearly be distinguished. This image is then analysed by the computer software, which digitalises the image in 256 grey level pixel by pixel, where 255 resembles the colour white and 0 resembles the colour black (Courage & Khazaka electronic GmbH, 2016b; Ferreira *et al.*, 2010:444).



Figure E.2: Measurement with the Visioscan® VC 98 (Adapted from Courage & Khazaka electronic GmbH, 2016b).

The skin was clearly marked with a skin marker and stencil to locate the exact spot for each measurement. The parameters calculated by the Visioscan® VC 98 and used during the anti-ageing study are summarised in Table E.3.

Table E.3: The SELS parameters used and calculated with the Visioscan VC® 98 (Courage & Khazaka electronic GmbH; 2016b) adapted from Van der Walt (2016:221).

SELS Parameter	Visioscan VC® 98 analysis	Interpretation
Scaliness (SEsc)	Computer software analysis of the image quantifies the desquamation of the stratum corneum	A decrease in this value means a decrease in the flaky skin cells of the stratum corneum, therefore meaning the skins moisture increased (↓ SEsc value = ↑ skin moisture) ^{a, b}
Smoothness (SEsm)	Computer software computes the image, using the average width of the wrinkles	An increase in this value means the total skin moisture of the skin increased (↑ SEms value = ↑ skin moisture) ^a
Roughness (SEr)	Computer software uses the comparison in grey colour between that above the threshold and the image as a whole	A decrease in this value indicates a decrease in the roughness of the skin (↓ SEr = ↓ roughness of the skin) ^{a, b}
Wrinkles (SEw)	Computer software determines wrinkle formation by dividing the average number of wrinkles by the average width	An increase in this value indicates an increase in the number of wrinkles (↑ Sew = ↑ number of wrinkles) ^{a, b, c}

a) Courage and Khazaka electronic GmbH, 2016b; b) Ferreira *et al.*, 2010:446; c) Choi *et al.*, 2012:351

E.2.2.3 Skin viscoelasticity measurements

The Cutometer® MPA 580 (Courage & Khazaka electronic GmbH, 2016c) is designed to deform the upper layer of the skin mechanically, using negative pressure. A pressure of 400 mbar is applied for 2 sec, which pulls the skin into the 2 mm probe aperture (Figure E.3). When the skin is sucked into the probe, its resistance (firmness) is measured and then its ability to return to its original position (elasticity) is measured thereafter. These values are calculated by a non-contact optical measuring system, which consists of a light source and receptor and two prisms facing one another, which projects the light from the transmitter to the receptor. The measurement values are displayed as a deformation curve (penetration depth in mm/time) and are used to objectively quantify skin ageing by using these elastic and mechanical properties (Courage & Khazaka electronic GmbH, 2016c).

When measurements were taken, the probe was kept still to avoid any pressure loss and applied to the skin at a right angle. If the probe is pressed too tightly onto the skin, it could disturb the blood circulation and influence the measurement. To evaluate the elasticity of the skin, the mechanical R-parameters are generally used, which describe the elastic properties of the skin. The R-parameters are categorised into viscoelastic and elastic groups and can be further subdivided into relative and absolute parameters (Akhtar *et al.*, 2011:69). A relatively new set of parameters, i.e. Q-parameters, are found to be simple but more accurate when

measuring the skins' viscoelastic properties (Qu *et al.*, 2006:198; Qu & Seehra 2016:43). Table E.4 summarises the four Q-parameters that will be used during the anti-ageing study.



Figure E.3: Measurement with the Cutometer® MPA 580 (Adapted from Courage & Khazaka electronic GmbH, 2016c).

Table E.4: Summary of the Q-parameters measured by the Cutometer® MPA 580 (Courage & Khazaka electronic GmbH, 2016c).

Parameter	Description	Interpretation
Q0	Maximum recovery area	A decrease in this value indicates an increase in the skins firmness ^{a, b, c}
Q1	Elastic recovery	An increase in this value means the elasticity of skin increased ^{a, b, c}
Q2	Viscous recovery	An increase in this value represents an increased viscous recovery of the skin ^{a, b, c}
Q3	Viscoelastic recovery (overall elasticity)	An increase in this value indicates more elasticity of the skin ^{a, b, c}

a) Courage and Khazaka electronic GmbH, 2016b; b) Qu *et al.*, 2006:197-198; c) Qu & Seehra, 2016:41-43

E.2.2.4 Skin erythema measurements

The Mexameter® MX 18 (Courage & Khazaka electronic GmbH, 2016d), designed to measure both melanin and haemoglobin (erythema) content in the skin, was used to measure the erythema levels of the volunteers. The probe emits a pre-determined quantified light band and then a receiver measures the reflected light from the skin. Thus, the quantity of light being absorbed from the skin can be subtracted from the initial quantity of light that the probe emitted, thereafter, the results are shown as indices on the screen ranging from 0 to 999 (Courage & Khazaka electronic GmbH, 2016d).



Figure E.4: Measurements with the Mexameter® MX 18 (Adapted from Courage & Khazaka electronic GmbH, 2016d).

E.2.3 General protocol design

During the clinical efficacy studies, all the protocols used were in accordance with the European Group for Efficacy Measurements on Cosmetics and Other Topical Products (EEMCO), as well as with the International Conference on Harmonization (ICH), Good Clinical Practice (GCP), World Medical Association (WMA, 2013) and the European Cosmetic, Toiletry and Perfumery Association (COLIPA) guidelines for the efficacy evaluation of cosmetic products. Prior to the studies, the volunteers were asked to sign an informed consent form and advised they could discontinue their participation at any time (Berardesca *et al.*, 1997:129; Danby *et al.*, 2013:42; Dupont *et al.*, 2012:207; Katsarou *et al.*, 2000:85; Li *et al.*, 2001:24; Zhai *et al.*, 2000:77).

The studies have been carried out in accordance with the WMA, Declaration of Helsinki (Ethical principles of medical research involving human subjects), under the umbrella application titled, “(In vivo) cosmetic efficacy studies” (NWU-00097-10-A5). The clinical efficacy studies were

carried out in the Cosmetics Efficacy Laboratory (CEL), an independent clinical cosmetic testing lab at the Centre for Pharmaceutical and Biomedical Services (North-West University, South Africa).

All the measurements taken during the studies were performed in a controlled environment, where the humidity and temperature were maintained between 40 - 60% relative humidity (RH) and 20 - 25 °C, respectively. Prior to all measurements, each volunteer were allowed to acclimatise for a period of 15 min (Berardesca *et al.*, 1997:129; Wunderlich, 2011:70).

E.2.3.1 Volunteers

Inclusion and exclusion criteria were included in the protocol design to minimise the variables between volunteers. The criteria related to age, gender, ethnicity, skin type and health conditions (Tables E.5 and E.6). Only female volunteers were allowed to participate in the studies due to the structural skin differences and dermal thickness variations between genders (Berardesca, 2011:93, 94; Darlenski & Fluhr, 2011:135).

Only volunteers with a specific skin type, Caucasian (Fitzpatrick skin type I-III), were enrolled to avoid inter-racial variables in the study. No enrolled volunteer was pregnant or breast-feeding and all appeared to be in good physical health (Darlenski & Fluhr, 2012:270; Serup, 2001:143, 148).

Table E.5: Inclusion criteria used to screen and enrol prospective volunteers for the clinical efficacy studies

Inclusion criteria	
1	Ethnicity/Race: Caucasian
2	Skin type: Fitzpatrick skin type I to III
3	Age group:35 – 60 years (Anti-ageing) and 20 – 60 years (anti-inflammatory)
4	Good physical health, with no known illness
5	No pathological events for the period immediately before and during the study
6	Willing to avoid prolonged sun or artificial UVR exposure, sun bathing and swimming during the study
7	Committed to follow study directions, attending all scheduled visits and applying the given products to the skin test sites indicated only
8	Able to effectively communicate with study personnel
9	Understands the informed consent document and all its content, provides consent to participate in the study by means of her signature to the consent document

Volunteers were instructed to only use the supplied Dove® soap for a washout period (seven days prior to the commencement of the studies) and for the whole duration of the studies. The

volunteers were allowed to follow their normal skin cleansing routines, but not on the specified skin areas. Also, due to the anti-ageing and anti-inflammatory action of α -lipoic acid being tested, the volunteers were not allowed to topically apply or ingest products containing corticosteroids or vitamin A, within three months before the studies started (Rawlings *et al.*, 2012:26; Shlivko *et al.*, 2014:137; Zussman *et al.*, 2010:511). No other lotion, soap, perfume or cosmetic was allowed on the specified skin areas during the washout period and throughout the studies.

Table E.6: Exclusion criteria used to screen and enrol prospective volunteers in the clinical efficacy studies

Exclusion criteria	
1	Not currently pregnant or breastfeeding
2	No known allergies to cosmetics and/or skincare products or other substances (specify)
3	Does not have irritated skin, lesions, open wounds or blemishes on test sites
4	Have not had cancer treatment for any type of cancer within the last two years
5	Does not have a history of dermatological disease or conditions, such as eczema, psoriasis, vitiligo, atopic dermatitis or any condition that alters the skins' appearance or physiologic response, e.g. porphyria
6	Not using topical or systemic drugs that would possibly affect the results of the study, e.g. topical anti-inflammatories, corticosteroids etc.
7	Does not have a history of endocrinopathies, e.g. thyroid or diabetes treatment
8	Did not ingest oral corticosteroids within three months of commencement of the study
9	Did not ingest oral vitamin A acid (or any derivative) less than three months before the study
10	Not currently participating in another clinical investigation or been involved within a period of 90 days before the admission of this study
11	Not currently using (or three months prior to the study) any skin lightening products or self-tanning products on the forearms
12	Does not have excessive hair growth on the tested sites

E.2.3.2 Statistical analysis

The raw data collected for each parameter was statistically analysed. To calculate the %change in each parameter relative to the baseline values (T0) measured; Equation E.1 was used.

$$\text{Percentage change (\%)} = ((T_x - T_0) / T_0) \times 100 \quad \text{Equation E.1}$$

Where:

T0 = Baseline value

Tx = Values measured at different time intervals during the anti-ageing and erythema studies.

Both the anti-ageing study and erythema patch study were performed to evaluate the anti-ageing and anti-inflammatory properties of the ATF, compared to control formulations, at 95% probability ($p < 0.05$) as a function of time for duration of treatment.

The primary objective of the studies was to evaluate the *in vivo* anti-ageing and anti-inflammatory effect of a test formulation as a function of treatment time. The secondary objective was to assess the clinical change in skin hydration, topography, elasticity and erythema for test products, compared to the control areas.

Statistical analyses were conducted using IBM SPSS Statistics Version 23. To assess the influence of topical treatment and time on the different clinical markers tested, such as the hydration level, SELS parameters, elasticity (Q-parameters) and erythema levels, linear mixed models were used. An unstructured or first-order autoregressive (AR(1)) covariance structure was used. Volunteers were treated as a Random effect and the topical treatment and time were treated as Fixed effects. The Type III Test for Fixed effects were performed and resulted in various p-values obtained; a Bonferroni adjustment for multiple comparisons was also made. The practical significance of the results was evaluated using Cohen's d-values. An effect size (d) ≈ 0.20 is indicative of a small effect or practical non-significant difference, $d \approx 0.50$ points towards a medium effect or practical visible difference, whereas $d \approx 0.80$ signifies a large effect or practical significant difference (Ellis & Steyn, 2003:52). The statistical analysis tests were performed on all the parameters measured during the anti-ageing and erythema studies.

E.2.4 Treatment protocol

Both the anti-ageing and anti-inflammatory studies were non-randomised, single blind studies and had 24 female, Caucasian volunteers participating in each study. The duration of the anti-ageing study was 28 days and the anti-inflammatory study was eight days.

E.2.4.1 Anti-ageing study

The aim of this study was to examine the effects that α -lipoic acid has on the inevitable ageing of skin. The treatment areas were the volar forearms of both the dominant and non-dominant arms, to limit the hydration differences between contra-lateral anatomical sites (Berardesca *et al.*, 1997:129). Furthermore, it is known that skin thickness, stratum corneum composition and the capillary network in the epidermis differs between anatomical locations (WHO, 2006:18, 23; Wiechers, 2008:318), therefore, all the volunteers received a personalised stencil to measure the precise application areas, to ensure the exact same skin areas were treated and measured throughout the studies.

The amount of formulation that was applied per application was pre-calculated to be within the range of $1 - 3 \mu\text{l}/\text{cm}^2$ (mg/cm^2) and volunteers were instructed to apply the two formulations twice daily (in the morning between 06:00 – 08:00 and in the evening between 18:00 – 20:00). Due to the small amounts required per application, the ATF and placebo formulation were given to the volunteers in pre-filled syringes and colour coded to easily distinguish between the two formulations (Figure E.5).



Figure E.5: Pre-filled syringes containing ATF (red) and placebo (blue) formulation

Each volunteer's initial baseline measurements were used as a control to evaluate the relative change in skin hydration, topography and elasticity over time. The treated skin areas were measured using the Corneometer® CM 825 (Courage & Khazaka electronic GmbH, 2016a), the

Visioscan® VC 98 (Courage & Khazaka electronic GmbH, 2016b) and the Cutometer® MPA 580 (Courage & Khazaka electronic GmbH, 2016c).

E.2.4.2 Erythema study

The following trial design was selected; neither parallel nor crossover group design was followed. Single-blind test products were applied on pre-determined, non-randomised skin areas. The ATF and control product responses were evaluated following multiple test product applications on the pre-treated skin areas.

The initial baseline values of the erythema study were taken on untreated skin areas, which were clearly marked with a skin marker and stencil. All the volunteers had five test areas on their non-dominant arm, which was measured and used to determine each volunteers' natural baseline redness or erythema levels.

The 1% SLS (w/v) was used to induce the erythema in the skin areas. Four Finn Chambers® were filled with 1% SLS and the last chamber was filled with deionised water. The patch was applied to each volunteers' inner forearm and left for 24 h. Over this period, the volunteers had to refrain from excessive exercise and had to keep the patch dry to avoid any possible leakage.

After the 24 h occlusion time, the Finn Chambers® were inspected and removed. Volunteers were instructed to return to the lab after another 24 h. Therefore, days 1 and 2 of the study were utilised to induce skin erythema. T1 was measured 48 h after the initial baseline T0 measurements, to determine whether statistical significant erythema was produced. The different test products were thereafter applied to the pre-determined skin areas, as seen in Table E.7.

Table E.7: Different skin areas with the pre-determined products applied three times daily

Skin area	Pre-treatment	Test Product applied
1	1% SLS treatment area	Positive control – 1% cortisone cream
2	1% SLS pre-treatment	Placebo formulation
3	1% SLS pre-treatment	ATF
4	1% SLS pre-treatment	Negative control – No product applied
5	No SLS pre-treatment (deionised water)	Untreated skin

Skin areas 4 and 5 were not treated with any test products. The skins' natural healing process was measured on skin area 4, hence skin area 4 was irritated with 1% SLS but not treated with any product. Skin area 5 represented the skins' natural condition, where the skin was subjected

to the occlusion process of the Finn Chamber[®] containing deionised water, but neither pre-treatment with 1% SLS, nor any test product was applied throughout the study.

The volunteers were instructed to apply the supplied test products three times a day for a period of five days. T2 measurements were taken 48 h after T1 and T3 measurements were taken 72 h after T2. To document the application times, each volunteer was given a customised diary and stencil to indicate the precise test areas. The test products were given to the volunteers in syringes due to the small quantities applied per application (as seen in Figure E.6). To measure the erythema levels, the Mexameter[®] MX 18 (Courage & Khazaka electronic GmbH, 2016d) was utilised.

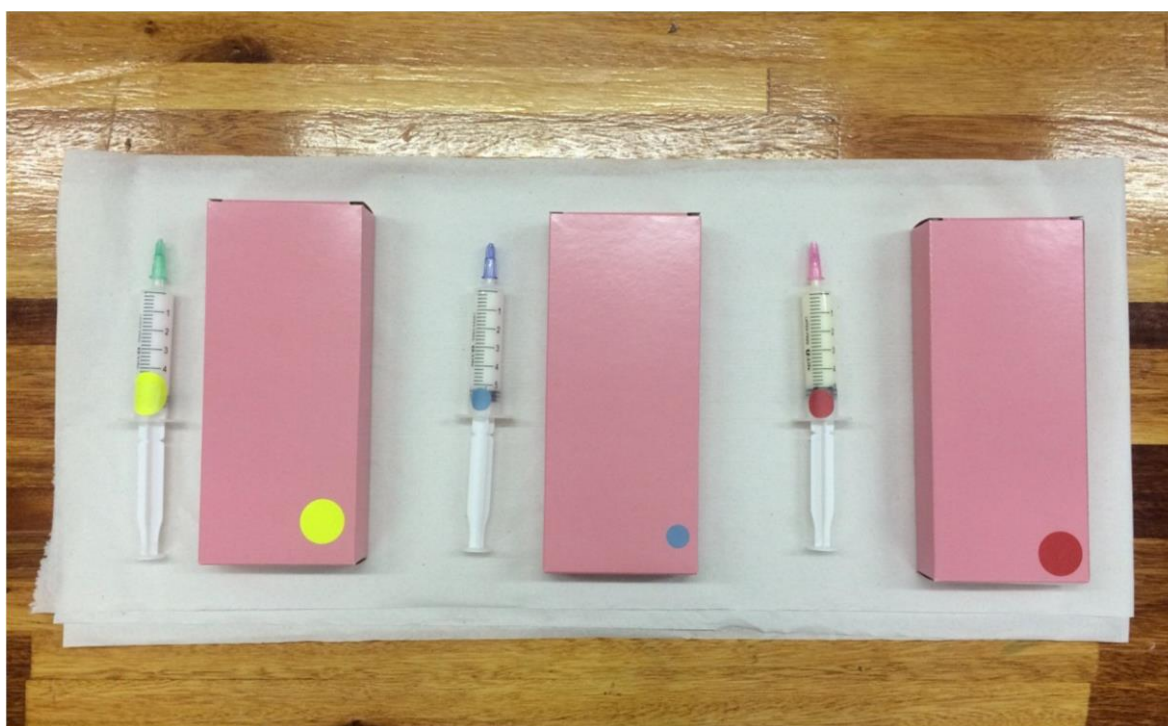


Figure E.6: Pre-filled syringes containing 1% cortisone cream (w/w) (yellow) placebo formulation (blue) and ATF (red)

E.3 Results and discussion

E.3.1 Anti-ageing study

The clinical efficacy of the ATF was evaluated by measuring several parameters, such as skin hydration, skin topography (SELS parameters) and skin elasticity (Q-parameters).

E.3.1.1 Skin hydration

The hydration levels increased after the four week treatment period from both tested formulations, but unfortunately to no statistical or practical significant degree (as seen in

Table E.8). Topical treatment with the ATF did therefore not result in statistical significant differences, according to the Bonferroni tests, but did cause an increase in the overall hydration of the tested sites, from $22.94 (\pm 6.09)$ to $24.92 \pm (6.40)$. The %changes over time (listed in Table E.8) for each formulation at the different measurements were calculated using Equation E.1 and graphically illustrated in Figure E.7. The mean T0 values, measured before any topical treatment, were measured for both the ATF (22.94 ± 6.09) and the placebo formulation (22.88 ± 7.06). From the results obtained, it is evident the placebo formulation improved the stratum corneum hydration levels by 23.75% compared to the ATF with a 13.00% increase in hydration levels.

Table E.8: Mean Corneometer® CM 825 (Courage & Khazaka electronic GmbH, 2016a) values for skin hydration with the %change over time for both formulations over four weeks treatment

Treatment	Baseline changes (Tx - T0)	Corneometer® CM 825 (mean $\pm$ SD)	%Change over time (%)*
ATF	T0	22.94 ± 6.09	-
	T1	21.49 ± 5.60	-3.20%
	T2	21.68 ± 6.35	-1.57%
	T3	24.30 ± 7.62	7.21%
	T4	24.92 ± 6.40	13.00%
Placebo formulation	T0	22.88 ± 7.06	-
	T1	20.99 ± 6.87	-3.82%
	T2	23.17 ± 4.91	9.27%
	T3	23.54 ± 6.97	7.61%
	T4	26.94 ± 6.49	23.75%

*Calculated using Equation E.1

From Table E.9, it can be seen that most measurements taken throughout the four week period did not differ to a statistical significant degree. The last measurement taken on T4 for the placebo formulation was identified as a statistical significant increase ($p = 0.003$) in hydration value. The Cohen test also identified the increase as a medium effect of the placebo formulation.

Table E.9: Results obtained for changes in skin hydration levels from the Bonferroni and Cohen's tests

Treatment	Baseline changes (Tx - T0)	p-Value	Statistical significance	Cohen's d-value	Effect size: Practical significance
ATF	T1	0.800	Not significant	0.239	Small effect
	T2	1.000	Not significant	0.199	Small effect
	T3	0.934	Not significant	0.177	Small effect
	T4	0.333	Not significant	0.308	Small effect
Placebo formulation	T1	0.421	Not significant	0.267	Small effect
	T2	1.000	Not significant	0.041	Small effect
	T3	1.000	Not significant	0.093	Small effect
	T4	0.003*	Significant	0.575	Medium effect

Statistical significant when $p < 0.05$; Cohen's d-value: $d \approx 0.20$ is indicative of a small effect, $d \approx 0.50$ points towards a medium effect, whereas $d \approx 0.80$ signifies a large effect

The Type III test for Fixed effects revealed statistical significant differences for both the ATF ($p = 0.008$) and placebo formulation ($p = 0.001$). Additionally, the practical significance that was examined for the ATF treatment did not result in any significant differences. Figure E.7 shows the %change over the four week treatment period from both the ATF and placebo formulations.

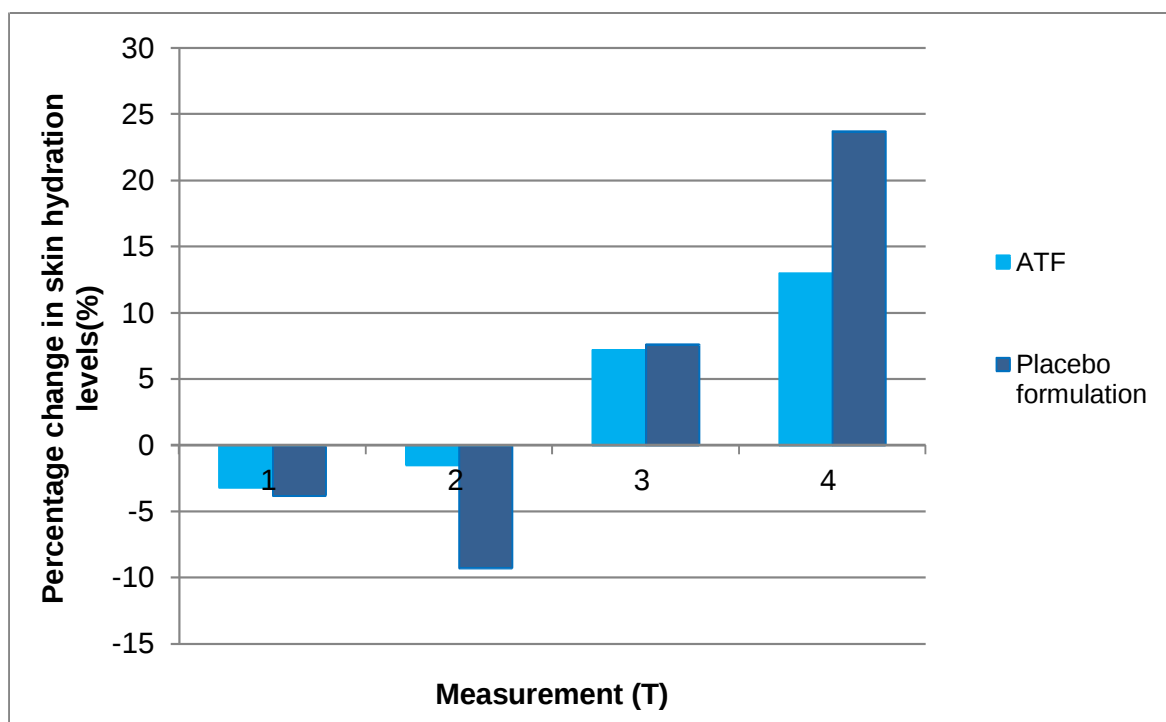


Figure E.7: %Change in the skin hydration over four weeks for both tested formulations

To conclude, both formulations increased the skins hydration levels compared to the respective T0 measurements. The ATF improved the skins' hydration by 13.00% and the placebo formulation by 23.75%. When comparing the two formulations, the placebo formulation had a higher efficacy in hydration properties than the ATF.

E.3.1.2 Skin topography results

Any changes in the skins' topography after the application of both formulations were evaluated using the Visioscan® VC 98 (Courage & Khazaka electronic GmbH, 2016b). The SELS parameters investigated were SEr (roughness), SEsc (scaliness), SEsm (smoothness) and SEw (wrinkling).

E.3.1.2.1 Roughness (SEr)

Data obtained from the Visioscan® VC 98 (Courage & Khazaka electronic GmbH, 2016b) were analysed and the only visible difference/increase in the data for skin roughness appeared to be at T2 for the placebo formulation ($d = 0.520$). The mean baseline measurement taken on T0 for the ATF was found to be 3.16 ± 1.11 and at T3, the value was 3.12 ± 0.68 . The placebo formulation had a mean baseline value of 2.76 ± 0.65 , with a slight increase to 2.92 ± 0.69 at T3 (as seen in Table E.10).

Table E.10: Mean Visioscan® VC 98 (Courage & Khazaka electronic GmbH, 2016b) values for skin roughness with the %change over time for both formulations over four weeks treatment

Treatment	Baseline changes (Tx - T0)	Visioscan® VC 98 (mean $\pm$ SD)	%Change over time (%)*
ATF	T0	3.16 ± 1.11	-
	T1	3.30 ± 0.94	13.83%
	T2	2.94 ± 0.70	-0.16%
	T3	3.12 ± 0.68	6.76%
	T4	2.97 ± 0.69	4.92%
Placebo formulation	T0	2.76 ± 0.65	-
	T1	2.95 ± 0.67	11.81%
	T2	3.10 ± 0.65	16.56%
	T3	2.84 ± 0.75	7.81%
	T4	2.92 ± 0.69	11.68%

*Calculated using Equation E.1

Table E.11: Results obtained for skin roughness changes from the Bonferroni and Cohen's tests

Treatment	Baseline changes (Tx - T0)	p-Value	Statistical significance	Cohen's d-value	Effect size: Practical significance
ATF	T1	0.496	Not significant	0.131	Small effect
	T2	0.312	Not significant	0.194	Small effect
	T3	0.880	Not significant	0.028	Small effect
	T4	0.446	Not significant	0.169	Small effect
Placebo formulation	T1	0.857	Not significant	0.272	Small effect
	T2	0.158	Not significant	0.520	Medium effect
	T3	1.000	Not significant	0.103	Small effect
	T4	1.000	Not significant	0.225	Small effect

Statistical significant when $p < 0.05$; Cohen's d-value: $d \approx 0.20$ is indicative of a small effect, $d \approx 0.50$ points towards a medium effect; whereas $d \approx 0.80$ signifies a large effect

Results for the skin roughness changes over the four week treatment period revealed the treatment from both the ATF and placebo formulation increased the roughness of the skin sites. Type III test for Fixed effects revealed the increase in skin roughness was not statistically significant for neither the ATF ($p = 0.469$) nor the placebo formulation ($p = 0.235$) after the treatment period. Results for the tests used to examine the skin roughness are summarised in Table E.11.

The increase in skin roughness after application of the placebo formulation was slightly higher when compared to the ATF. The only apparent difference is the T2 measurement on the ATF tested sites that indicated the ATF moisturised the skin areas with a 0.16% difference in roughness from the baseline measurements. Thereafter, all further measurements revealed both tested formulations increased the overall skin roughness. The %differences are graphically depicted in Figure E.8.

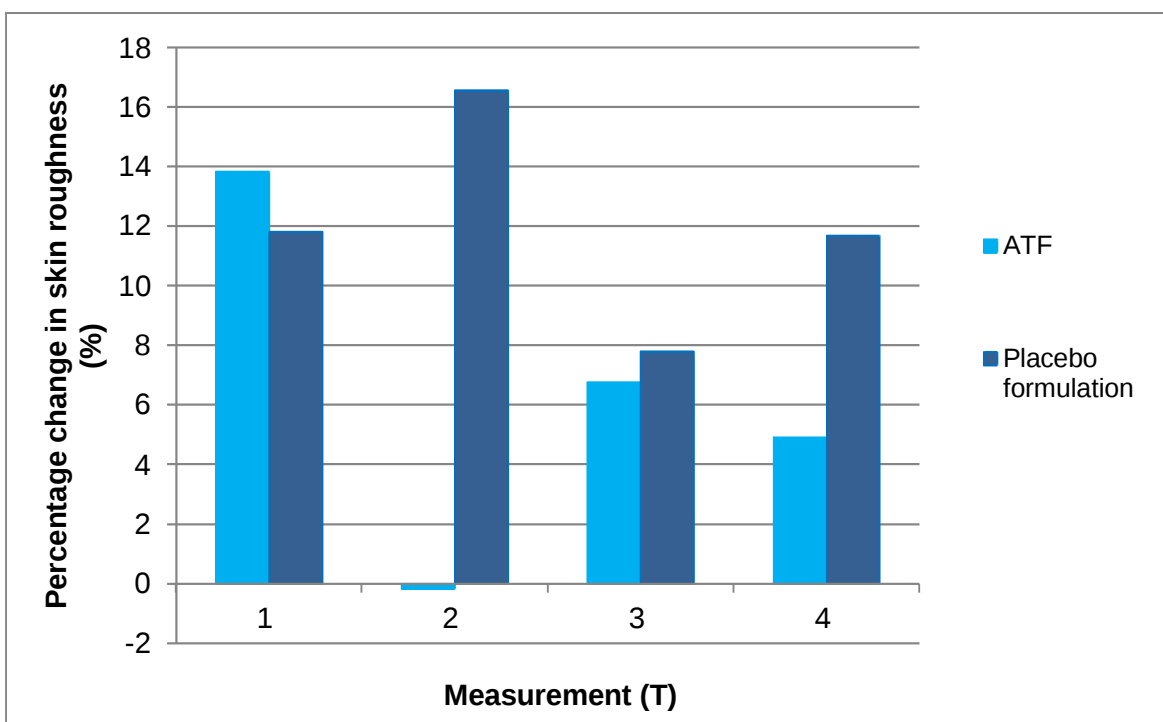


Figure E.8: %Change in the skin roughness over four weeks for both tested formulations

After four weeks of application, the ATF resulted in a 4.92% increase in the skins' roughness, which was slightly less than the result of the placebo formulation, which increased the roughness by 11.68%.

E.3.1.2.2 Scaliness (SEsc)

The ATF decreased skin scaliness by 9.98%, as shown in Table E.12, with an initial mean value of 0.78 ± 0.34 and a T4 mean value of 0.63 ± 0.20 . The placebo formulation decreased the mean value at the baseline from 0.85 ± 0.51 to a value of 0.66 ± 0.31 at T4. The mean measured values and %changes over time are shown in Table E.12.

Table E.12: Mean Visioscan® VC 98 (Courage & Khazaka electronic GmbH, 2016b) values for skin scaliness with the %change over time for both formulations over four weeks treatment

Treatment	Baseline changes (Tx - T0)	Visioscan® VC 98 (mean ± SD)	%Change from baseline(%)*
ATF	T0	0.78 ± 0.34	-
	T1	0.91 ± 0.47	37.62%
	T2	0.66 ± 0.22	-2.93%
	T3	0.83 ± 0.49	-18.87%
	T4	0.63 ± 0.20	-9.98%
Placebo formulation	T0	0.85 ± 0.51	-
	T1	0.90 ± 0.55	21.60%
	T2	0.71 ± 0.33	-2.40%
	T3	0.74 ± 0.42	-3.97%
	T4	0.66 ± 0.31	-12.15%

*Calculated using Equation E.1

The evaluation of the data revealed the ATF decreased the skins' scaliness over the treatment period of four weeks, but with practical significant differences ($d = 0.402$). The placebo formulation showed a slightly smaller effect ($d = 0.372$), but also resulted in a decrease in the measured skin scaliness. Cohen's tests were performed to determine the practical significance between the values obtained for both formulations (as seen in Table E.13).

Table E.13: Results obtained for skin scaliness changes from the Bonferroni and Cohen's tests

Treatment	Baseline changes (Tx - T0)	p-Value	Statistical significance	Cohen's d-value	Effect size: Practical significance
ATF	T1	0.895	Not significant	0.275	Small effect
	T2	1.000	Not significant	0.328	Small effect
	T3	1.000	Not significant	0.106	Small effect
	T4	0.845	Not significant	0.402	Small effect
Placebo formulation	T1	0.575	Not significant	0.094	Small effect
	T2	0.134	Not significant	0.271	Small effect
	T3	0.238	Not significant	0.213	Small effect
	T4	0.041*	Significant	0.372	Small effect

Statistical significant when $p < 0.05$; Cohen's d-value: $d \approx 0.20$ is indicative of a small effect, $d \approx 0.50$ points towards a medium effect; whereas $d \approx 0.80$ signifies a large effect

The ATF ($p = 0.07$) and placebo formulation ($p = 0.058$) showed no statistical significant changes in the data from the baseline measurements to the T4 measurements, according to the

Type III test for Fixed effects. The only statistical significant difference in the data, identified by the Bonferroni test, was the T4 value of the placebo treated skin sites ($p = 0.041$).

Both the ATF and placebo formulation resulted in reduced skin scaliness over the treatment period of four weeks. The %changes in skin scaliness are graphically illustrated in Figure E.9. From the figure, it is evident that the placebo formulation showed a greater effect in reducing the skin's scaliness, but both formulations resulted in an overall decrease in scaliness.

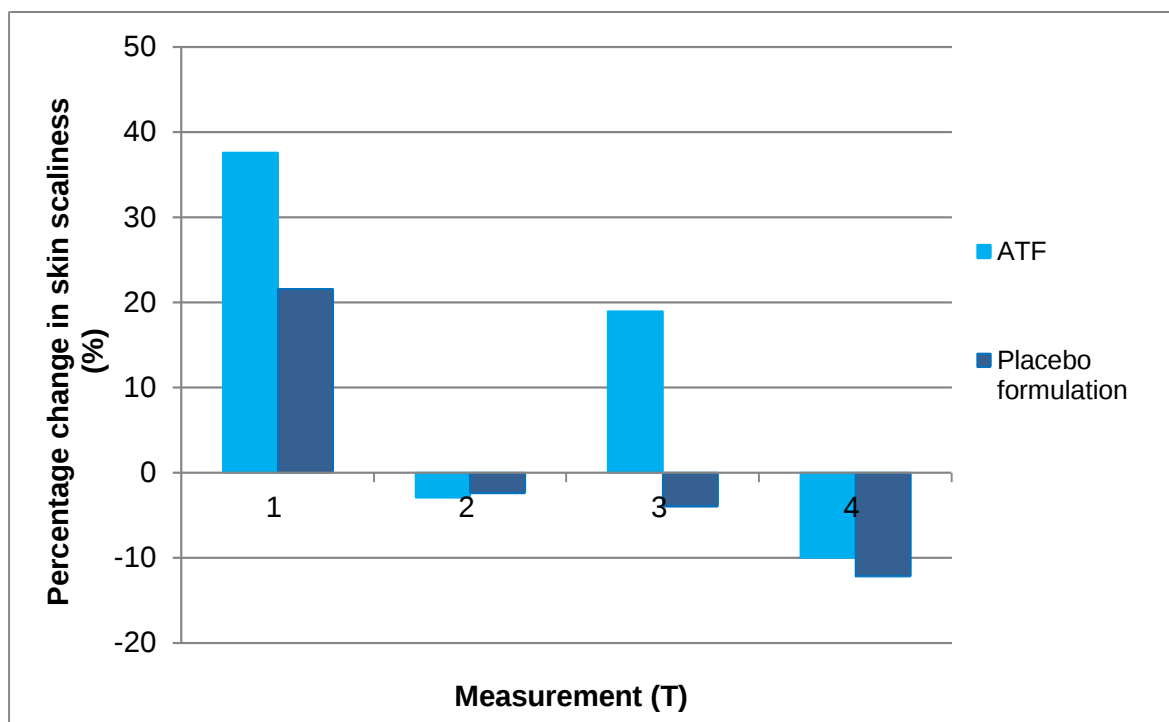


Figure E.9: %Change in the skin scaliness over four weeks for both tested formulations

Both formulations resulted in the ultimate decrease in skin scaliness. After the treatment period, the mean measured values obtained for the ATF reduced by 9.98% and for the placebo formulation, 12.15%.

E.3.1.2.3 Smoothness (SEsm)

Results obtained from the Type III test for Fixed effects revealed the smoothness of the skin decreased after applications of both tested formulations. Table E.14 summarises the mean measurements taken each week for both the ATF and placebo formulations. The initial mean baseline measurements were 138.78 ± 26.04 for the ATF and 125.18 ± 26.71 for the placebo formulation, with a final reduction to 132.40 ± 31.33 and 110.30 ± 18.22 , respectively. The %change in skin smoothness over time is shown in Table E.14 and graphically illustrated in Figure E.10.

Table E.14: Mean Visioscan® VC 98 (Courage & Khazaka electronic GmbH, 2016b) values for skin smoothness with the %change over time for both formulations over four weeks treatment

Treatment	Baseline changes (Tx - T0)	Visioscan® VC 98 (mean ± SD)	%Change from baseline (%)*
ATF	T0	138.78 ± 26.04	-
	T1	132.98 ± 35.97	-3.77%
	T2	124.61 ± 19.87	-9.04%
	T3	132.80 ± 29.51	-3.08%
	T4	132.40 ± 31.33	-3.62%
Placebo formulation	T0	125.18 ± 26.71	-
	T1	127.61 ± 41.58	3.81%
	T2	119.11 ± 20.49	-2.92%
	T3	116.46 ± 18.33	-4.83%
	T4	110.30 ± 18.22	-9.62%

*Calculated using Equation E.1

After the treatment period, only the placebo formulation reduced the smoothness to a statistically significant degree ($p = 0.039$). The ATF also reduced overall skin smoothness, but the differences in the measured values were not statistically significant ($p = 0.193$). Further investigations revealed the ATF reduced skin smoothness significantly ($p = 0.015$) after two weeks treatment. As for the placebo formulation, a statistical significant decrease ($p = 0.015$) in skin smoothness was only apparent after the total four weeks treatment. Table E.15 summarises the results calculated using the Bonferroni and Cohen's tests.

Table E.15: Results obtained for skin smoothness changes from the Bonferroni and Cohen's tests

Treatment	Baseline changes (Tx - T0)	p-Value	Statistical significance	Cohen's d-value	Effect size: Practical significance
ATF	T1	0.313	Not significant	0.161	Small effect
	T2	0.015*	Significant	0.544	Medium effect
	T3	0.298	Not significant	0.202	Small effect
	T4	0.267	Not significant	0.203	Small effect
Placebo formulation	T1	0.686	Not significant	0.058	Small effect
	T2	0.316	Not significant	0.227	Small effect
	T3	0.151	Not significant	0.326	Small effect
	T4	0.015*	Significant	0.556	Medium effect

Statistical significant when $p < 0.05$; Cohen's d-value: $d \approx 0.20$ is indicative of a small effect, $d \approx 0.50$ points towards a medium effect; whereas $d \approx 0.80$ signifies a large effect

The practical significance between the measurements was examined by performing the Cohen's test, which confirmed the results obtained from the Type III test for Fixed effects. The differences in skins' smoothness after application of the ATF was visibly significant ($d = 0.544$) after two weeks of treatment. After four weeks of treatment with the placebo formulation, a visible difference ($d = 0.556$) was revealed in the measurements taken.

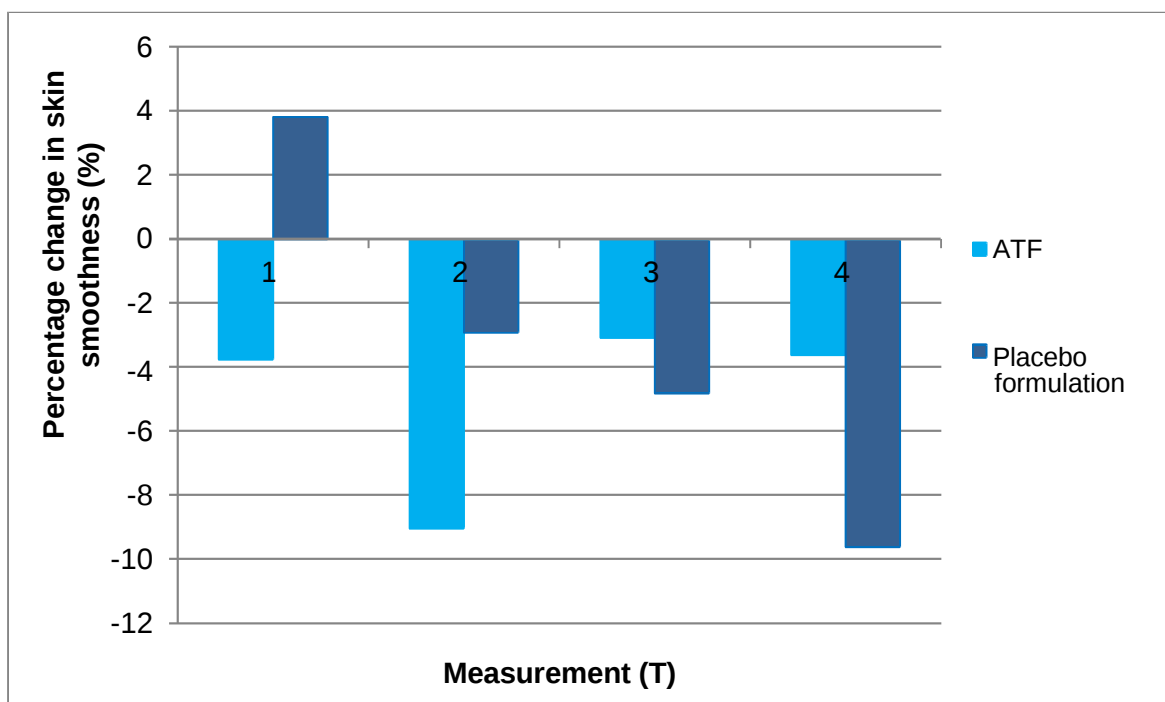


Figure E.10: %Change in the skin smoothness over four weeks for both tested formulations

When comparing the ATF and the placebo formulation on their effect on the skins' smoothness, the placebo formulation largely decreased the smoothness. The %change over four weeks after application of the placebo formulation was 9.62%, whereas the application of the ATF resulted in a 3.62% decrease in overall skin smoothness.

E.3.1.2.4 Wrinkling (Sew)

Results obtained showed the application of both tested formulations caused the wrinkle appearance of skin to increase. Cohen's tests revealed no practical significance in the measurements, with all calculated values being smaller than $d = 0.2$. The initial baseline mean measurements for wrinkling were found to be 48.28 ± 7.62 for the ATF and 49.15 ± 9.33 for the placebo formulation. The %changes in wrinkle appearance over time are tabled in Table E.16.

Table E.16: Mean Visioscan® VC 98 (Courage & Khazaka electronic GmbH, 2016b) values for wrinkle appearance with the %change over time for both formulations over four weeks treatment

Treatment	Baseline changes (Tx - T0)	Visioscan® VC 98 (mean $\pm$ SD)	%Change over time (%)*
ATF	T0	48.28 ± 7.62	-
	T1	50.31 ± 8.93	4.60%
	T2	50.10 ± 8.00	4.70%
	T3	52.53 ± 12.89	8.51%
	T4	52.99 ± 10.41	10.21%
Placebo formulation	T0	49.15 ± 9.33	-
	T1	50.82 ± 12.99	4.25%
	T2	51.40 ± 8.68	5.78%
	T3	50.10 ± 9.10	3.23%
	T4	48.71 ± 9.73	0.10%

*Calculated using Equation E.1

According to the results obtained from the Type III test for Fixed effects, both formulations did not have a statistical significant effect on the skin's wrinkle appearance after a four week treatment period. The ATF slightly increased the appearance of wrinkles, but to no statistical significant degree ($p = 0.549$), whereas the placebo formulation obtained a value of $p = 0.071$, also indicative of no statistical significant changes. Further statistical tests were performed and results are listed in Table E.17.

Table E.17: Results obtained for skin wrinkling changes from the Bonferroni and Cohen's tests

Treatment	Baseline changes (Tx - T0)	p-Value	Statistical significance	Cohen's d-value	Effect size: Practical significance
ATF	T1	0.412	Not significant	0.227	Small effect
	T2	0.781	Not significant	0.227	Small effect
	T3	0.793	Not significant	0.330	Small effect
	T4	0.403	Not significant	0.453	Small effect
Placebo formulation	T1	1.000	Not significant	0.147	Small effect
	T2	1.000	Not significant	0.046	Small effect
	T3	0.088	Not significant	0.043	Small effect
	T4	0.046*	Significant	0.138	Small effect

Statistical significant when $p < 0.05$; Cohen's d-value: $d \approx 0.20$ is indicative of a small effect, $d \approx 0.50$ points towards a medium effect; whereas $d \approx 0.80$ signifies a large effect

As seen in Table E.16, the percentage increases from the application of both formulations, followed a similar pattern. The last measurement after four weeks treatment showed a difference between the effects of the formulations, with a 10.21% increase in wrinkle appearance from the ATF compared to the 0.10% increase by the placebo formulation.

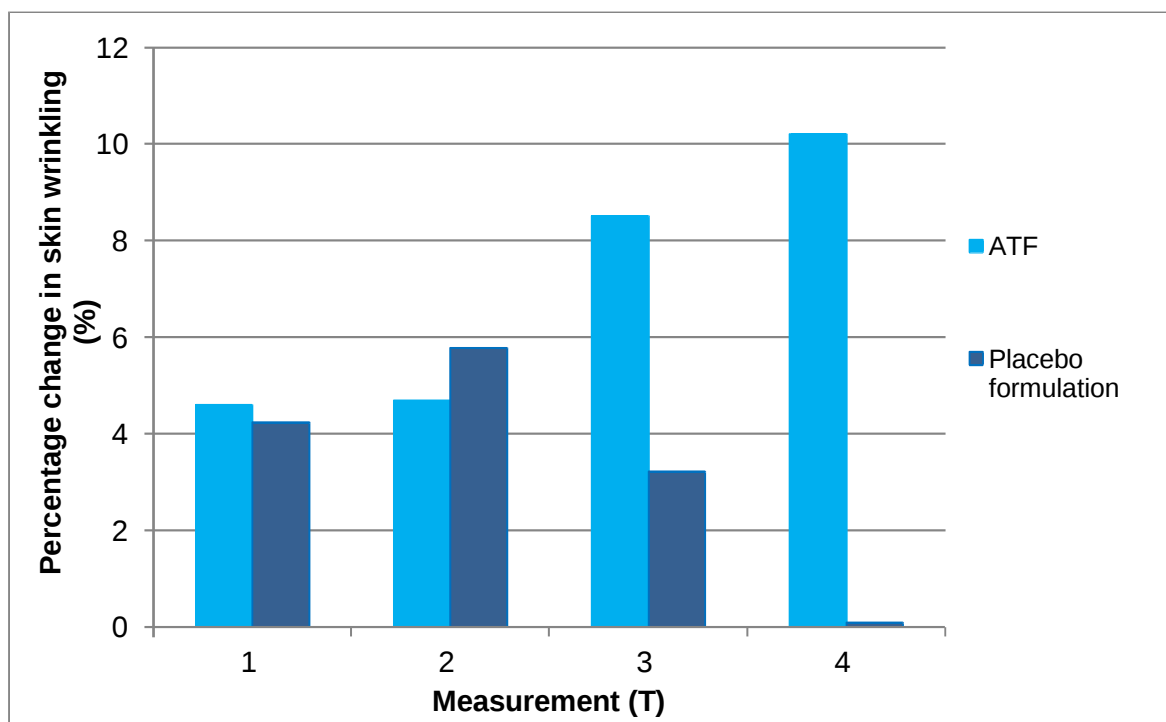


Figure E.11: %Change in the skin wrinkling over four weeks for both tested formulations

The %changes over time are graphically illustrated in Figure E.11 and clearly show the initial similarity and difference after T3 between the tested formulations. The application of both formulations initially resulted in the increase in wrinkle appearance on the tested skin sites. After the four week treatment period, the ATF resulted in a 10.21% increase in the wrinkle value and the placebo formulation decreased the value by 0.10%.

E.3.1.3 Skin viscoelastic results

The Cutometer[®] MPA 580 (Courage & Khazaka electronic GmbH, 2016c) was used to analyse the skins' viscoelasticity changes using the Q-parameters developed by Qu (Qu *et al.*, 2006:198; Qu & Seehra 2016:43), namely maximum recovery area (Q0), elastic recovery (Q1), viscous recovery (Q2) and viscoelastic recovery (Q3).

The results indicated that Q0 and Q1 both showed improvement over the period of 28 days. Only the Q0 parameter indicated a statistical significant difference in the effect ($p = 0.005$) compared to the difference of Q1 ($p = 0.085$); Q2 and Q3 did not result in any statistical significant differences in the values over the period of 28 days.

E.3.1.3.1 Maximum recovery area (Q0)

Measurement value for the Q0 parameter showed an overall decrease after application of both formulations. Table E.18 lists the changes in the mean measured values and clearly illustrates the slight decrease in Q0 values over time. The initial baseline value for the ATF was found to be 71.52 ± 5.47 and ultimately decreased to a mean value of 66.13 ± 6.67 . The mean baseline value measured for the placebo arm site was 70.89 ± 6.39 , which reduced over the four week treatment period to a value of 67.46 ± 6.78 .

From Table E.18, it is clear the application of both tested formulations over a period of four weeks resulted in a reduction of the Q0 parameter. Figure E.12 graphically illustrates the %change over time for the measured values of the Q0 parameter. The ATF resulted in a 7.24% reduction from the baseline value, while the application of the placebo formulation resulted in a 4.35% reduction.

By performing the Type III test for Fixed effects, only the ATF showed statistically significant differences ($p = 0.005$) in the measured values from T0 to T4. The placebo formulation obtained a value of $p = 0.085$, indicating the decrease in measurement values was not statistically significant.

Furthermore, results obtained from the Bonferroni tests (Table E.19) showed only a statistical significant difference in the measurement values for the ATF at T4 ($p = 0.011$). The placebo formulation also obtained only one value of significance at T2 ($p = 0.049$).

Table E.18: Mean Cutometer® MPA 580 (Courage & Khazaka electronic GmbH, 2016c) values with the %change over time for the Q0 parameter mean measurements for both formulations over four weeks treatment

Treatment	Baseline changes (Tx - T0)	Cutometer® MPA 580 (mean $\pm$ SD)	%Change over time (%)*
ATF	T0	71.52 $\pm$ 5.47	-
	T1	71.56 $\pm$ 6.91	0.27%
	T2	67.79 $\pm$ 5.85	-4.86%
	T3	69.05 $\pm$ 7.79	-3.40%
	T4	66.13 $\pm$ 6.67	-7.24%
Placebo formulation	T0	70.89 $\pm$ 6.39	-
	T1	69.01 $\pm$ 4.91	-1.12%
	T2	67.01 $\pm$ 5.46	-4.65%
	T3	68.25 $\pm$ 6.49	-3.30%
	T4	67.46 $\pm$ 6.78	-4.35%

*Calculated using Equation E.1

Table E.19: Results obtained for Q0 parameter changes over time evaluated by the Bonferroni and Cohen's tests

Treatment	Baseline changes (Tx - T0)	p-Value	Statistical significance	Cohen's d-value	Effect size: Practical significance
ATF	T1	1.000	Not significant	0.036	Small effect
	T2	0.057	Not significant	0.457	Small effect
	T3	0.167	Not significant	0.354	Small effect
	T4	0.011*	Significant	0.562	Medium effect
Placebo formulation	T1	1.000	Not significant	0.294	Small effect
	T2	0.049*	Significant	0.607	Medium effect
	T3	0.339	Not significant	0.407	Small effect
	T4	0.106	Not significant	0.505	Medium effect

Statistical significant when $p < 0.05$; Cohen's d-value: $d \approx 0.20$ is indicative of a small effect, $d \approx 0.50$ points towards a medium effect; whereas $d \approx 0.80$ signifies a large effect

The practical significance was evaluated (as seen in Table E.19) and visible differences between the measured values were identified for the ATF at T4 ($d = 0.562$) and for the placebo

formulation at T2 ($d = 0.607$) and at T4 ($d = 0.505$). Therefore, either tested formulations resulted in visible differences or decreases in Q0 values over four weeks.

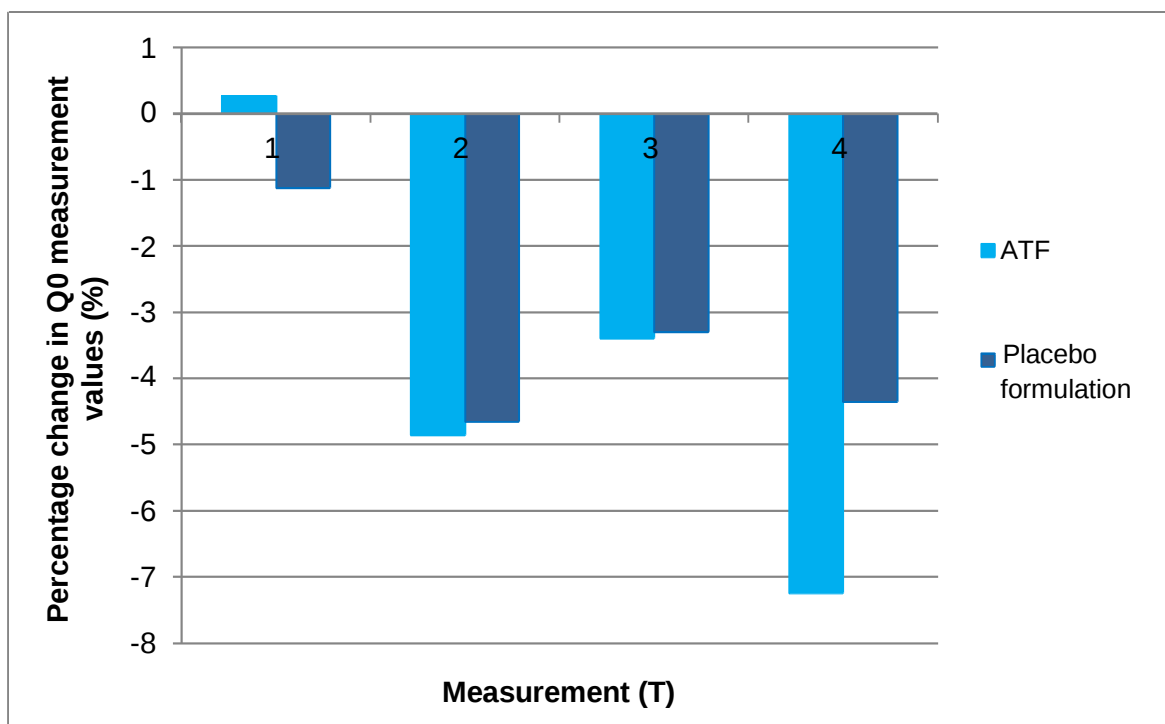


Figure E.12: %Change in the Q0 parameter over four weeks for both tested formulations

According to Courage & Khazaka (electronic GmbH, 2016c), a decrease in the Q0 parameter is indicative of the skins' firmness that increased, therefore, it is clear from Figure E.12 that over a four week treatment period the application of ATF provided a more effective skin firming action. Comparing the efficacy of the ATF to the placebo formulation, it is evident that both formulations possess skin-firming properties, but the ATF produced a more efficient action (7.24%) than the placebo formulation (4.35%).

E.3.1.3.2 Elastic recovery (Q1)

An increase in the elastic recovery was found after four week treatment with both the ATF and placebo formulation. The results obtained from further Cohen's tests showed no practical significant changes in the Q1 measured values over time. When comparing the initial baseline measurements to the measured values after four week treatment, it is clear no great changes occurred. The mean baseline value obtained for the ATF skin site was 0.77 ± 0.06 and increased to 0.79 ± 0.03 ; the initial baseline value of the placebo skin site was 0.76 ± 0.08 and increased to 0.77 ± 0.08 (as seen in Table E.20).

Table E.20: Mean Cutometer[®] MPA 580 (Courage & Khazaka electronic GmbH, 2016c) values with the %change over time for the Q1 parameter mean measurements for both formulations over four weeks treatment

Treatment	Baseline changes (Tx - T0)	Cutometer [®] MPA 580 (mean $\pm$ SD)	%Change over time (%)*
ATF	T1	0.76 $\pm$ 0.05	-0.16%
	T2	0.76 $\pm$ 0.06	-0.08%
	T3	0.78 $\pm$ 0.05	1.94%
	T4	0.79 $\pm$ 0.03	2.26%
Placebo formulation	T1	0.78 $\pm$ 0.05	3.37%
	T2	0.76 $\pm$ 0.06	1.12%
	T3	0.76 $\pm$ 0.08	1.05%
	T4	0.77 $\pm$ 0.08	2.57%

*Calculated using Equation E.1

From Table E.20, the %changes over time can also be seen. There was an initial reduction from the application of ATF in the Q1 parameter (T0 - T2), thereafter the measured values increased to a final percentage increase of 2.26% for the ATF and 2.57% for the placebo formulation. Results obtained from further test performed are listed in Table E.21.

Table E.21: Results obtained for Q1 parameter changes over time evaluated by the Bonferroni and Cohen's tests

Treatment	Baseline changes (Tx - T0)	p-Value	Statistical significance	Cohen's d-value	Effect size: Practical significance
ATF	T1	1.000	Not significant	0.038	Small effect
	T2	1.000	Not significant	0.028	Small effect
	T3	0.749	Not significant	0.199	Small effect
	T4	0.451	Not significant	0.329	Small effect
Placebo formulation	T1	0.680	Not significant	0.242	Small effect
	T2	1.000	Not significant	0.055	Small effect
	T3	1.000	Not significant	0.059	Small effect
	T4	1.000	Not significant	0.174	Small effect

Statistical significant when $p < 0.05$; Cohen's d-value: $d \approx 0.20$ is indicative of a small effect, $d \approx 0.50$ points towards a medium effect; whereas $d \approx 0.80$ signifies a large effect

The Type III for Fixed effects revealed no statistical significant differences in the values measured for either the ATF or the placebo formulation regarding the Q1 parameter. The difference in values resulted in a value of $p = 0.182$ for the ATF and $p = 0.636$ for the placebo formulation. Figure E.13 graphically shows the %changes in the Q1 parameter over time.

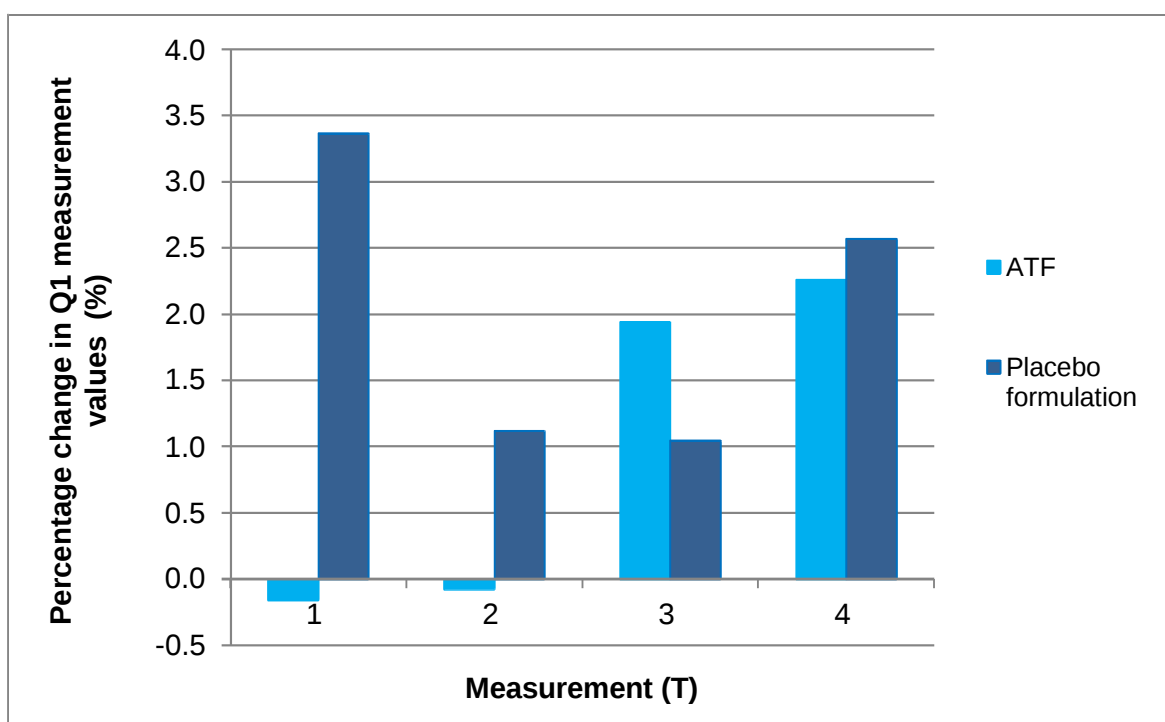


Figure E.13: %Change in the Q1 parameter over four weeks for both tested formulations

An increase in the Q1 parameter means the elasticity of the skin increased (Courage and Khazaka electronic GmbH, 2016b). Both the ATF and placebo formulations therefore increased the elasticity of the skin with a similar efficacy after a four week treatment period. From the data obtained, the placebo formulation constantly produced increased values compared to the ATF, which only increased the Q1 values from T3.

E.3.1.3.3 Viscous recovery (Q2)

According to the results obtained, the viscous recovery increased after four weeks' application of both the ATF and the placebo formulations; Cohen's tests also indicated that no practical significant changes were present between the measured values for both formulations at any point in the treatment time. Comparison between the initial baseline measurements and the final mean measurements taken on T4 showed the increase in Q2 measurements, for both formulations, applied. The mean baseline measurement for the ATF tested site was 0.63 ± 0.07 and the value increased to 0.66 ± 0.04 after the treatment period. The mean baseline measurement for the placebo formulations' tested site was 0.62 ± 0.08 , and increased over four weeks treatment to a final value of 0.64 ± 0.08 (as seen in Table E.22).

Table E.22: Mean Cutometer[®] MPA 580 (Courage & Khazaka electronic GmbH, 2016c) values with the %change over time for the Q2 parameter mean measurements for both formulations over four weeks treatment

Treatment	Baseline changes (Tx - T0)	Cutometer [®] MPA 580 (mean $\pm$ SD)	%Change over time (%)*
ATF	T0	0.63 $\pm$ 0.07	-
	T1	0.63 $\pm$ 0.07	-0.05%
	T2	0.64 $\pm$ 0.07	1.48%
	T3	0.65 $\pm$ 0.07	2.98%
	T4	0.66 $\pm$ 0.04	3.83%
Placebo formulation	T0	0.62 $\pm$ 0.08	-
	T1	0.64 $\pm$ 0.07	4.00%
	T2	0.63 $\pm$ 0.06	2.13%
	T3	0.62 $\pm$ 0.09	1.08%
	T4	0.64 $\pm$ 0.08	3.23%

*Calculated using Equation E.1

The %changes in the Q2 parameter measured over the four week treatment period is tabled in Table E.22 and graphically depicted in Figure E.14. The ATF showed a constant increase in the measurement values from T2 up to T4. The placebo formulation initially had the highest increase in value and thereafter fluctuated in the measured values. The outcome after four weeks of applications was an increase of 3.83% for the ATF and 3.23% for the placebo formulation.

Table E.23: Results obtained for Q2 parameter changes over time evaluated by the Bonferroni and Cohen's tests

Treatment	Baseline changes (Tx - T0)	p-Value	Statistical significance	Cohen's d-value	Effect size: Practical significance
ATF	T1	1.000	Not significant	0.031	Small effect
	T2	1.000	Not significant	0.103	Small effect
	T3	0.396	Not significant	0.228	Small effect
	T4	0.160	Not significant	0.385	Small effect
Placebo formulation	T1	0.626	Not significant	0.212	Small effect
	T2	1.000	Not significant	0.083	Small effect
	T3	1.000	Not significant	0.029	Small effect
	T4	1.000	Not significant	0.151	Small effect

Statistical significant when $p < 0.05$; Cohen's d-value: $d \approx 0.20$ is indicative of a small effect, $d \approx 0.50$ points towards a medium effect; whereas $d \approx 0.80$ signifies a large effect

The measured values were not identified as statistical significant for the ATF ($p = 0.096$), nor the placebo formulation ($p = 0.602$). Results obtained from performing the Bonferroni test, identified no statistical significant differences either, as reported for the Type III test for Fixed effects. The results are shown in Table E.23.

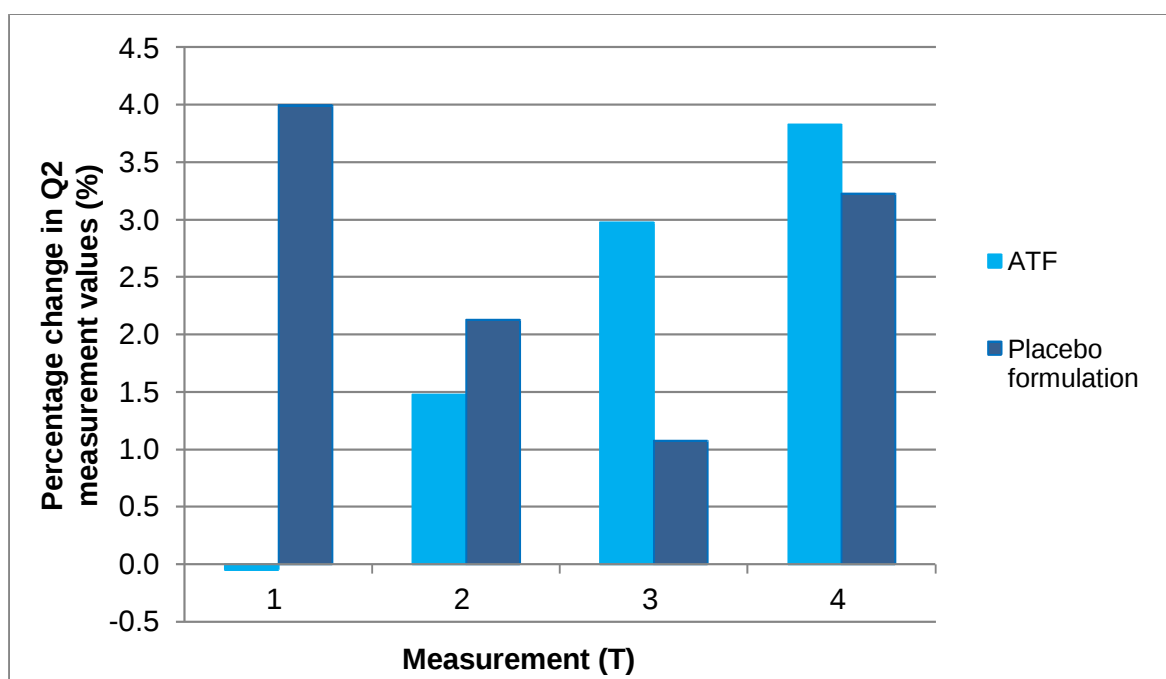


Figure E.14: %Change in the Q2 parameter over four weeks for both tested formulations

The ATF initially had a delayed effect on the Q2 measurement, but T2 constantly increased the Q2 parameter, and therefore improved the viscous recovery by 3.83%. The placebo formulation also increased the skins' viscous recovery to a slightly smaller effect (3.23%) compared to the ATF (Courage and Khazaka electronic GmbH, 2016b).

E.3.1.3.4 Viscoelastic recovery (Q3)

The results obtained from the measurement values for the viscoelastic recovery, identified that the application of ATF resulted in a decrease in the Q3 parameter over four weeks. No statistical or practical differences were identified by the Bonferroni and Cohen's tests performed. From the mean baseline measurement value of the ATF tested site (0.136 ± 0.02), it can be seen that the measured value slightly decreased to 0.131 ± 0.02 ; the placebo tested site increased from a mean baseline value of 0.133 ± 0.02 to a value of 0.134 ± 0.02 (as seen in Table E.24).

Table E.24: Mean Cutometer® MPA 580 (Courage & Khazaka electronic GmbH, 2016c) values with the %change over time for the Q3 parameter mean measurements for both formulations over four weeks treatment

Treatment	Baseline changes (Tx - T0)	Cutometer® MPA 580 (mean ± SD)	%Change over time (%)*
ATF	T0	0.136 ± 0.02	-
	T1	0.132 ± 0.02	-1.03%
	T2	0.127 ± 0.02	-4.74%
	T3	0.132 ± 0.02	-1.57%
	T4	0.131 ± 0.02	-1.49%
Placebo formulation	T0	0.133 ± 0.02	-
	T1	0.133 ± 0.02	0.79%
	T2	0.130 ± 0.02	-0.21%
	T3	0.135 ± 0.02	3.58%
	T4	0.134 ± 0.02	1.57%

*Calculated using Equation E.1

The %changes in the measurement for the Q3 parameter are listed in Table E.24 and graphically illustrated in Figure E.15. It is clear from the graph that the ATF and placebo formulations had different effects on the viscoelasticity of the skin areas tested. Further statistical tests were performed on the data to examine the differences in changes over time (summarised in Table E.25)

Table E.25: Results obtained for Q3 parameter changes over time evaluated by the Bonferroni and Cohen's tests

Treatment	Baseline changes (Tx - T0)	p-Value	Statistical significance	Cohen's d-value	Effect size: Practical significance
ATF	T1	1.000	Not significant	0.157	Small effect
	T2	0.236	Not significant	0.381	Small effect
	T3	1.000	Not significant	0.178	Small effect
	T4	0.993	Not significant	0.231	Small effect
Placebo formulation	T1	1.000	Not significant	0.131	Small effect
	T2	1.000	Not significant	0.194	Small effect
	T3	1.000	Not significant	0.028	Small effect
	T4	1.000	Not significant	0.169	Small effect

Statistical significant when $p < 0.05$; Cohen's d-value: $d \approx 0.20$ is indicative of a small effect, $d \approx 0.50$ points towards a medium effect; whereas $d \approx 0.80$ signifies a large effect

According to the Type III test for fixed effects, the changes in the measurement values were not statistically significant with a value of $p = 0.438$. After the four week treatment with the placebo formulation, the Q3 values increased but also to no statistical significant degree ($p = 0.851$).

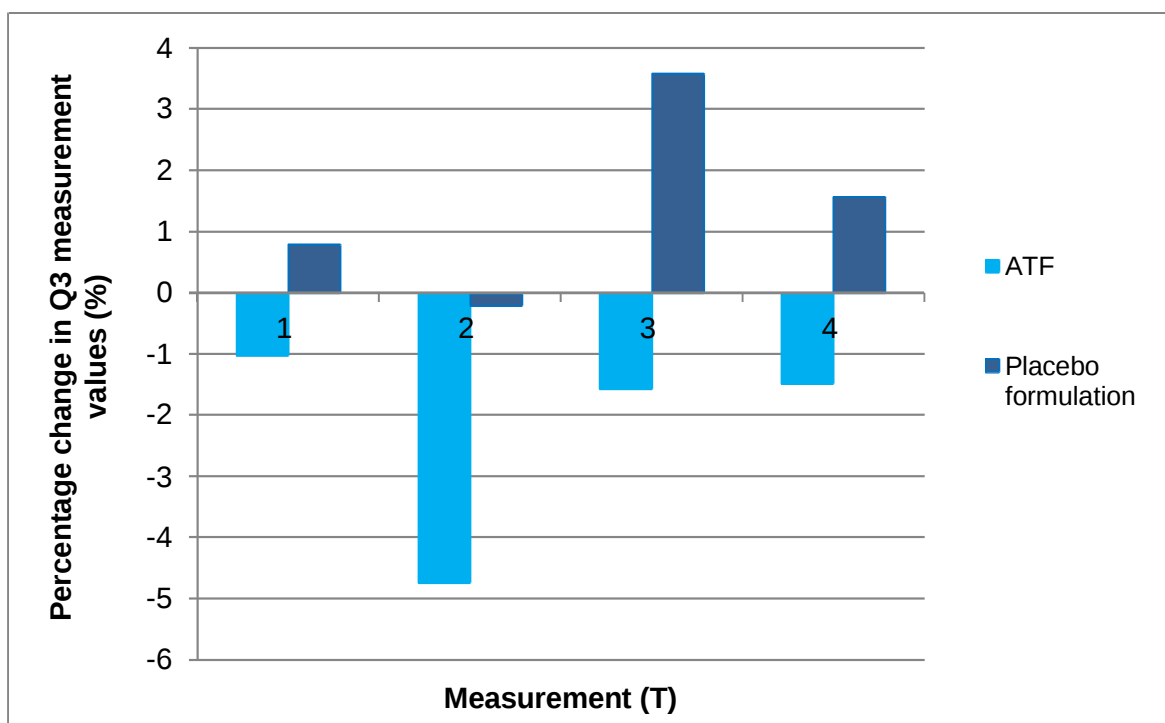


Figure E.15: %Change in the Q3 parameter over four weeks for both tested formulations

The ATF decreased the Q3 parameter values by 1.49% over a treatment time of four weeks. The placebo formulation had the opposite effect on the viscoelastic recovery of the skin, increasing the Q3 value with 1.57%. The higher the Q3 parameter value, the higher the overall viscoelasticity of the skin (Courage and Khazaka electronic GmbH, 2016b).

E.3.2 Erythema study

One enrolled volunteer was not able to attend the clinical measurement phase due to personal reasons; this volunteer was not replaced. One volunteer dropout was recorded due to a volunteer presenting with an unidentified (possibly unrelated) rash. This volunteer was advised to cease participation in the clinical trial and treatment was stopped.

Table E.26: The mean Mexameter® MX 18 (Courage & Khazaka electronic GmbH, 2016d) values measured and average %change in erythema levels for each tested skin site over 5 days of treatment

Treatment	Time point	Baseline changes (Tx - T0)	
		Mexameter® MX 18 (mean ± SD)	%Change over time (%)*
Positive control: 1% hydrocortisone cream (w/w)	T0	341.30 ± 78.13	-
	T1	338.69 ± 65.65	-0.4%
	T2	322.12 ± 64.36	-4.7%
	T3	280.56 ± 54.65	-16.6%
Placebo formulation	T0	306.21 ± 66.82	-
	T1	297.53 ± 62.85	-2.1%
	T2	283.40 ± 55.90	-6.6%
	T3	261.22 ± 50.98	-13.8%
ATF	T0	304.90 ± 59.18	-
	T1	298.51 ± 63.94	-1.5%
	T2	289.12 ± 51.65	-4.6%
	T3	260.42 ± 48.16	-13.9%
Negative control	T0	304.54 ± 60.24	-
	T1	289.84 ± 60.21	-4.4%
	T2	287.31 ± 55.82	-5.1%
	T3	274.45 ± 56.44	-9.4%
Untreated skin	T0	231.62 ± 55.09	-
	T1	219.90 ± 51.59	-3.9%
	T2	218.01 ± 54.15	-5.0%
	T3	215.57 ± 45.20	-5.4%

*Calculated using Equation E.1

The results obtained from the erythema study, as described in Section E.2.4.2, was analysed by using both the Type III Test for Fixed Effects and the Cohen's tests to examine the practical significance between values. The percentage improvement for each measurement (as seen in Table E.26) was determined to evaluate the overall anti-inflammatory action over the treatment period. The results indicated, as expected, the positive control (1% hydrocortisone cream) had the best anti-inflammatory action and decreased the erythema levels by 16.6% over a treatment time of five days, showing an initial delay period of 48 h. The skin's natural healing process (negative control) decreased the inflammation slightly less than the positive control, but showed constant increase from T1 through to T3, with no initial delay in action.

Figure E.16 graphically shows the %change over time for all the tested skin areas, illustrating the overall anti-inflammatory action over the treatment time of five days. It is evident that the positive control had the best anti-inflammatory action (16.6%) after the five-day treatment, but initially had the smallest improvement (0%). Both the ATF (13.9%) and placebo formulations (13.8%) decreased the erythema levels, indicating the anti-inflammatory action of the active ingredient showed no significance compared to its control. The untreated skin also showed improvement (5%) in erythema levels, although no inflammation was induced to the tested skin site. Results for all tests are summarised in Table E.27.

Table E.27: Results obtained from the Post-Hoc test with Bonferroni correction for multiple comparisons and the Cohen's test for practical significant differences for the erythema study

Treatment	Baseline changes (Tx - T0)	p-Value	Statistical significance	Cohen's d-value	Effect size: Practical significance
Positive control: 1% hydrocortisone cream (w/w)	T1	1.000	Not significant	0.033	Small effect
	T2	0.099	Not significant	0.245	Small effect
	T3	0.001*	Significant	0.777	Medium effect
Placebo formulation	T1	0.527	Not significant	0.130	Small effect
	T2	0.002*	Significant	0.341	Small effect
	T3	0.001*	Significant	0.673	Medium effect
ATF	T1	0.397	Not significant	0.100	Small effect
	T2	0.039*	Significant	0.267	Small effect
	T3	0.001*	Significant	0.752	Medium effect
Negative control	T1	0.016*	Significant	0.244	Small effect
	T2	0.005*	Significant	0.286	Small effect
	T3	0.001*	Significant	0.499	Small effect
Untreated skin	T1	0.118	Not significant	0.213	Small effect
	T2	0.071	Not significant	0.247	Small effect
	T3	0.034*	Significant	0.291	Small effect

Statistical significant when $p < 0.05$; Cohen's d-value: $d \approx 0.20$ is indicative of a small effect, $d \approx 0.50$ points towards a medium effect; whereas $d \approx 0.80$ signifies a large effect

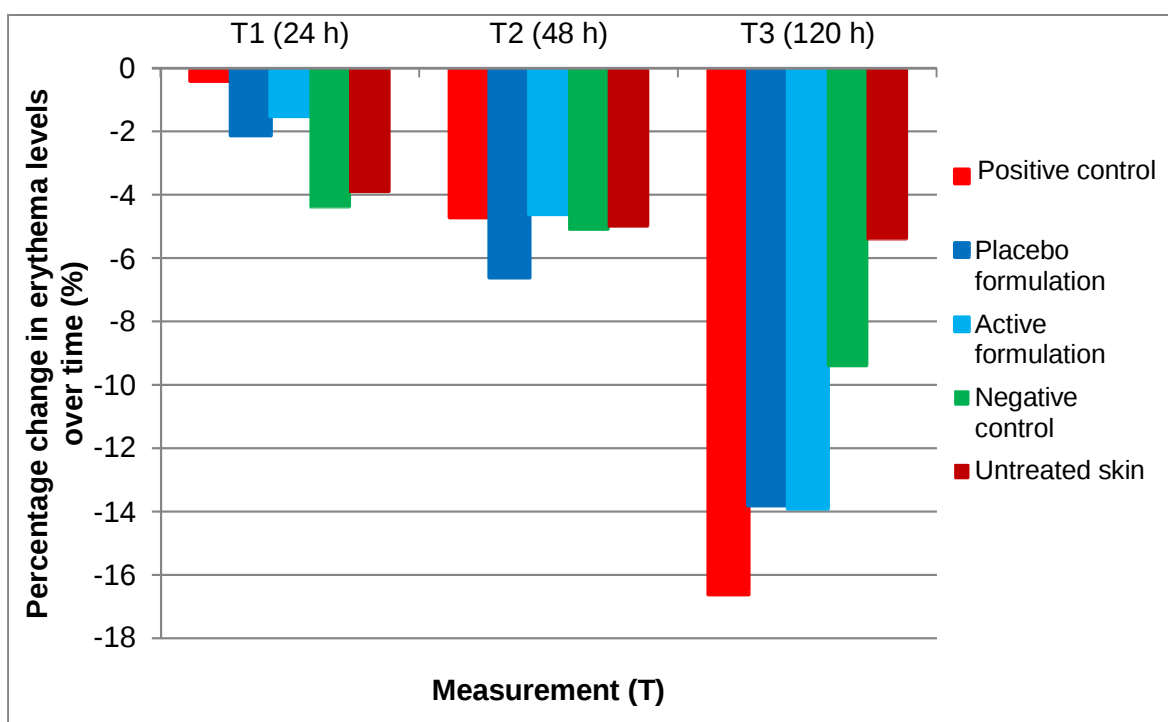


Figure E.16: Anti-inflammatory action measured on each individual skin area over five days of treatment

Results for the Type III test for fixed effects revealed all the treated skin areas (1 to 3) showed statistical significant ($p < 0.05$) changes or improvement in the erythema levels over a period of five days (T0 to T3). The negative control (4) had a value of $p = 0.001$, representing a statistical significant decrease in skin erythema levels, furthermore, the untreated skin (5) showed no statistical significant changes over the treatment time of five days with p -values of $p = 0.147$. To investigate the primary objective further and to determine where the statistically significant changes in values occurred relative to the T0 measurements, a Post-Hoc test was conducted with a Bonferroni correction for multiple comparisons. Results indicated only the natural healing response of the skin showed significant improvement on T1 relative to T0, and both the ATF and placebo formulations only showed statistical significant differences from T2 relative to T0. The positive control (1), 1% hydrocortisone cream (w/w), only showed statistical significant differences between T3, in relation to the T0 measurements, indicating a possible delayed effect. Conversely, the negative control (4) showed statistical significance in the differences between all three measurements, indicating the constant and immediate effect of the skins' natural healing process. It is also evident, from Table E.27, that both the ATF and placebo formulations had a slight delay in anti-inflammatory action, with significant improvement only from 48 h (T0 to T2).

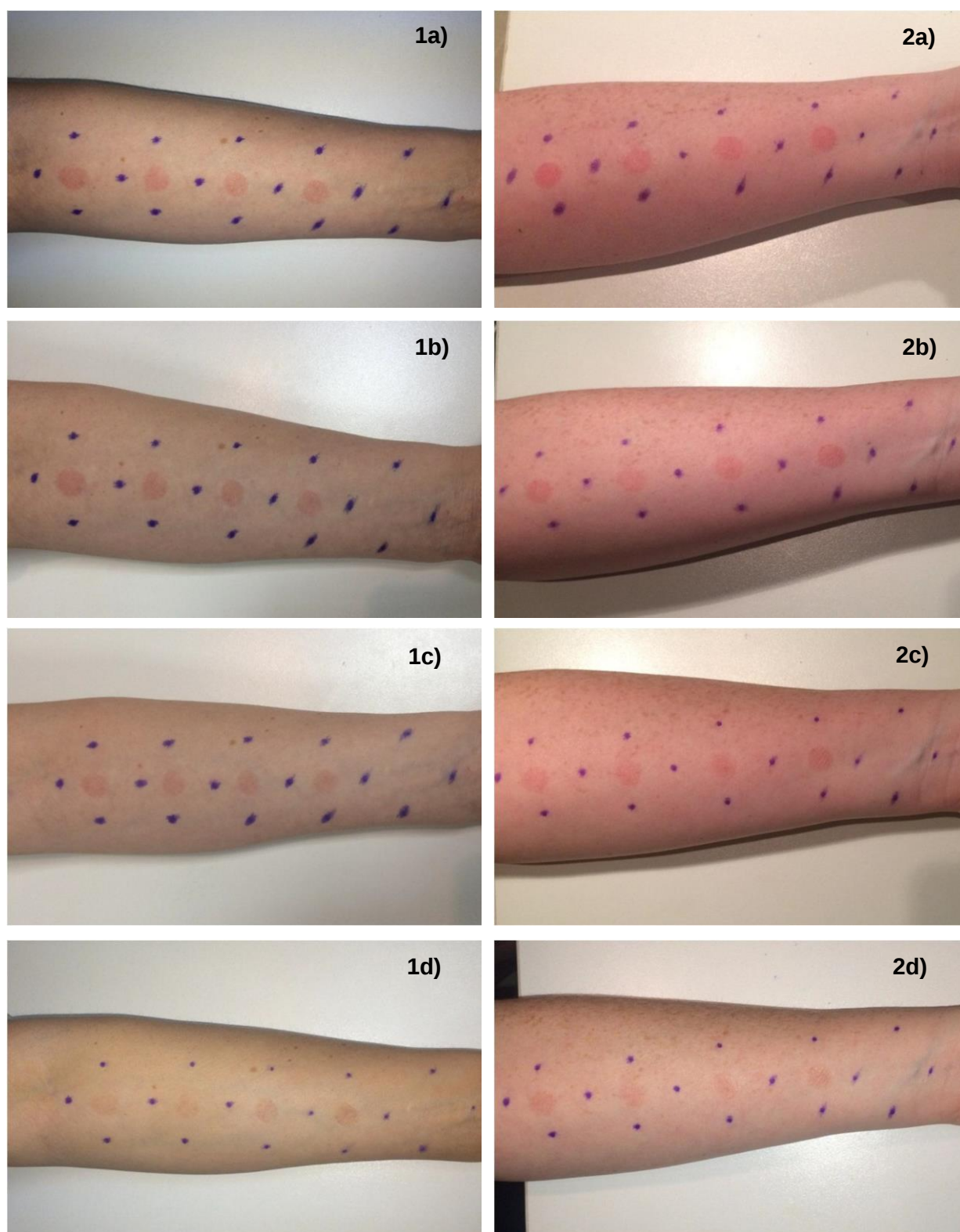


Figure E.17: Images taken on T0 (a), T1 (b), T2 (c) and T3 (d) for two volunteers during the erythema study.

Results for Cohen's test revealed that after a treatment period of 120 h (T0 to T3), the d-values for the positive control ($d = 0.777$), placebo formulation ($d = 0.673$) and the ATF ($d = 0.752$)

showed practical significant improvement in erythema levels in the skin sites. Figure E.17 shows two volunteers' arms photographed during the erythema study, with the skin marker indicating the precise area to be measured.

To conclude, no difference in the anti-inflammatory activity of ATF compared to the placebo formulation was observed over a treatment time of five days. Both formulations showed statistical significant and practical significant differences in erythema values after 48 h of treatment and ultimately obtained an improvement of 5% compared to the skin's natural healing process. The positive control still showed the best anti-inflammatory activity with an initial delay in action (T0 to T1), but resulted in a 16.6% improvement in the erythema levels for the tested skin site.

E.4 Conclusion

The obtained results for the anti-ageing and anti-inflammatory clinical studies demonstrated that both the ATF and placebo formulation improved the skins' hydration, scaliness and Q1 and Q2 parameters for elasticity and erythema levels. Unfortunately, the results obtained for the skin roughness, smoothness and wrinkling did not deem the ATF an effective topical formulation as an anti-ageing treatment.

E.4.1 Anti-ageing study

The hydration levels measured with the Corneometer® CM 825 (Courage & Khazaka electronic GmbH, 2016a) during the four week study proved the application of both the ATF and the placebo formulation resulted in statistical significant increases. Both tested formulations initially decreased the hydration levels, but after two weeks, the placebo formulation increased the hydration and a week later, the ATF started increasing the hydration as well. The final measurements revealed the placebo formulation had a 10.75% better hydrating effect when compared to the ATF.

Using the Visioscan® VC 98 (Courage & Khazaka electronic GmbH, 2016b) clinical SELS parameters, the skin surface changes were evaluated and efficacy of the ATF tested. If the skin's condition is improved, the resulting measurements should have indicated a decrease in roughness, scaliness and wrinkling, and an increase in skin smoothness.

The SELS parameters were evaluated after the four week treatment and are changes are describes as follow:

-
- Roughness (SEr): Both the ATF and placebo formulations resulted in an increase in the measured values for skin roughness. The effect of the ATF was slightly less (4.92%) when compared to the placebo formulation.
 - Scaliness (SEsc): The ATF and placebo formulation resulted in the decrease in the scaliness of the tested skin sites. Once again, the effect delivered by the ATF was smaller (2.17%) compared to the placebo formulation.
 - Smoothness (SEsm): The placebo formulation significantly decreased the smoothness of the skin compared to the ATF with a less significant effect (6%).
 - Wrinkling (SEw): Both the ATF and placebo formulation resulted in an increased appearance of wrinkles. The effect brought about by the ATF was greater (10.11%) than the effect caused by the placebo formulation.

Therefore, the application of ATF increased the hydration levels of the skin. It also resulted in the decrease in skin scaliness, but no topographic properties improved significantly and where negatively influenced the roughness, smoothness and wrinkling of the tested skin sites.

The Cutometer[®] MPA 580 (Courage & Khazaka electronic GmbH, 2016c) was used to measure the viscoelastic properties of the skin. The Q-parameters used to calculate the changes over time are known as the maximum recovery area (Q0), elastic recovery (Q1), viscous recovery (Q2) and viscoelastic recovery (Q3). For the overall elasticity of the skin to improve, the Q0 parameter must decrease and the Q1, Q2 and Q3.

- Q0: The skin firmness increased significantly ($p = 0.005$) after four weeks treatment with the ATF. The placebo formulation also decreased the Q0 parameter value, but with a smaller efficacy.
- Q1: The evaluation of the Q1 parameter revealed both the ATF and placebo formulation improved the elasticity of the skin by increasing the Q2 parameter value. In comparison, the ATF increased the value by 2.26% and the placebo by 2.57% after a four weeks treatment period.
- Q2: The ATF increased the viscous recovery of the tested skin sites by 3.83% and the placebo formulation by 3.23%. The Type III test for Fixed effects identified no statistical significance differences in the measured values on T0 and T4.
- Q3: After evaluation, results revealed the ATF decreased the viscoelastic recovery of the tested skin sites by 1.49%. In contradiction to this, the application of the placebo formulation resulted in a 1.57% increase in this parameter. The differences in measured values were not found to be statistically significant.

E.4.2 Erythema study

The results for the erythema study revealed both the ATF and placebo formulation produced statistically significant differences in the measured values and ultimately resulted in a 13.9% and 13.8% reduction in the erythema levels after the treatment period, respectively. Therefore, it can be said that no significant difference between the anti-inflammatory actions of the ATF compared to the placebo formulation was observed. The positive control had the best anti-inflammatory action, but initially had a delayed effect for 24 h before decreasing the erythema levels by 16.6% after five days treatment. The negative control and untreated skin was evaluated and no statistical significant differences in the measured values were identified. The value obtained for the untreated skin is a validation of the results as no change was expected, because no irritant or treatment was applied to this area.

To conclude, with regard to the anti-inflammatory activity of the ATF, no difference was identified between the ATF and the placebo formulation over a treatment time of five days. Both formulations showed statistical significant differences in erythema values after 48 h of treatment and obtained an improvement of 5% compared to the skin's natural healing process. A possible explanation could be the amount of active ingredient present in the ATF, which was not adequate to produce the anti-inflammatory activity desired.

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Appendix F

AUTHORS GUIDE: INTERNATIONAL JOURNAL OF PHARMACEUTICS

Introduction

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Appendix G

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LANGUAGE EDITOR DECLARATION

I hereby declare that I completed the text editing on the abstract [Afrikaans] of the mini-dissertation, entitled *Formulation and topical delivery of niosomes and proniosomes containing α -Lipoic acid*, submitted in partial fulfilment of the requirements for the degree **Masters in Pharmaceutical Science (MSc Pharmaceutics)** at the North-West University, for Tanja Meyer.



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Date	28/11/2016
Subject	Clinical efficacy of topical formulations containing α -lipoic acid in the treatment of skin ageing and inflammation.
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I, Gill Smithies, certify that I have proofed the following,

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