Formulation and topical delivery of niosomes and proniosomes containing carnosine

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“For beautiful eyes, look for the good in others; for beautiful lips, speak only words of kindness; and for poise, walk with the knowledge that you are never alone.”

Audrey Hepburn

This dissertation is presented in the so-called article format, which includes sub-chapters, one article for publication in a pharmaceutical journal and appendices containing experimental results and discussion. The article for publication has specific author guidelines for publishing in Appendix H.

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outliers (o). The following key can be used for the different preparations, N: niosomes, PN: proniosomes, G: gel and C: cream

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EE (%) = Cr / Ct x 100

APPENDIX A

Equation A.1

y = mx + c

APPENDIX B

Equation B.1

EE (%) = Cr / Ct x 100

APPENDIX C

Equation C.1

EE (%) = Cr / Ct x 100

APPENDIX E

Equation E.1

%Change = (T0 - Tx) / T0 x 100

ABSTRACT

Wiechers (2008:1-18) explains that the efficacy of a biologically active cosmetic product depends on both the intrinsic activity of the active, as well as the delivery of the active. This study aimed to deliver the cosmeceutical active, carnosine, topically.

Carnosine is a naturally occurring compound with both anti-oxidation and anti-glycation properties (Kyriazis, 2010:45-49). The biological functions of carnosine vary amongst different tissues, as well as within one kind of tissue (Boldyrev *et al.,* 2013:1820). Both oxidation and glycation are associated with ageing in skin, and with carnosine's aforementioned properties, it could possibly provide the skin with anti-ageing benefits (Hipkiss, 1998:864).

The outermost layer of the skin is the most important physical barrier to overcome during topical delivery. This layer, the stratum corneum, is also considered as the rate-limiting barrier (El Maghraby *et al.,* 2008:203-206; Wickett & Visscher, 2006:98-106). The stratum corneum consists of intercellular spaces filled with a lipophilic matrix and corneocytes aligned in a scaffold-like framework, which contribute to the difficulty of delivering hydrophilic compounds topically (Goebel *et al.,* 2012:281-286; Venus *et al.,* 2011:471-474).

Unfortunately, carnosine is a hydrophilic compound and has unfavourable properties when considering the relevant physico-chemical properties for skin diffusion. The important physicochemical properties of the active to consider for skin permeation include the octanol-water partition coefficient (log P), molecular size and aqueous solubility (Benson & Watkinson, 2012). The ideal log P of an active for topical delivery is 1 to 3, whereas the general rule is normally for molecular size and aqueous solubility to be less than 500 Da and more than 1 mg/ml, respectively (Khalid *et al.,* 2016:129; Naik *et al.,* 2000:319). According to the Material Safety Data Sheet (MSDS, 2013), carnosine has a molecular weight of 226.23 Da. The aqueous solubility was determined as 122.804 ± 0.716 mg/ml in phosphate buffer solution (PBS) at pH 7.4 and 25 °C, whilst the octanol-buffer distribution coefficient (log D) was determined as - 2.891 ± 0.013 in PBS (pH 7.4).

Due to carnosine's unfavourable log D, poor skin diffusion can be predicted and external interventions might be necessary to enhance skin permeation. Vesicle systems have been used since the 80s to improve skin permeation (Nasir *et al.,* 2012:484). Vesicles are colloidal particles consisting of a hydrophilic headgroup and a hydrophobic tail (Honeywell-Nguyen & Bouwstra, 2005:67-74). Non-ionic surfactant based vesicles, better known as niosomes, can encapsulate both amphiphilic and lipophilic actives (Nasir *et al.,* 2012:479-487; Uchegbu & Vyas, 1998:33-70). The use of niosomes to encapsulate drug molecules provides a number of advantages for topical delivery. These advantages include, but are not limited to, increased

penetration of the stratum corneum, prolonged residence time of active ingredients in the skin and reduced systemic absorption (Mali *et al.,* 2013:587). In cosmetic delivery, niosomes also increase the stability and improve the bioavailability of the active (Nasir *et al.,* 2012:484). Proniosomes are a dry form of niosomes, which are more stable than niosomes, but easily hydrated with an aqueous phase upon use (Kumar & Rajeshwarrao, 2011:214; Marianecci *et al.,* 2013:71).

Niosomes can only be considered as a pre-formulation due to problems such as instability, visual appearance and mostly the very low viscosity. According to Barry (2007:595), topical preparations must be acceptable for patients. Patient preference generally includes products, which are easily transferred from the container, spread freely to leave no residue and are not difficult to remove from the skin. In order to benefit from the advantages of niosomes and increase patient compliance, a proper semi-solid dosage form is necessary. Stahl (2015:209-218) classifies semi-solid dosage forms into gels, creams, ointments and pastes. These dosage forms have sufficient viscosity to stay on the skin for a prolonged time, resulting in an increased chance for diffusion of the active ingredient through the formulation into the skin (Stahl, 2015:209-218).

In this study, niosomes and proniosomes were used as pre-formulations. After a trial and error approach, the ideal carnosine concentration to be encapsulated was determined as 3%. Tests were performed on the two final pre-formulations to characterise them and ensure the quality of the dispersions. The characteristics tested included vesicle size, polydispersity index (PdI), zeta-potential, pH, viscosity and entrapment efficiency (EE%). Except for the low viscosities, the pre-formulations had overall good characteristics. The niosomes were chosen to formulate the semi-solids, since they had better overall characteristics and were easier and quicker to prepare prepared to the proniosomes. Two semi-solid formulations, a gel and a cream containing carnosine encapsulated in niosomes, were then formulated.

Membrane release experiments, followed by transdermal diffusion studies and tape stripping experiments were performed on all four of the preparations. The membrane release experiments proved that carnosine was released from all four of the preparations. The niosomes had the best median flux (1 139.10 µg/cm^2.h) of the four preparations, followed by the proniosomes, the gel and finally the cream. A slight negative effect of the formulations on the release from the pre-formulations was noticed. None of the samples had carnosine in the receptor phase after transdermal diffusion studies; whilst all four of the experiments successfully delivered carnosine to the stratum corneum-epidermis (SCE) and epidermis-dermis (ED). The gel delivered the highest median concentration carnosine (2.458 µg/ml) to the SCE, followed by niosomes, proniosomes and finally the cream. The niosomes delivered the highest median concentration to the ED (2.465 µg/ml), followed by the gel, the cream and finally the

proniosomes. The niosomes and the gel were the best pre-formulation and semi-solid formulation considering topical delivery of carnosine. The median values were preferred, as they represented the skewed data more accurately than the average values (Dawson & Trapp, 2001:30; Gerber *et al*., 2008:190).

The two semi-solid formulations underwent accelerated stability tests for three months. The stability tests were performed following the International Conference on Harmonisation (ICH) Guidelines. The formulation changes were assessed during accelerated (40 ± 2 °C/75 ± 5% RH (relative humidity)), intermediate (30 ± 2 °C/60 ± 5% RH) and long-term (25 ± 2 °C/60 ± 5% RH) storage conditions (ICH, 2003:3). The stability tests included concentration assays on the active and preservatives, pH, conductivity, viscosity and zeta-potential measurements, mass loss determination, as well as a microscopic and macroscopic examination. Neither of the products was considered stable after three months and was not suitable for manufacture.

Key words: Carnosine, niosomes, semi-solids, stability, skin, *in vitro*

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UITTREKSEL

Wiechers (2008:1-18) verduidelik dat die effektiwiteit van ’n biologiese aktiewe kosmetiese produk hoofsaaklik berus op die aktiewe bestanddeel se intrinsieke aktiwiteit en die aflewering van die aktiewe bestanddeel. Hierdie studie het ten doel gehad om die kosmetiese aktiewe bestanddeel, karnosien, topikaal af te lewer.

Karnosien is ’n verbinding wat natuurlik voorkom met anti-oksidasie en anti-glikasie eienskappe (Kyriazis, 2010:45-49). Die verbinding het verskillende biologiese funksies in verskillende tipes weefsel, sowel as in dieselfde weefsel (Boldyrev *et al.,* 2013:1820). Beide oksidasie en glikasie word verbind met veroudering, en as gevolg van karnosien se voorgenoemde eienskappe, hou die topikale gebruik daarvan moontlike anti-verouderings voordele vir die vel in (Hipkiss, 1998:864).

Die buitenste laag van die vel is egter die belangrikste fisiese versperring vir topikale aflewering. Hierdie laag staan bekend as die stratum corneum en word dikwels na verwys as die tempo-bepalende versperring (El Maghraby *et al.,* 2008:203-206; Wickett & Visscher, 2006:98-106). Die intersellulêre spasies gevul met ‘n lipofiele matriks en korneosiete in die stratum corneum dra by tot die uitdaging om ‘n hidrofiele aktiewe bestanddeel topikaal af te lewer (Goebel *et al.,* 2012:281-286; Venus *et al.,* 2011:471-474).

Karnosien is ongelukkig ’n hidrofiele verbinding met ongunstige eienskappe vir topikale aflewering. Die belangrikste fisies-chemiese eienskappe vir suksesvolle topikale aflewering sluit die oktanol-water verdelingskoëffisiënt (log P), molekulêre grootte en wateroplosbaarheid van die verbinding in (Benson & Watkinson, 2012). Die ideale eienskappe vir suksesvolle topikale aflewering is ’n log P van 1 tot 3, ’n molekulêre grootte van minder as 500 Da en ’n wateroplosbaarheid van meer as 1 mg/ml (Khalid *et al.,* 2016:129; Naik *et al.,* 2000:319). Karnosien het ’n molekulêre grootte van 226.23 Da (MSDS, 2013). Die wateroplosbaarheid in fosfaatbufferoplossing (PBS, pH 7.4) by 25 °C is bepaal as 122.804 ± 0.716 mg/ml, terwyl die oktanol-buffer verdelingskoëffisiënt (log D) bepaal is as - 2.891 ± 0.013 in PBS (pH 7.4).

As gevolg van karnosien se ongunstige log D, word ongunstige veldiffusie voorspel en bykomende metodes word genoodsaak om permeasie te verbeter. Vesikelsisteme is reeds vanaf die 80s gebruik om deurlaatbaarheid deur die vel te verbeter (Nasir *et al.,* 2012:484). Vesikelsisteme bestaan uit kolloïdale deeltjies met ’n hidrofiele kop en ’n hidrofobiese stert (Honeywell-Nguyen & Bouwstra, 2005:67-74). Nie-ioniese surfaktant gebaseerde vesikels, ook bekend as niosome, is vesikels wat beide hidrofiele en lipofiele aktiewe bestanddele kan enkapsuleer (Nasir *et al.,* 2012:479-487; Uchegbu & Vyas, 1998:33-70). Die gebruik van niosome bied heelwat voordele vir die topikale aflewering van aktiewe bestanddele. Hierdie

voordele sluit verhoogde permeasie van die stratum corneum, verlengde deponering in die vel en verminderde sistemiese absorpsie in (Mali *et al.,* 2013:587). Tydens die aflewering van kosmetiese verbindings, verbeter niosome ook die stabiliteit en biobeskikbaarheid van die verbindings (Nasir *et al.,* 2012:484). Proniosome is die droë vorm van niosome en is meer stabiel (Kumar & Rajeshwarrao, 2011:214). Die poeier word gewoonlik maklik gehidreer met die waterfase indien dit benodig word (Marianecci *et al.,* 2013:71).

Niosome kan ongelukkig slegs as 'n pre-formulering gebruik word as gevolg van onstabiliteit, swak visuele voorkoms en lae viskositeit. Volgens Barry (2007:595), moet topikale produkte aanvaarbaar wees vir pasiënte. Pasiënte verkies gewoonlik produkte wat maklik oorgedra word na die vel, maklik versprei oor die vel, geen residu agterlaat en laastens, maklik verwyder kan word. Om ten volle te baat by die voordele en om pasiëntmeewerkendheid te verseker, moet die niosome in 'n waardige semi-soliede doseervorm gebruik word. Stahl (2015:209-218) klassifiseer semi-soliede doseervorms as jelle, rome, salwe en pastas. Hierdie doseervorme het 'n beter viskositeit en sal vir 'n langer tydperk op die vel bly; gevolglik sal die kans vir diffusie van die aktiewe bestanddeel deur die formulering in die vel in vergroot word (Stahl, 2015:209-218).

Niosome en proniosome is as pre-formulerings gebruik tydens hierdie studie. Die optimale karnosienkonsentrasie vir die gebruik in die pre-formulerings iss bepaal as 3%. Die eienskappe van die pre-formulerings is bepaal om kwaliteit te verseker. Die vesikelgrootte, polidispersie indeks (PdI), zeta-potensiaal, pH, viskositeit en enkapsuleringseffektiwiteit (EE%) is onder andere bepaal. Behalwe vir die lae viskositeit, het die pre-formulerings algehele goeie eienskappe gehad. Twee semi-soliede formulerings, 'n jel en 'n room, is gevolglik geformuleer vanuit die niosome. Die niosome was die verkose pre-formulering as gevolg van algehele beter karakteristieke en 'n makliker en vinniger voorbereidingsmetode.

Membraanvrystellingseksperimente, transdermale diffusie eksperimente en kleefbandafstropingseksperimente is op al vier die formulerings uitgevoer. Die membraanvrystellingseksperimente het bewys dat karnosien vrygestel is uit al die formulerings. Die niosome het die hoogste mediaan konsentrasie vloed waarde (1 139.10 µg/cm^2.h), gevolg deur die proniosome, die jel en laastens die room. 'n Effens negatiewe invloed van die formulerings op die aflewering vanuit die pre-formulerings is opgemerk. Na afloop van die transdermale diffusie eksperimente, is gevind dat karnosien nie deur die vel gediffundeer het nie, maar wel in die stratum korneum-epidermis (SCE) en epidermis-dermis (ED) gedeponeer het. Die jel het die grootste mediaan konsentrasie karnosien (2.458 µg/ml) gedeponeer in die SCE, gevolg deur die niosome, proniosome en laastens die room. Die niosome het die grootste konsentrasie (2.465 µg/ml) karnosine in die ED afgelewer, gevolg deur die jel, room en laastens die proniosome. Die niosome was die voorstaande pre-formulering en die jel die voorstaande

semi-soliede formulering. Die mediaan waardes is verkies bo die gemiddelde waardes omdat dit die data beter voorstel en nie deur uitskieters beïnvloed word nie (Dawson & Trapp, 2001:30; Gerber *et al*., 2008:190).

Die twee semi-soliede doseervorms het ook versnelde stabiliteitstoetse vir drie maande ondergaan. Die *International Conference on Harmonisation* (ICH) se riglyne is gevolg tydens die stabiliteitstoetse. Veranderinge is beoordeel tydens versnelde (40 ± 2 °C/75 ± 5% RH (relatiewe humiditeit)), intermediêre (30 ± 2 °C/60 ± 5% RH) en lang termyn (25 ± 2 °C/60 ± 5% RH) bergingstoestande (ICH, 2003:3). Die maandelikse stabiliteitstoetse het konsentrasiebepalings van die aktiewe bestanddeel en preserveermiddels, pH, konduktiwiteit, viskositeit en zeta-potensiaalmetings, massaverliesbepalings en mikroskopiese en makroskopiese ondersoeke ingesluit. Na afloop van die stabiliteitstoetse is gevind dat nie een van die twee produkte voldoende stabiliteit het om vervaardig te word nie.

Sleutel woorde: Karnosine, niosome, semi-soliede, stabiliteit, vel, *in vitro*

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CHAPTER 1

INTRODUCTION AND PROBLEM STATEMENT

1.1 Introduction

L-carnosine was first described by Gulevitsch and Amiradgibi in the 1900s (Fedorova *et al.,* 2009:62). The dipeptide of the amino-acids β-alanyl and L-histidine is a compound with numerous clinical benefits due to its antioxidative, antiglycating and neuroprotective properties (Hipkiss *et al.,* 2002:285-294; Kyriazis, 2010:45). This compound is normally found in muscle and brain tissue and is destroyed by an enzyme called carnosinase (Kyriazis, 2010:45).

According to Bellia *et al.* (2014:2299-2316) carnosinase is the catalyst during the hydrolysis reactions of carnosine and homocarnosine. This enzyme was first described and named in 1949 by Hanson and Smith after being isolated from porcine kidney. Two types of this enzyme, human serum carnosinase and human tissue carnosinase, were identified in 2003. Carnosine mainly serves as a substrate for human serum carnosinase under normal physiological conditions.

In this study, the research focus will be the formulation and topical delivery of niosomes containing carnosine. Successful topical delivery of carnosine can have possible anti-ageing benefits for the skin. Cellular ageing is a process often characterised by multiple physiological changes (Hipkiss *et al.,* 2002:285; Kyriazis, 2010:45-47). Oxidation, glycation and asparagines deamination are all part of the modifications that result in ageing proteins and it has been proved that carnosine may be involved in all three of these changes (Hipkiss *et al.,* 2002:285-291).

Although some antioxidant mechanisms of carnosine are still being studied, it was found carnosine has a chelating activity on metals, an inactivating effect on reactive oxygen species (ROS) and can also scavenge free radicals (Babizhayev, 2006:2343-2355; Kyriazis, 2010:45-47). A positive effect on human fibroblasts was also discovered when carnosine increased their lifespan and reversed the appearance of senescent cells (Babizhayev, 2006:2344).

Besides the strong antioxidant properties of carnosine, it also shows a number of outstanding antiglycating properties (Kyriazis, 2010:45-47). Due to the preferred glycation positions in its structure, carnosine is capable of decreasing and scavenging protein carbonyl groups and malondialdehyde, therefore reducing the number of advance glycation end-products (AGEs) formed (Hipkiss *et al.,* 2002:285-292; Kyriazis, 2010:45-47).

The word integument originated from '*integere'*, the Latin for 'to cover' (Venus *et al.,* 2011:471). The skin, formally known as the integument, is the body's largest organ making up 16% of a person's body weight (Goebel *et al.,* 2012:281; Venus *et al.,* 2011:471). Covering the whole body, it has two major functions, namely to protect the body against the outside environment and to prevent loss of water resulting in dehydration (Wickett & Visscher, 2006:98-100). The skin consists of three major layers, namely the hypodermis, the dermis and the layered avascular epidermis (El Maghraby *et al.,* 2008:203-206). The epidermis can be divided into the stratum basale, stratum spinosum, stratum granulosum and the stratum corneum (Venus *et al.,* 2011:471-474).

For the purpose of this study and thus the topical delivery of carnosine, the most important barrier to overcome is the stratum corneum, or the outermost layer. This layer is also considered the rate-limiting barrier (El Maghraby *et al.,* 2008:203-206; Wickett & Visscher, 2006:98-106). The structure of the stratum corneum is often best described by the brick-and-mortar model in which the corneocytes are the bricks and the layered lipids between the cells are the mortar (Wickett & Visscher, 2006:99-102). Both the intercellular spaces, filled with a lipophilic matrix and the corneocytes aligned in a scaffold-like framework, contribute to the significant challenge of topical delivery of hydrophilic compounds (Goebel *et al.,* 2012:281-286; Venus *et al.,* 2011:471-474).

According to Ademola (1997:511-534), topical formulations include gels, foams, sprays, creams, ointments, etc. These formulations are used to deliver active ingredients, in this case, cosmeceuticals, directly to the tissue under or around the application site. Local skin diseases are usually treated via the topical route. The bioavailability of topically applied active ingredients varies between 1% and 15%, and although systemic uptake is not ideal, it is normally unavoidable for most topical formulations. The application frequency cannot be determined precisely because of all the external factors affecting the amount of cosmeceutical delivered topically. These factors include inter-patient variations in rubbing or applying techniques, clothes removing some of the ingredients, evaporation of the ingredients and exposure to the environment. The release of active ingredients from the formulation can be summarised by partitioning of the active from the vesicle and passive diffusion of the active through the skin. Both the physicochemical properties of the vesicle and the skin and the interactions between the active and the skin can influence the release of the active ingredient.

According to Benson and Watkinson (2012), the physicochemical properties of the active to be considered for successful topical delivery include the octanol-water partition coefficient (log P), molecular size and aqueous solubility. The hydration status of the skin can also influence topical delivery. The ideal log P of an active for topical delivery is 1 to 3, whereas the general rule for molecular size is normally less than 500 Dalton (Da). For the topical delivery of a

cosmeceutical active, both the lipophilic and hydrophilic properties are important. The active needs to have efficient lipophilic properties to cross the stratum corneum and limited hydrophilic properties for targeted topical delivery. It has also been proved that a hydrated skin will usually be permeated more easily than dry skin. Carnosine has a log P of - 2.972 ± 0.436 and a molecular weight of 226.23 Da (Goebel *et al.,* 2012:281-287). Although the exact aqueous solubility is not yet determined, it is known that carnosine is soluble in cold water (MSDS, 2013).

As a result of the water solubility, the topical delivery of carnosine will be however challenging. This hydrophilic compound must therefore be incorporated into a vesicle in order to cross the lipophilic stratum corneum. Vesicles, often used as delivery systems, are colloidal particles consisting of a hydrophilic headgroup and a hydrophobic tail (Honeywell-Nguyen & Bouwstra, 2008:205-208). In this case, non-ionic surfactant based vesicles (niosomes) will be used to ease the topical delivery of carnosine.

Non-ionic surfactants were first brought together in vesicles by cosmetic researchers of L'Oreal in the seventies (Uchegbu & Vyas, 1998:33-70; Nasir *et al.,* 2012:479-487). Since then niosomes have been studied for their potential to act as drug carriers (Uchegbu & Vyas, 1998:33-70). Niosomes are analogous to liposomes and can encapsulate amphiphilic or lipophilic actives (Nasir *et al.,* 2012:479-487; Uchegbu & Vyas, 1998:33-70). These multilamellar or unilamellar vesicles are obtained on hydration of non-ionic surfactants to encapsulate the aqueous solution in a bilayer (Nasir *et al.,* 2012:479-487; Uchegbu & Vyas, 1998:33-70). The bilayer of niosomes and liposomes differ because niosomes are formed from non-ionic surfactants and liposomes are formed from double-chain phospholipids (Marianecci *et al.*, 2013:65-90). Cholesterol is sometimes added to either of them to ensure rigidity and shape (Tangri & Khurana, 2011:47-53).

Advantages of niosomes include (Nasir *et al.,* 2012:479-487; Tangri & Khurana, 2011:47-53):

- niosomes contain biocompatible and biodegradable surfactants;
- these surfactants used increase the stability of the vesicle, which then contributes to the stability of the active;
- niosomes can accommodate both amphiphilic and lipophilic actives;
- they are excellent vesicles to enhance skin penetration of the active; and
- they ensure targeted delivery of the active, function as depots for controlled release and improve performance of the active.

According to Marianecci *et al.* (2013:65-90), proniosomes are niosomes in a powder form. When non-ionic surfactants are used to coat water-soluble carriers, a dry formulation

(proniosomes) is obtained. Proniosomes are more stable than niosomes, resulting in easier storage for longer time-periods and are easily hydrated before use.

1.2 Research problem

The research problem of this study is best described by two different aspects: the hydrophilic properties of the active and vesicle systems only regarded as pre-formulations.

The problem described by the hydrophilic and lipophilic differences between the active ingredient and the stratum corneum is essential in this study. The active will only be of value if the delivery is successful. As stated above, the stratum corneum is a lipophilic barrier, which prevents hydrophilic compounds crossing the skin. Although carnosine has an optimal molecular size of 226.23 Da for topical delivery, it is water soluble and does not have an optimal log P (- 2.972 ± 0.436) (Goebel *et al.,* 2012:281-287). The active will be formulated into vesicles (niosomes and proniosomes) to investigate whether topical delivery will be improved. The vesicles will have both hydrophilic and lipophilic properties to ensure target site (dermis) delivery of the active.

Furthermore, the low viscosity, instability and poor visual appearance of vesicle systems increase the need for proper semi-solid dosage forms. Two semi-solid formulations will be formulated to enhance the visual appearance and to alter the viscosity and stability of the niosomes. The topical delivery from the formulations will be investigated and compared to the delivery from the pre-formulations.

1.3 Aim and objectives

The aim of this study is to incorporate carnosine into niosomes and proniosomes for topical delivery.

The objectives are as follow:

- Development and validation of a high performance liquid chromatography (HPLC) method to quantitatively determine the concentration of carnosine at different stages during the study;
- determine the aqueous solubility and octanol-buffer distribution coefficient (log D) of carnosine;
- formulation of niosomes and proniosomes as vesicles to entrap carnosine effectively for topical delivery;
- determine the characteristics of the vesicles;
- perform membrane studies to determine the release of carnosine from the vesicles;

- perform diffusion studies with skin, followed by tape stripping to determine if carnosine diffuses through and/or into the skin, respectively;
- formulation of semi-solid dosage forms including a niosome containing cream and a niosome containing gel;
- perform stability tests where the semi-solid formulations will be stored at long-term conditions (25 ± 2 °C/60 ± 5% RH (relative humidity)), intermediate storing conditions (30 ± 2 °C/60 ± 5% RH) and accelerated storage conditions (40 ± 2 °C/75 ±5 % RH).
- HPLC methods will be developed and validated to determine the concentrations of all the excipients together with the active ingredient used in the formulations.
- Evaluations including concentration assays, pH, conductivity, viscosity, zeta-potential, mass loss, microscopic analysis and macroscopic analysis will be conducted at zero, one, two and three months;
- perform membrane studies to determine the release of carnosine from the semi-solid formulations; and
- perform diffusion studies with skin followed by tape stripping to determine if carnosine diffuses through and/or into the skin, respectively.

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CHAPTER 2

FORMULATION AND TOPICAL DELIVERY OF SEMI-SOLID DOSAGE FORMS CONTAINING CARNOSINE ENCAPSULATED IN NIOSOMES

2.1 Introduction

The skin is often seen as an overall reflection of health and emotional well-being of a person and cosmetic products have been used from as early as the time of Cleopatra to beautify the skin (Schneider, 2008:462). According to *Transparency Market Research* (2014), the projected global anti-ageing market growth rate for 2013 to 2019 is 7.8%. This market will be worth approximately USD 191.7 billion by 2019. In the report, they also explain the large market for anti-ageing products and the great opportunities to introduce new highly efficient products in the near future. There is a fast growing eternal desire of human beings to increase their time on earth and to stay young for longer, or at least to continue blooming and looking younger (Binic *et al.,* 2013:1).

According to Wiechers (2008:1-18), there has been a remarkable trend since the 1990s to create biologically active cosmetic products. The efficacy of these products mainly depends on the intrinsic activity of the active, as well as the delivery of the active. The intrinsic activity is in general the functionality profile of the active, but it will only be guaranteed by successful delivery. Topical delivery of a product refers to the transport process of an active ingredient from the formulation to the site of action, which can be achieved by using a delivery system. The term “delivery system”, in general, refers to a method of transporting, holding and carrying an active ingredient (Schneider, 2008:462). Delivery is thought to be successful when sufficient concentrations of the active are delivered to the target site for a prolonged time-period for the transport process to take place. The intrinsic activity and the delivery are therefore of equal importance in the process of developing a biologically active and effective product.

The term cosmeceutical active is an informal term used to describe a substance that is applied on external parts of the body to provide a number of benefits (Schneider, 2008:462); this term is not recognised by the European Union Commission (EU) or the Food and Drug Administration (FDA) (Schneider, 2008:466). The benefits provided include, but are not limited to, cleansing, beautifying, altering appearance, perfuming and of course improving attractiveness (Schneider, 2008:463). Cosmeceuticals can be used on the nails, hair, skin and lips (Schneider, 2008:463).

Carnosine, with its anti-oxidation and anti-glycation properties, is the cosmeceutical active, which will be investigated during this study (Babizhayev, 2006:2344; Kyriazis 2010:46; McFarland & Holliday, 1999:36). It is a hydrophilic dipeptide with a very high functionality profile

to provide the skin with possible anti-ageing benefits. The hydrophilic properties result in a very low affinity for the skin and despite the low molecular mass of the molecule, it does not show good skin penetration (Wiechers, 2008:5). The possible benefits for the skin will be of no value if proper skin penetration cannot be reached.

Skin penetration can be enhanced in various ways, for instance formulating a pro-drug, using penetration enhancers or incorporating the active into a vesicle system. In this study, the focus will be to enhance penetration by making use of a vesicle system and by formulating a delivery vehicle containing the vesicles.

Non-ionic surfactant based vesicles, better known as niosomes, will be formulated in this study. Niosomes are microscopic vesicles with sizes ranging in the nanometric scale. Cosmetic researchers have focused on niosomes since the seventies and vesicle systems have been used commercially from as early as the late 1980s to improve skin delivery (Cevc, 2003:676; Nasir *et al.,* 2012:484). Niosomes are only considered as a pre-formulation used to incorporate into a semi-solid topical formulation, or final product.

Well-known topical delivery vehicles include gels, foams, creams, sprays, ointments etc. (Ademola, 1997:511-534). Amongst these vehicles, the creams and ointments are more traditional, but because of their greasiness and thick consistency, this has led to patient dissatisfaction (Kurian & Barankin, 2011:4). Newer vehicles such as sprays, foams and gels are being developed in order to improve efficacy, patient satisfaction and compliance and result in fewer side effects (Kurian & Barankin, 2011:4). Some of the rather important considerations when choosing a vehicle include skin type, patient preference and formulation aspects (Kurian & Barankin, 2011:4).

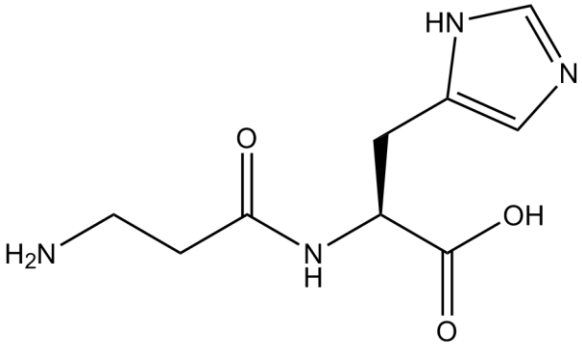
2.2 Carnosine

β-alanyl-L-histidine, better known as L-carnosine, is a dipeptide with the chemical formula $C_9H_{14}N_4O_3$ *(*MSDS, 2013). This naturally occurring compound was first described in the 1900s by two Russian scientists, Gulevitsch and Amiradgibi (Fedorova *et al.,* 2009:62). It is synthesised by carnosine synthase and is normally found in mammalian muscle and brain tissue (Hipkiss, 1998:863; McFarland & Holliday, 1999:35). Carnosinase is the enzyme responsible for the degradation of carnosine under normal physiological conditions (Hipkiss, 1998:863).

2.2.1 Physical and chemical properties of carnosine

The important physical chemical properties of carnosine follow in Table 2.1.

Table 2.1: The physical and chemical properties of carnosine retrieved from Goebel *et al.,* 2012:281-287, MSDS, 2013, Sigma-Aldrich, 2016 and Singh *et al.*, 2009:734.

Description	Specification
Physical appearance and state	Powdered white solid
Molecular formula	$C_9H_{14}N_4O_3$
Molecular structure	
Molecular weight (g/mol)	226.23
Partition coefficient (log P)	- 2.972 ± 0.436
Solubility (mol/l)	0.91 at 25 °C

2.2.2 Metabolic pathways of carnosine

According to Hipkiss (1998:863), carnosine is synthesised by carnosine synthase from β-alanyl and l-histidine. This is an adenosine triphosphate-dependant (ATP) reaction, which mainly takes place in brain and skeletal muscle cells, which explains the high concentration of carnosine present in these tissues. Furthermore, carnosinase is responsible for the degradation of carnosine and high concentrations of this enzyme are present in the serum and brain tissue. It is evident that the concentration of carnosinase in the body increases with age (Bellia *et al.,* 2014:2300). Carnosine, homocarnosine and anserine are the main substrates for carnosinase (Bellia *et al.,* 2014:2299).

Carnosinase is not a regular dipeptidase enzyme and forms part of the M20/M28 metalloproteases family (Boldyrev *et al.,* 2013:1817). As stated by Bellia *et al.* (2014:2299-2305), two isoforms namely, human serum carnosinase and human tissue carnosinase, are present in the human body. Human serum carnosinase has a higher specificity towards carnosine and homocarnosine, compared to the broad specificity of human tissue carnosinase towards various dipeptides. Carnosine will only serve as a substrate for human tissue carnosinase under non-physiological conditions, such as at pH 9.5.

According to Goebel *et al.* (2012:282), carnosine also functions as a substrate for a number of other enzymes in tissue. These enzymes are responsible for the biotransformation of carnosine into related substances via decarboxylation, methylation and acetylation. Carcinine, n-acetyl-l-carnosine and free amino acids (β-alanyl and l-histidine) are related to carnosine but do not function as substrates for carnosinase. Figure 2.1 is a representation of the synthesis and degradation reactions of carnosine (Bellia *et al.,* 2014:2301).

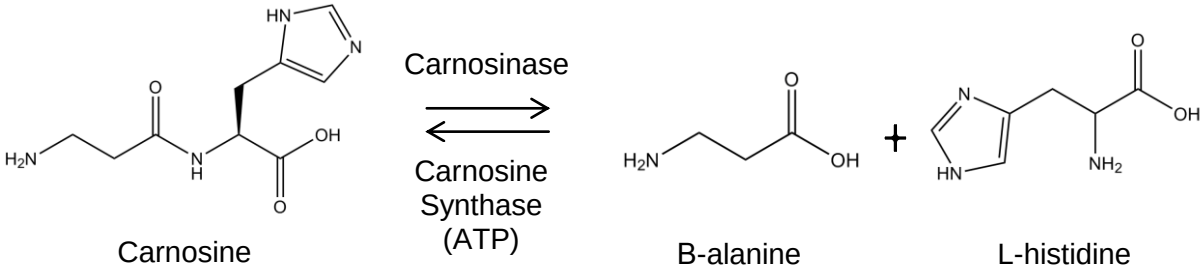


Figure 2.1: A schematic representation of the synthesis and degradation reactions of carnosine under normal physiological conditions (Bellia *et al.,* 2014:2301).

On-going research suggests it is possible to stabilise carnosine against degradation reactions (Kyriazis, 2010:45), for instance combining carnosine with vitamin E derivatives (Kyriazis, 2010:45). Carnosinase normally acts quickly to reduce the levels of carnosine in the serum, but there is very little evidence of the activity of carnosinase in the skin (Babizhayev, 2006:2343; Kyriazis, 2010:45).

2.2.3 Biological function of carnosine

Little was known about the numerous biological functions of carnosine at the time of first discovery (Kyriazis, 2010:45). Most of the functions have only been confirmed after extensive research from 1990 to 2010 and only now is carnosine considered as a compound with a wide variety of benefits for various areas of health (Kyriazis, 2010:45). Despite the differences in significance of the biological functions of carnosine, it is still generally recognised as a pluripotent compound (Kyriazis, 2010:48).

According to Babizhayev (2006:2343-2344), the main biological functions of carnosine include pH buffering, immunomodulation (mostly stimulation) and the ability to regulate enzymes - more specifically, carnosine buffer pH in muscles to prevent metabolic acidosis and enhance endurance during exercise (Kyriazis, 2010:46). Carnosine also consists of anti-oxidation and anti-glycation properties (Hipkiss, 1998:863; Kyriazis, 2010:45). The ability of carnosine to bind metals such as calcium, zinc and copper, contributes to the potential involvement in signal transduction and gene regulation (Hipkiss, 1998:864).

Due to the aforementioned functions, carnosine is being used commercially to protect the skin against the environment and as an overall anti-ageing supplement (Kyriazis, 2010:45). Possible benefits for the digestive system, cataracts and other degenerative diseases, such as ischemia and cardiovascular disease, neurodegeneration and loss of functional cells, have been reported (Kyriazis, 2010:45-48). Carnosine also enhances wound healing by, on the one hand, functioning as a histamine precursor and, on the other, by the stimulation of collagen biosynthesis (Boldyrev *et al.,* 2013:1827-1828). Furthermore, carnosine has potential therapeutic or prophylactic applications in Alzheimer's disease, cancer, liver cirrhosis and the secondary complications of diabetes, such as peripheral neuropathy, cataracts and kidney failure (Hipkiss, 1998:863-866).

According to Boldyrev *et al.* (2013:1820), it is evident that the biological functions of carnosine are not only different within one kind of tissue, but also differ amongst different tissues. The focus in this study will be on the anti-ageing function of carnosine in the skin when applied topically. Both oxidation and glycation are associated with ageing and therefore both the anti-oxidation and anti-glycation properties play an important role in the anti-ageing functions (Hipkiss, 1998:864).

2.3 Ageing

Ageing is a result of damage over time and usually leads to disturbed cellular, tissue and organ functions (Gkogkolou & Böhm, 2012:259). Cellular ageing, is a process often characterised by multiple physiological changes and can easily be identified by the increase in protein carbonyl groups originating from oxidation and glycation, as well as a decrease in proteasome activity (Hipkiss *et al.,* 2002:285-294; Kyriazis, 2010:45-49).

The multisystem degenerative ageing process starts from the moment humans are born and the skin is involved in this process (Sjerobabski-Masnec & Situm, 2010:515-519). Both the internal processes involved, as well as external stressors, contribute to ageing in skin (Gkogkolou & Böhm, 2012:259). Over time, the cell regeneration of the epidermal cells will slow down and the skin cells will become senescent (Khalid *et al.,* 2016:127). Wrinkles on our most visible organ are often a constant reminder of ageing (Binic *et al.,* 2013:1; Sjerobabski-Masnec & Situm, 2010:515-519). Despite the visible changes in appearance, ageing also affects the physiological functions, such as vitamin D synthesis, immune function and sweat production of the skin (Gkogkolou & Böhm, 2012:259). General skin care is becoming increasingly important to prevent cancer development, improve appearance as well as confidence; and due to all the processes involved in ageing, any anti-ageing compound must target several changes in order to be effective (Lorencini *et al.,* 2014:100-115; Kyriazis, 2010:45-49).

According to Sjerobabski-Masnec and Situm (2010:515-519), ageing can be divided into intrinsic ageing and extrinsic ageing. They described the two types as follow: the natural ageing process (intrinsic ageing) is a continuous process that usually starts in the mid-twenties, although it will not be visible yet. During the process, the collagen production in the skin slows down, elasticity is lost and the replacement of dead skin cells with new skin cells will also slow down. Visible signs of intrinsic ageing include fine wrinkles, thinning of the skin, lesser subcutaneous fat, dry skin, hair loss and unwanted hair, as well as insufficient sweat production due to a decrease in glands. Extrinsic ageing is the unnatural ageing process and is caused by numerous external factors, which include facial expressions and the movement of muscles, gravity, smoking, the way humans sleep and most importantly, sun exposure. With the loss of elasticity, fine lines form more easily which causes the skin to stop returning to the original state and visible wrinkles occur. Excessive exposure to the sun is the leading cause of photoageing, which is characterised by remarkable textural and pigmentary changes, which occur upon chronic exposure to sun. One of the first documented sun exposure effects was when the necks of sailors were compared to the necks of nuns (Giacomoni & Rein, 2004:180).

Visible ageing can therefore result from both oxidation and glycation; and a flattened dermal-epidermal junction is the most distinct biological feature of aged skin (Binic *et al.,* 2013:1; Sjerobabski-Masnec & Situm, 2010:515-519). The clinical presentations and typical appearance of chronologically (intrinsic) aged and photoaged (extrinsic) skin are easily distinguishable, but it is evident that the two processes share some substantial molecular features (Binic *et al.,* 2013:2). These features include changes in signal transduction, damage to connective tissue and a decrease in collagen synthesis (Binic *et al.,* 2013:2).

2.3.1 The role of oxidative stress in ageing

Preiser (2012:147) defines oxidative stress as an imbalance between decreased anti-oxidant activity and increased levels of reactive oxygen species (ROS). ROS, such as hydrogen peroxide, singlet oxygen and hypochlorous acid, are well known oxidative stressors. The different mechanisms involved in the production of ROS are represented in Figure 2.2.

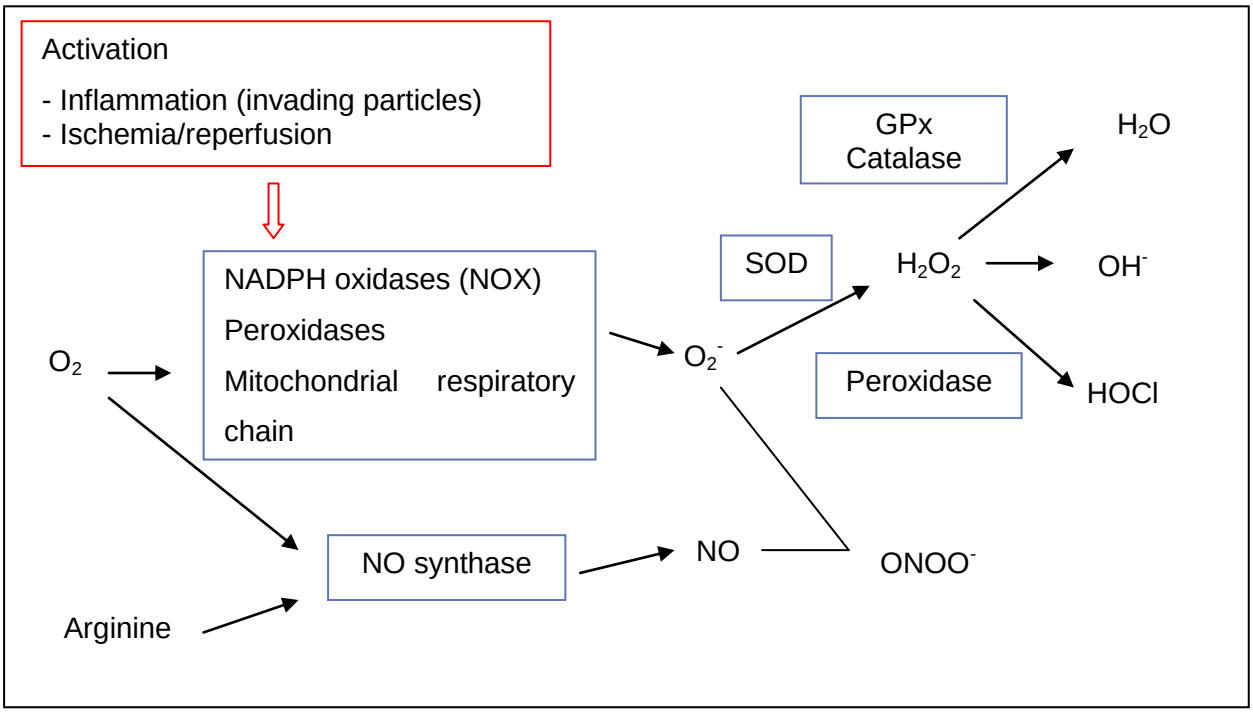


Figure 2.2: A schematic representation of the different mechanisms involved in the production of reactive oxygen species. Oxygen (O_2) is converted by one of the enzymes in the upper rectangle to superoxide. Hydrogen peroxide (H_2O_2) is then formed spontaneously by dismutation of superoxide via superoxide dismutase enzyme (SOD). Hydrogen peroxide can then be converted to water (H_2O), hydroxyl (OH^-) or hypochlorous acid (HOCl), depending on the reaction conditions and catalysts. Nitric oxide (NO^-) is formed when oxygen and arginine are combined by NO synthase. The powerful ROS, preoxynitrite ($ONOO^-$), is formed after a spontaneous reaction between superoxide and nitric oxide (Preiser, 2012:147).

Oxidative stress is one of the leading causes of ageing in skin. During photoageing, ultraviolet (UV) light penetrates the skin to have an effect on different cells depending on the wavelength of the light and the depth of the cells (Berneburg *et al.,* 2000:240). Shorter UV wavelengths (UVB, 280 – 320 nm) penetrate the epidermis to compromise epidermal cells and longer wavelengths (UVA 320 – 400 nm) penetrate deeper to affect both the dermal fibroblasts and epidermal cells (Berneburg *et al.,* 2000:240). The UV light can also be absorbed by melanin

pigmentation and as a result, individuals with darker skin will be more protected against photo damage than their fair-skinned counterparts (Berneburg *et al.,* 2000:240).

UV radiation (280 – 400 nm), due to sun exposure, is the predominate cause of oxidative stress, but other environmental factors, such as pollution and food preservatives, also leads to ROS synthesis (Binic *et al.,* 2013; Kozina *et al.,* 2013:18-22; Terra *et al.,* 2012:34-41). Both UVA and UVB light are responsible for ROS synthesis, but UVB light also interacts directly with deoxyribonucleic acid (DNA) to cause damage (Berneburg *et al.,* 2000:240).

Uncontrolled ROS production easily leads to levels that exceed the skin's own anti-oxidant systems which then causes excessive ROS to build up in the skin (Kozina *et al.,* 2013:18-22; Ou-Yang *et al.*, 2009:65). Increased levels of ROS can cause cellular damage by reacting with collagen cross-links (Ou-Yang *et al.,* 2009:65). ROS therefore plays a major role in UV-induced skin damage, such as photoageing, melanogenesis, immunomodulation and photocarcinogenesis (Peres *et al.,* 2011:93-97).

2.3.2 The role of carnosine as an anti-oxidant

An anti-oxidant is any agent with the ability to inhibit oxidation reactions (Oxford English Dictionary). According to Babizhayev (2006:2344) and Kyriazis (2010:46), carnosine is a molecule with outstanding anti-oxidation properties and is involved in a number of different anti-oxidation mechanisms. These mechanisms include the chelating activity on pro-oxidative metals and the ability to scavenge ROS and free radicals. Carnosine is also able to scavenge products of lipid peroxidation and when added to previously oxidised lipids, it reduces the concentration of thiobarbituric acid reactive substances (TBARS) (Babizhayev, 2006:2344; Kyriazis, 2010:46).

Ageing is generally correlated with oxidation-induced damage on a macromolecular level and as a result, this strong anti-oxidant substance can protect biological tissue and reverse cell senescence (Babizhayev, 2006:2344; Hipkiss *et al.,* 2002:286). It is also evident that carnosine can increase the lifespan and extend the Hayflick limit of cultured fibroblasts (Babizhayev, 2006:2344; Hipkiss, 1998:864). The Hayflick limit refers to the maximum limit of cell division (Hipkiss, 1998:864).

2.3.3 The role of glycation in aging

Kyriazis (2010:45-49) defines the non-enzymatic glycosylation process (glycation) as a reaction between aldehyde molecules or aldoses and free amino groups in protein. It occurs in the following three steps: cross linking of aldoses to proteins or DNA to form either abnormal protein-protein bonds or protein-DNA bonds, the formation of AGEs, due to accumulation of abnormal proteins and finally the reaction of AGEs with free radicals to result in degenerative

diseases related to ageing. The cross-links between aldoses and proteins, which form during glycation, affect the suppleness of the dermis and cause increased stiffness of the skin (Gasser *et al.,* 2011:366; Pageon *et al.,* 2014:169).

The formation of AGEs due to glycation of proteins is a complex multistep reaction also known as the Maillard reaction (Gkogkolou & Böhm, 2012:259-265). During this reaction, the carbonyl groups in sugars react with amino groups of amino acids to form an unstable Schiff base, which can rearrange to form a more stable Amadori product (Gkogkolou & Böhm, 2012:260). This Amadori product is a ketoamine (Gkogkolou & Böhm, 2012:260). The formation of Schiff bases and Amadori products are reversible reactions, but a further irreversible reaction with amino acid residues of proteins leads to the formation of AGEs, which usually gather in collagen and elastin molecules in the extracellular matrix of the skin (Gkogkolou & Böhm, 2012:260; Pageon *et al.,* 2014:169).

Glycation is recognised as an important mechanism of ageing in skin because it affects the slow-renewing tissue in the dermis to alter skin homeostasis (Pageon *et al.,* 2014:169). The altered homeostasis can lead to cell senescence, as well as altered gene expression and protein synthesis (Pageon *et al.,* 2014:169). According to Draelos and Pugliese (2011:444), glycation can be correlated with accelerated ageing in skin, yellowing of the skin, poor circulation and decreased suppleness of the dermis.

2.3.4 The role of carnosine as an anti-glycation agent

Carnosine is an anti-glycating agent with the ability to inhibit non-enzymatic glycosylation of proteins (McFarland & Holliday, 1999:36). The amino groups with proximal carboxyl and/or imidazole groups in their structure act as preferred glycation sites (Hipkiss 1998:864; Hipkiss *et al.,* 2002:286). During the glycation reaction, carnosine reacts with keto- or aldehyde groups to remove sugars and prevent further reactions with terminal amino groups in proteins to prevent the formation of AGEs (Kyriazis, 2010:46; McFarland & Holliday, 1999:36). Protein-carbonyl groups and cross-links that form during glycation reactions are well known age-related modifications in the skin (Hipkiss 1998:864). It is evident that glycated carnosine is not a mutagenic agent and will not cause harm (Hipkiss 1998:864). Figure 2.3 explain possible outcomes of the reactions between carnosine and glycated proteins.

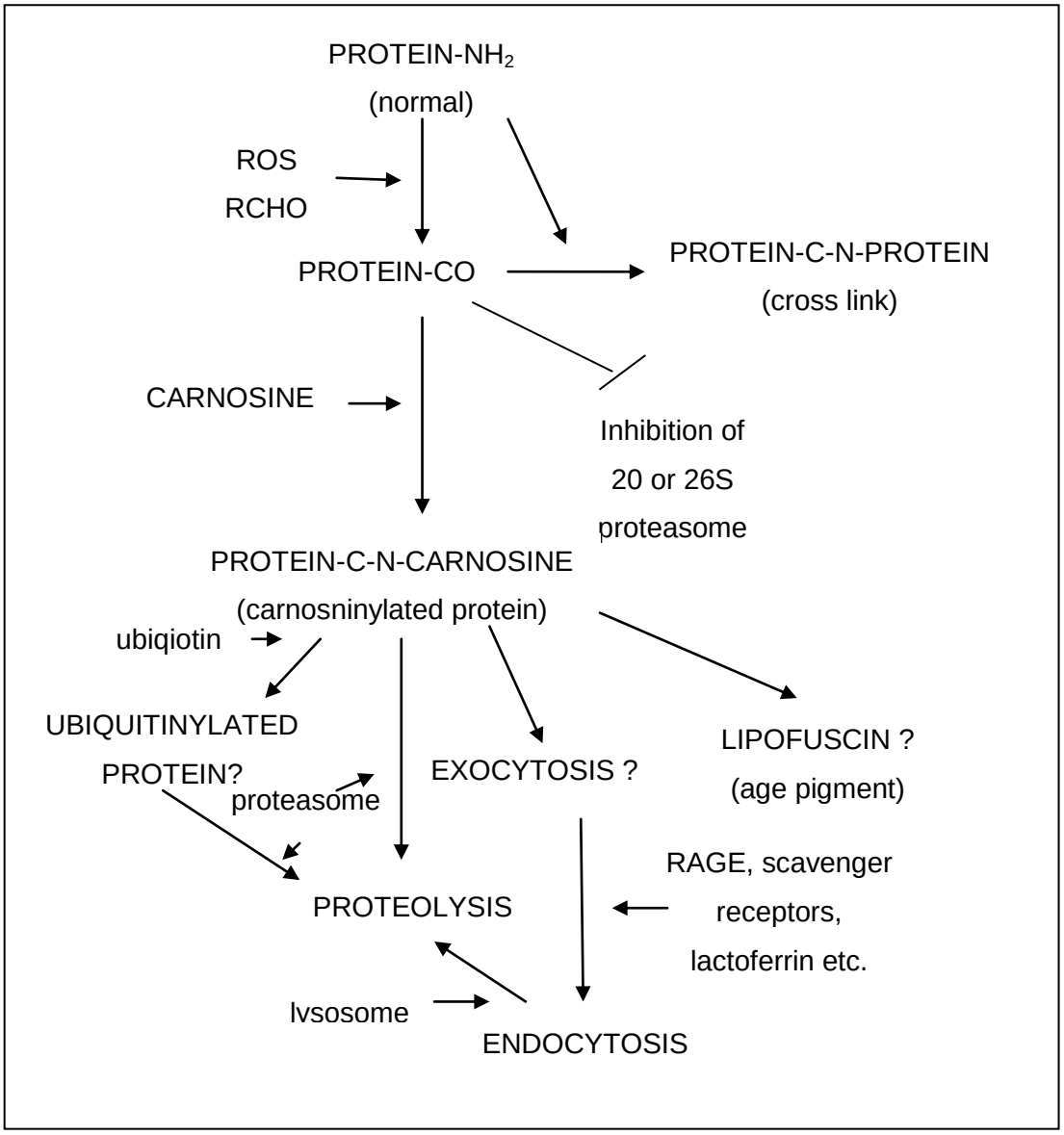


Figure 2.3: A schematic representation of the possible outcomes of reactions between carnosine and glycated proteins. ROS: reactive oxygen species; RCHO: reactive aldehydes and ketones; Protein-Co: protein carbonyl (Hipkiss & Brownson, 2000:220).

2.4 The skin

The skin is the largest organ of the human body and forms part of the integumentary system, the organ system responsible for protection of the body (Baroni *et al.*, 2012:257-262; Oláh *et al.*, 2012:67-75). Covering the whole body, it makes up approximately 16% of the total body weight (Wickett & Visscher, 2006:98-110; Oláh *et al.*, 2012:67-75). According to Wickett and Visscher (2006:98), the skin has two major functions, namely to prevent dehydration due to water loss and to protect the body against various external agents. Other important functions include sensory and immune functions, regulation of body temperature and vitamin D synthesis.

2.4.1 Layers of the skin

Varying between 0.05 and 2 mm in thickness, the human skin is divided in several different layers (Foldvari, 2000:417). The three well-known layers include the hypodermis, which is a subcutaneous layer of adipose tissue; the dermis mostly consisting of connective tissue; and the epidermis composed out of terminally differentiated squamous epithelium (El Maghraby *et al.,* 2008:203-206). The different layers of the skin are illustrated in Figure 2.4. Various different cell-types contribute to different functions in each layer of the skin (Ng & Lau, 2015:3).

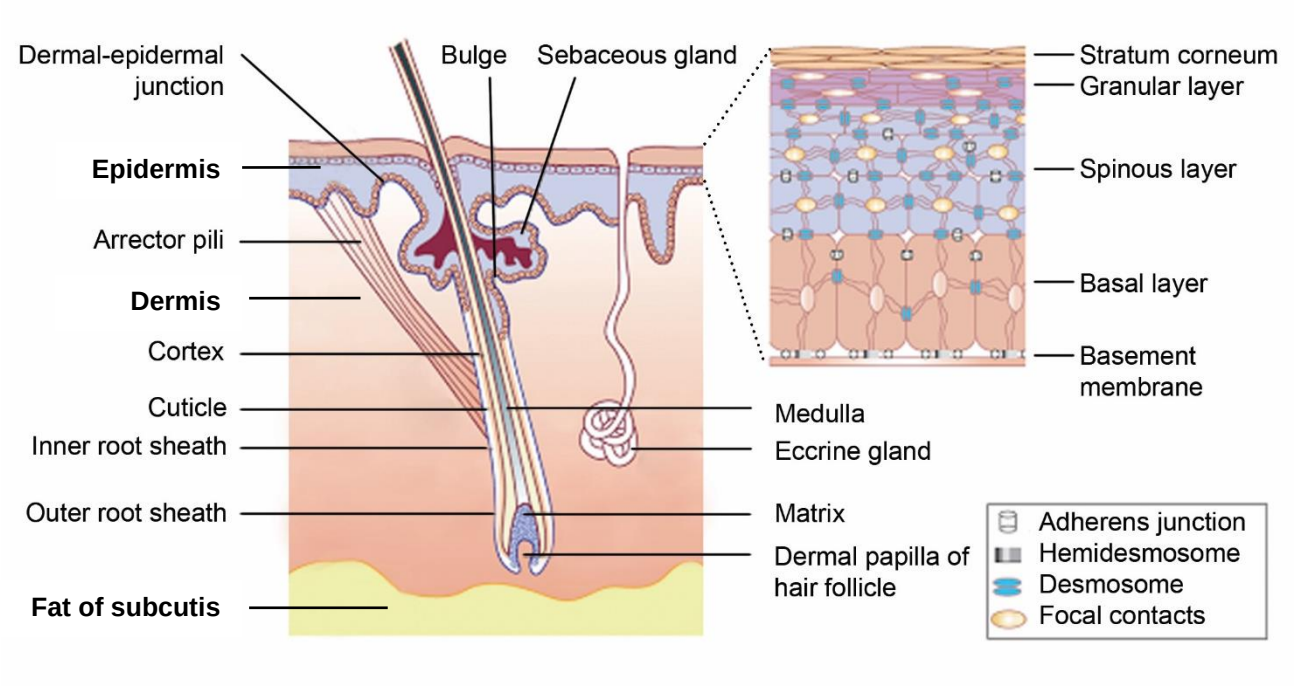


Figure 2.4: An illustration of the different layers in the skin with its appendages (McGrath *et al.,* 2004).

2.4.1.1 The dermis and hypodermis

According to Baroni *et al.* (2012:260), the dermis and hypodermis are the most important skin layers to prevent thermal and traumatic abuse. The dermis is an elastic acellular layer containing multiple blood vessels, nerve endings and lymphatic vessels and hosts the sweat glands, sebaceous glands and hair follicles. Protein fibres, such as collagen and elastin, are mostly responsible for the suppleness and elasticity of this layer. The main cell types present in this layer are fibroblasts, which are responsible for continuous renewal of the extracellular matrix, and macrophage, which eliminates foreign bodies. The hypodermis is the subcutaneous fat layer and is important in energy storage and metabolism.

2.4.1.2 The epidermis

The epidermis is the top multi-layered area of the skin (Wickett & Visscher, 2006:99). Keratinocytes are the main cell type of the epidermis and have two main functions: synthesising keratin and producing cytokines in case of injury (Venus *et al.,* 2011:471-474). Keratin is described in the Medical Dictionary Online (2012) as a type of fibrous protein representing, amongst others, the primary constituent of the nails, hair and epidermis. Other cell types in the epidermis include pigment donating melanocytes, Langerhans' cells consisting of immunological functions, epidermotropic lymphocytes, dendritic T cells and Merkel cells (Foldvari, 2000:418; McGrath *et al.,* 2004). The epidermis can be divided into the stratum basale, stratum spinosum, stratum granulosum, stratum lucid and the stratum corneum (Wickett & Visscher, 2006:98-110).

The stratum granulosum is an essential component of the epidermis and some key transformations take place in this layer to form the stratum corneum barrier (Wickett & Visscher, 2006:98-110). Keratohyalin granules filled with protein and lamellar bodies containing lipids are the two types of granules formed in the stratum granulosum (Wickett & Visscher, 2006:98-110). Cells in the stratum granulosum are then transformed into corneocytes, also known as squames, to form the stratum corneum (Wickett & Visscher, 2006:98-110).

2.4.2 Barrier function of the stratum corneum

The stratum corneum is the outermost layer of the epidermis and forms the main physical barrier, whereas chemical barriers include hydrolytic enzymes, acids, lipids and antimicrobial peptides (Baroni *et al.,* 2012:258; Jepps *et al.*, 2013:152-168). According to Ng and Lau (2015:3), the stratum corneum is approximately 10 to 20 µm thick and consists of flattened corneocytes, or squames, that lack nuclei and cytoplasmic organelles. The 'brick and mortar' model is often used to describe the structure and barrier function of the stratum corneum. In this model, the corneocytes filled with keratin filaments are the bricks and the extracellular matrix, consisting of lipids, peptides and proteins, is the mortar.

Menon *et al.* (2012:4) describe several different components that form part of the stratum corneum barrier. These crucial components include:

- corneocytes which function as the main physical barrier;
- corneodesmosomes responsible for connecting and linking the corneocytes;
- lipids between the corneocytes consisting of cholesterol, ceramides and fatty acids which function as the permeability barrier;
- proteolytic and lipolytic enzymes responsible for degradation reactions and other biochemical activities; and

- pro-barrier lipids between the stratum granulosum and stratum corneum.

Despite all the barrier properties, it is still possible for active ingredients to overcome the stratum corneum and penetrate into or through the skin (Zhang *et al.,* 2009:227). An important condition for successful transdermal delivery is to understand the different penetration mechanisms and the methods to enhance penetration (Benson & Watkinson, 2012).

2.4.3 Penetration pathways through the skin

According to Bhowmick and Sengodan (2013:636-638), there are two main penetration pathways through the skin, namely the epidermal route and the trans-follicular pathway. The choice of pathway mainly depends on the characteristics of the formulation and the physicochemical properties of the active.

Bhowmick and Sengodan (2013:636-638) describe the routes as follow:
The epidermal route is directly across the intact stratum corneum via transcellular or paracellular mechanisms. Transcellular mechanisms include passive transport, active transport, endocytosis and transcytosis of molecules through the cells, depending on the molecular size and polarity of the molecules. Intercellular and intracellular routes form part of the paracellular route, depending on the partition coefficient of the molecules.
The trans-follicular route is the transport of molecules through appendages such sweat glands, hair follicles and sebaceous glands. This is not a remarkable route, for only 0.1% of the skin consists of glands and follicles, but recent studies showed potential in the absorption of nano-carriers through these appendages.

2.4.4 Important physico-chemical properties for skin penetration

According to Bhowmick and Sengodan (2013:638), there are a series of steps contributing to the permeation process of an active ingredient through the skin. The steps are as follow: release of the active from the dosage form, partitioning into the stratum corneum followed by diffusion through the stratum corneum, partitioning into the epidermis and finally diffusion into cutaneous layers. This whole process is highly dependent on the physicochemical properties of the active as well as some important biological factors.

The four main physicochemical factors are the log P of the active, the molecular mass of the active and the number of hydrogen-bond donors and hydrogen-bond acceptors of the active (Bolzinger *et al.,* 2012:157). The log P represents partitioning between the stratum corneum and water, whereas the molecular mass will determine the diffusion coefficient (Bolzinger *et al.,* 2012:157). Furthermore, the hydrogen-bond donors and -acceptors control interactions between the active and the surface of the skin (Bolzinger *et al.,* 2012:157). According to

Pouillot *et al.* (2008:146), the main biological factors include skin hydration, age and skin metabolism.

Molecules with a lower mass (less than 600 Da) and optimal log P (2 to 3) will penetrate the skin more easily (Barry, 2001:102; Benson & Watkinson, 2012). It is also important for the formulation to contain a high concentration of the active to ensure a maximum diffusion driving force, and for the skin to be hydrated to enhance permeation (Barry, 2001:102; Benson & Watkinson, 2012). There is evidence that the amount of lipids in the skin decreases with age and hydrophilic compounds can penetrate aged skin more easily (Khalid *et al.,* 2016:128). Delivery of the compound contributes to the efficacy of the product and optimisation of the formulation is of great importance.

2.5 Topical delivery of carnosine

Honeywell-Nguyen and Bouwstra (2005:67) define dermal delivery as the topical application of active ingredients to treat skin diseases. Dermal delivery leads to the advantage of localising increased drug concentrations and preventing systemic uptake leading to systemic adverse effects. Other advantages, such as increased patient compliance, easy access to this non-invasive route, pain avoidance and quick withdrawal of the drug in case of adverse effects, contribute to the importance of the transdermal route (Jepps *et al.,* 2013:153; Zhang *et al.,* 2009:227).

Transdermal penetration is one of the most important considerations to ensure the therapeutic usefulness of topical peptides such as carnosine (Khalid *et al.,* 2016:128). Due to the significant functionality profile of carnosine, the focus in this study will be to enhance skin penetration during topical delivery. Increased concentrations of the active localised in the skin will result in the prevention and treatment of skin ageing. There are some physical properties considered rather important to allow the penetration of peptides into the skin (Table 2.2).

Table 2.2: The physical properties of proteins to allow skin permeation compared to the properties of carnosine (Goebel *et al.,* 2012:281-287; Khalid *et al.,* 2016:129; MSDS, 2013; Singh *et al.*, 2009:734).

Property	Ideal range	Carnosine
Molecular weight	< 500.00 Da	226.23 Da
Log P	1 - 3	- 2.972 ± 0.436
Melting point	< 200 °C	253 °C
Aqueous solubility	> 1 mg/ml	0.91 mol/l at 25 °C (205.869 mg/ml)
Hydrogen bonding groups	< 4	7

As seen in Table 2.2, the molecular weight and aqueous solubility of carnosine will promote skin permeation, but the log P, melting point and hydrogen-bonding groups may cause some trouble. A vesicle system will be used to overcome the possible challenges.

2.6 Vesicle systems

2.6.1 Niosomes

The first therapeutic agent, econazole, entrapped in a vesicle for dermal delivery was commercialised in the late 1980s (Cevc, 2003:676). According to Honeywell-Nguyean and Bouwstra (2005:67-68), vesicular systems are one of the most controversial methods for dermal delivery. Vesicles are colloidal particles consisting of an amphiphilic bilayer with water-filled centres. Non-ionic surfactant based vesicles, better known as niosomes, which are similar to liposomes but more stable, are vesicular systems prepared from synthetic surfactants and other amphiphilic molecules, such as cholesterol. These vesicles can carry both hydrophilic and lipophilic active ingredients.

Nasir *et al.* (2012:480) described the membrane additives, non-ionic surfactants and cholesterol, as principal components during niosome preparation. Non-ionic surfactants, such as Tween® (Polyoxyethylenesorbitan monolaurate) and Span® (Sorbitan monostearate), are the most important components of niosomes and consist of a hydrophilic head and a hydrophobic tail. The waxy substance, cholesterol, enables vesicle formation and provides stability to the niosomes by reducing aggregation of particles. It is also used to provide the niosomal bilayer with orientational order and rigidity.

According to Mali *et al.* (2013:587), the use of niosomes to encapsulate drug molecules for topical delivery can increase penetration of the stratum corneum, prolong the residence time of active ingredients in the skin and reduce systemic absorption. In cosmetic delivery, niosomes also enhance skin penetration, increase the stability of the active and improve the bioavailability (Nasir *et al.,* 2012:484). These factors contribute to an increase in bioavailability and a decrease in side effects.

The effectiveness of a vesicle depends on the physicochemical properties, including size, thermodynamic state, bilayer elasticity, lamellarity and charge (Honeywell-Nguyen & Bouwstra, 2005:68). These properties can change according to the production method used (Azeem *et al.,* 2009:686-687).

According to Marianecci *et al.* (2014:188), a nano-carrier should meet the following requirements to be successful:

- contain only non-toxic biodegradable, biocompatible and bioexcretable substances;

- have the capacity to carry high drug loads;
- provide targeted delivery of active ingredients;
- prevent premature release of the active ingredient; and
- provide controlled release of the active ingredient at the targeted site.

Due to the stability, low cost, formulation with non-toxic substances and the ability to enhance skin permeation, as well as targeted delivery, niosomes show potential for the dermal delivery of cosmeceutical actives (Nasir *et al.,* 2012:479-489; Uchegbu & Vyas, 1998:33-70).

2.6.2 Proniosomes

Proniosomes are often described as a dry form of niosomes (Kumar &Rajeshwarrao, 2011:214) and are produced when water-soluble carriers are covered with non-ionic surfactants (Marianecci *et al.,* 2014:192). The surfactants include substances such as Span®, Tween® and sucrose stearate, whereas the water-soluble carriers are usually sugars such as glucose, lactose monohydrate, maltodextrin and sorbitol (Marianecci *et al.,* 2013:71). According to Marianecci *et al.* (2013:71), the proniosomes are easily hydrated with a warm aqueous phase and light agitation to produce niosomes. The hydration temperature needs to be higher than the phase transition temperature.

Advantages of proniosomes include (Kumar & Rajeshwarrao, 2011:214; Marianecci *et al.,* 2013:71; Marianecci *et al.,* 2014:192):

- they are more stable than niosomes and prevent fusion and aggregation of vesicles;
- they prevent leaking of active ingredients resulting in increased entrapment efficiency of active ingredients;
- they can be processed further into forms such as beads and tablets;
- they can be stored for longer time periods (in air-tight containers at 4 °C); and
- they are easily hydrated before use.

2.7 Semi-solid dosage formulations

Semi-solid preparations are often formulated to improve stability, compatibility and patient compliance (Barry, 2002:528). These preparations include gels, creams, ointments and lotions containing dissolved and suspended active ingredients (Mahato, 2007:183). Creams and ointments are often referred to as traditional formulations, while gels, foams and sprays are newer formulations developed to improve patient acceptability (Kurian & Barankin, 2011:4). Creams and gels are the semi-solid dosage forms chosen as delivery vehicles in this study.

Creams are semi-solid preparations for topical application (Barry, 2007:594-595). It can be prepared form either oil-in-water emulsions (o/w) or water-in-oil emulsions (w/o) (Mahato, 2007:186). Reflection of light from the emulsified phases results in the creamy appearance (Mahato, 2007:186). The o/w creams are well known as vanishing creams (Barry, 2007:594-595). These creams are rubbed into the skin, resulting in evaporation of the continuous phase and an increased concentration of the active ingredient in the film on the skin (Barry, 2007:594-595).

Gels are semi-solid preparations rich in liquid with a continuous structure providing solid-like features (Barry, 2007:593). Synthetic macromolecules, cellulose derivatives and natural gums are generally used as gelling agents (Mahato, 2007:186). Gels are prepared by adding the appropriate gelling agent to an aqueous solution of the active ingredient (Mahato, 2007:186).

2.8 Skin metabolism

Topically applied active ingredients will be subject to biotransformation due to all the active enzymes responsible for degradation of compounds in skin tissue (Zhang *et al.,* 2009:227). A number of cutaneous enzymes present in the skin are responsible for the degradation and metabolism of both endogenous and exogenous substances resulting in altered pharmacological effects (Zhang *et al.,* 2009:227). Examples of these enzymes are flavin-containing monooxygenases, proteases, esterases/amidases and of course, the main cytochrome P450 (CYPs) enzymes including, but not limited to, 1A1, 1A2, 2A6, 2B6 and 2C9 (Zhang *et al.,* 2009:228).

According to Zhang *et al.* (2009:227), the epidermis, with a lot of on-going cutaneous CYPs activity, has been identified as a primary area of metabolism in the skin. The most important Phase 1 CYP enzymes are localised in the keratinocytes, hair follicles and their associated sebaceous glands and are mainly responsible for catalysing hydrolysis, oxidation and reduction reactions. The hydrolysis reaction of carnosine by the enzyme known as carnosinase takes place under normal physiological conditions, although specific activity of this enzyme in the epidermis was not yet reported (Babizhayev, 2006:2343). It is important to remember the extent to which active ingredients become metabolised in the skin will be determined by both the type of substance and the enzymes involved (Zhang et al., 2009:227).

2.9 Summary

In this study, carnosine will be the active used for topical delivery. Due to the unfavourable physico-chemical properties poor skin permeation is expected and the active will be encapsulated into niosomes and proniosomes to attempt to enhance permeation. As a result of the instability, low viscosity and unattractive visual appearance, niosomes and proniosomes are

only considered as pre-formulations. A niosome cream and niosomes gel will be formulated as semi-solid formulations to attempt to improve these aforementioned properties and possibly increase patient compliance.

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CHAPTER 3

ARTICLE FOR PUBLICATION IN DIE PHARMAZIE

Chapter 3 is written in article format, for the purpose of submission for publication in **Die Pharmazie**. The author guidelines and restrictions of this journal followed are attached in Appendix H. The paragraphs were justified to ease the reading. This chapter is written in US English.

Formulation, stability testing and topical delivery of carnosine encapsulated in niosomes

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Abstract

The aim of this study was to evaluate the skin permeation of carnosine and the effect that different formulations containing carnosine have thereon. Carnosine was formulated into two pre-formulations (niosomes and proniosomes) and two semi-solid topical formulations (niosome gel and niosome cream). The niosomes were prepared according to two different methods, firstly by using the thin-film hydration method and secondly, by hydrating proniosomes. The optimal concentration carnosine was determined to be 3%. Membrane release studies proved that carnosine was released from all four of the preparations and would therefore be available for diffusion through the stratum corneum. The niosomes had the highest median flux (1 139.10 µg/cm^2.h), followed by the proniosomes (1 096.20 µg/cm^2.h), the gel (796.41 µg/cm^2.h) and finally, the cream (378.64 µg/cm^2.h). Transdermal diffusion and tape stripping experiments followed the membrane release experiments, proving that topical delivery was obtained and the four preparations targeted the stratum corneum-epidermis (SCE) and epidermis-dermis (ED). None of the preparations reached the receptor phase, which indicates the active was concentrated in the skin layers. The gel delivered the highest median concentration carnosine (2.46 µg/ml) to the SCE, followed by the niosomes (1.66 µg/ml), proniosomes (1.16 µg/ml) and finally, the cream (1.05 µg/ml). The niosomes delivered the highest median concentration carnosine (2.46 µg/ml) to the ED, followed by the gel (1.37 µg/ml), the proniosomes (0.96 µg/ml) and lastly, the cream (0.92 µg/ml). Another aim of this study was to perform accelerated stability

tests on the semi-solid formulations for a period of three months. The International Conference on Harmonisation (ICH) Guidelines were followed and formulation changes were assessed during accelerated (40 ± 2 °C/75 ± 5% RH (relative humidity)), intermediate (30 ± 2 °C/60 ± 5% RH) and long-term (25 ± 2 °C/60 ± 5% RH) storage conditions (ICH 2003). The semi-solid formulations did not meet the ICH requirements for stability, therefore could not be considered suitable for manufacturing.

Keywords: Carnosine, niosomes, proniosomes, semi-solids, stability, skin, *in vitro*

1 Introduction

Ageing is a multisystem degenerative process that starts from the moment humans are born (Sjerobabski-Masnec and Situm 2010). It affects the skin and since the skin is the most visible organ, humans are constantly aware of the ageing process (Binic *et al*. 2013). The scientific interest to reduce the appearance of ageing and the tendency to use less invasive procedures such as natural products, instead of laser rejuvenating techniques and plastic surgery, have increased in the last decade (Binic *et al*. 2013). Carnosine is a naturally occurring compound with possible anti-ageing benefits for the skin due to anti-oxidation and anti-glycation properties (Hipkiss 1998). It also has the ability to regulate epidermal protection against solar radiation (Lorencini *et al*. 2014). According to Wiechers (2008), the efficacy of the active ingredient depends on both the intrinsic activity and the delivery thereof. Successful topical delivery is therefore essential in order to benefit from its' anti-ageing properties.

The stratum corneum is the outermost layer of the skin and the main physical barrier to overcome during topical delivery (Baroni *et al*. 2012). It is generally considered as the rate-limiting barrier and consists of corneocytes aligned in a scaffold-like framework and lipid-enriched intercellular spaces (El Maghraby *et al*. 2008; Goebel *et al*. 2012; Venus *et al*. 2011; Wickett and Visscher 2006).

Three main physicochemical properties of the active that must be considered during topical delivery are the octanol-water partition coefficient (log P), molecular size and aqueous

solubility (Benson and Watkinson 2012). Carnosine is a hydrophilic active with a highly unfavorable log P (- 2.97 ± 0.46), and despite the favorable aqueous solubility (205.9 mg/ml at pH 7.0 and 25 °C) and molecular size (226.23 g/mol), poor skin permeation can be predicted (Goebel *et al.* 2012; MSDS 2013; Singh *et al.* 2009). One approach to improve dermal and transdermal delivery is by making use of vesicle systems (Honeywell-Nguyean and Bouwstra 2005).

Vesicle systems are pre-formulations, generally used to improve skin permeation. Niosomes are non-ionic surfactant based vesicles with the ability to encapsulate amphiphilic and lipophilic molecules (Nasir *et al*. 2012; Uchegbu and Vyas 1998). The use of niosomes as pre-formulations provide advantages, including increased penetration of the stratum corneum, prolonged residence time of the active in the skin and reduced systemic absorption of the active (Mali *et al*. 2013). In cosmetics, the stability and bioavailability of the active are also increased (Nasir *et al*. 2012). Proniosomes are a dry form of niosomes, produced by coating water-soluble carriers, such as sorbitol and maltodextrin with non-ionic surfactants (Kumar *et al*. 2011; Marianecci *et al*. 2014). They are easily hydrated upon usage with the aqueous phase (Marianecci *et al*. 2014).

Unfortunately, considering the low viscosity, instability and visual appearance, niosomes cannot be used as a dosage form. A proper semi-solid dosage form is needed to benefit from the aforementioned advantages and to increase the stability and patient compliance. According to Kurian *et al*. (2011), creams and ointments are traditional semi-solids, whilst gels, sprays and foams are newer formulations with improved patient satisfaction and compliance, improved efficacy and fewer side effects.

The aim of this study was to deliver carnosine topically successfully. Two pre-formulations, niosomes and proniosomes, and two semi-solid formulations, a cream and a gel containing carnosine encapsulated in niosomes, were prepared. The delivery of carnosine from the pre-formulations and semi-solid formulations was investigated and compared. Furthermore, the two formulations underwent stability tests, since stability is a major determinant before manufacture.

2 Investigations, results and discussions

2.1 Analytical method for the analysis of carnosine in study samples

The HPLC method was successfully validated in terms of linearity, accuracy, precision, ruggedness, robustness, specificity, limit of detection (LOD = 0.08675 µg/ml) and lower limit of quantification (LLOQ = 0.17350 µg/ml), range 0.17 – 141 µg/ml. The method proved to be adequately reliable and sensitive for the determination of carnosine in study samples.

2.2 Aqueous solubility of carnosine

The aqueous solubility of carnosine in phosphate buffer solution (PBS) at pH 7.4 and 25 °C was determined to be 122.8 ± 0.7 mg/ml. The aqueous solubility determined by Singh *et al*. (2009) was 205.9 mg/ml at 25 °C with pH 7.0 and 52.0 mg/ml at pH 8.0. The difference between the experimental value and the value obtained from literature could be due to the pH differences. A molecule with an aqueous solubility of at least 1 mg/ml will be able to undergo passive skin diffusion (Naik *et al.* 2000). Judged according to aqueous solubility, carnosine could be a good candidate for topical delivery, but unfortunately, a single parameter cannot be utilized to predict topical delivery.

2.3 Octanol-buffer distribution coefficient (log D) determination of carnosine

The log D of carnosine was determined to be - 2.89 ± 0.01 in PBS (pH 7.4) at 25 °C. This value correlates well with literature, as the log P value of carnosine retrieved from Goebel *et al*. (2012), was - 2.97 ± 0.46. Compounds with a log P of 1 to 3 are expected to travel across the skin easily (Khalid *et al*. 2016). The log D of carnosine (- 2.89) is unfavorable for skin permeation and interventions might be needed to improve skin permeation.

2.4 Preparation of the pre-formulations

The pre-formulations were prepared by the thin-film hydration method and from hydrating proniosomes. The niosomes was a visually attractive white milky dispersion. It was slightly oily and no large particles or sediment was visible. The proniosomes was also a white milky dispersion with a slightly thicker consistency compared to the niosomes, with no visible particles or sediment noticed. The formulas used are indicated in Table 1.

2.5 Characterization of the pre-formulations

The morphology of the vesicles includes the formation, shape and size of the droplets. The niosome and proniosome droplets were mostly spherical with sizes in the nanometric scale. The proniosome droplets were slightly larger than the niosome droplets and a smaller amount had formed compared to the vast amount of niosome droplets. The micrographs obtained from the transmission electron microscope (TEM) proved that vesicles had formed in both of the pre-formulations. The niosomes are seen in Fig. 1 and the proniosomes in Fig. 2. The characteristics of the niosomes and proniosomes are summarized in Table 2.

The average size of the niosome and proniosome droplets were 360.20 ± 15.5 nm and 391.77 ± 33.91 nm, respectively. The niosomes and proniosomes had an average Polydispersity Index (PdI) of 0.722 ± 0.031 and 0.590 ± 0.044, respectively. According to Marianecci *et al*. (2013), a PdI of less than 0.400 generally indicates a monodispersed formulation. The PdI of both dispersions was poor and above 0.400 (Marianecci *et al*. 2013). Despite these poor values, Gaumet *et al*. (2008) found no linear correlation between the PdI value and the monodispersity of the preparation.

According to Malvern Instruments (2015) and Marianecci *et al*. (2013) the zeta-potential is an indication of the surface charge of the droplets in dispersion. The surface charge will affect the dispersions' stability and in general, a zeta-potential of lower than - 30 mV or higher than + 30 mV indicates good stability. The average zeta-potential of the niosomes and proniosomes was - 59.80 ± 0.51 mV and - 45.73 ± 1.68 mV, respectively. Both of these values were below - 30 mV and indicated excellent stability (Marianecci *et al*. 2013).

The niosomes and proniosomes had an average pH of 8.08 and 8.11, respectively. The ideal pH for topical preparations is between five and nine, therefore the dispersions were regarded suitable for topical use as the pH values were within the acceptable range (Naik *et al*. 2000).

The viscosity of the niosomes and proniosomes were 3.0 cP and 4.3 cP, respectively. The vesicle systems had a very low, but constant viscosity. Since viscosity and rate of phase separation is inversely proportional, phase separation may possibly occur due to the low

viscosities, and these two preparations were incorporated into semi-solid formulations, which altered the viscosity and consequently increased the stability (Roland *et al*. 2003). The constant pH and viscosity values amongst preparations were also indicative of good quality of the vesicle systems (Roland *et al*. 2003).

The niosomes and proniosomes had a %entrapment efficiency (EE%) of 46.7 ± 1.0% and 44.5 ± 0.3%, respectively. According to literature, the EE% varies amongst studies. The niosomes prepared by Bayindir and Yuksel (2010) and proniosomes prepared by Ammar *et al*. (2011) had EE% ranging from 12.1% to 96.6% and 22.4% to 92.0%, respectively.

2.6 Formulation of semi-solids containing carnosine encapsulated in niosomes

The cream (see Table 3) had a homogenous texture and an off-white color with no particular smell. It applied easily and was not too oily, but needed some rubbing for complete evaporation. The gel (see Table 3) had a bright white color and applied easily, leaving the skin feeling moist and light. Almost no rubbing was necessary as the excess fluid evaporated swiftly. The gel had a characteristic odor.

2.7 Analytical method for assay analysis of active ingredient and excipients in the topical formulations

The HPLC method was validated in terms of linearity, accuracy, precision, ruggedness, robustness and specificity. The method proved to be reliable and sensitive for the determination of excipients during assays.

2.8 Stability testing of semi-solid formulations

The concentration assay results generated during the three months are summarized in Tables 4 and 5 for the cream and gel, respectively; the rest of the stability parameters follow in Tables 6 and 7.

According to Barnes (2007), starting with an excess of ingredients is a simple strategy to increase the shelf life of products. Two important requirements for using this strategy are the dose of the product must not be critical and the degradation products must not be toxic. Vitamins and other natural products generally meet these requirements. Since no monograph for a carnosine containing cream or gel was available, a USP monograph for

vitamin E was utilized. Vitamin E and carnosine are both naturally occurring compounds and are therefore comparable. The vitamin E monograph indicated that the final product must contain no less than 95% and not more than 120% of the labeled amount (USP 2016). The concentrations in both the cream and the gel fluctuated during the three months and followed more or less the same patterns. The fluctuations could be attributed to poor mixing during bulk formulation. The ingredients showed an overall decrease in concentrations, but because of the excess at the start of the testing period, the final concentrations of all the ingredients remained between 95% and 120% of the labeled amount, as required by USP (USP 2016) (See Tables 4 and 5).

According to Hach Company (2010), the pH value expresses the ratio between the hydrogen ions [H^+] and hydroxide ions [OH^-] in a solution. This ratio will remain constant in a stable solution; consequently, a change in the pH value of a formulation suggests a disturbance of the [H^+] and instability. The pH values of all the cream samples (see Table 6) decreased over time. The pH of the gel samples (see Table 7) also decreased but remained more stable than the cream. The decreases in pH are indicative of [H^+] and [OH^-] concentration variations, possibly due to water release from the formulations (condensation) (Hach Company 2010).

According to Lamba *et al*. (2015), electrical conductivity is associated with charged ions and indicates to what extent a preparation will allow an electrical current to pass through it. Ferreira *et al*. (2010), explains that the character of a formulation can be evaluated by the electrical conductivity procedure and formulations with an aqueous nature will present high conductivity values. Conductivity changes can be the result of any phase separation or release of encapsulated active ingredient (Lamba *et al*. 2015). The conductivity of the cream (see Table 6) showed an overall decrease after three months. The condensation that took place, possibly led to a smaller amount of free water in the formulation, and possibly contributed to the decrease in conductivity values (Korać *et al*. 2014). The conductivity of the gel (see Table 7) showed an overall increase after three months. This increase could possibly be attributed to the release of encapsulated carnosine or phase separation in the

formulation (Lamba *et al*. 2015). Furthermore, the pH changes suggesting a change in ion concentrations could possibly have led to a higher concentration of charged ions, resulting in the increase in conductivity values of the gel (Hach Company 2014-2015). The variance between the conductivity values of the cream (Table 6) and the gel (Table 7) reveal the aqueous nature of the gel (higher overall conductivity values) and the nature of the cream (lower overall conductivity values) (Ferreira *et al*. 2010). Despite the oily nature, the cream still consists of an aqueous phase enabling electrical conductivity.

All the samples revealed an increase in viscosity during the test period. In order to understand viscosity, other comparable viscosities must be brought into consideration. For instance, the viscosity of golden syrup is 100 000 cP and of honey, 10 000 cP (Barnes *et al*. 1989). The 90% difference between the two substances seems significant, but upon physical examination, the golden syrup is only slightly thicker than the honey. Therefore, after considering the aforementioned comparisons and upon physical examination of the cream and gel samples (see Tables 6 and 7) at all the time intervals, the viscosity did not differ notably during the three-month testing period.

According to Lamba *et al*. (2015), the zeta-potential indicates the surface charge of each particle in a formulation and determines whether the particles will repel each other with sufficient force to prevent aggregation. Consequently, zeta-potential determinations are an easy way to evaluate stability of formulations. Repulsive forces will exceed attractive forces of particles if a formulation has a highly positive or negative zeta-potential (Malvern Instruments, 2015; Marianecci *et al*. 2013). However, if the zeta-potential is low, aggregation might occur and particles will cluster together (Malvern Instruments 2015). A zeta-potential lower than - 30 mV or higher than + 30 mV is indicative of a stable formulation (Malvern Instruments 2015). All the samples' results remained highly negative (see Tables 6 and 7), suggesting the repulsive forces exceeded the attractive forces and that the particles in the formulations remained dispersed and deflocculated (Roland *et al*. 2003).

There were only small, but acceptable mass variations (see Tables 6 and 7), indicating that the containers used for storage of the products sealed well, therefore limiting evaporation and moisture absorption during the three month testing period.

The freshly prepared cream samples were smooth, off-white, semi-solid formulations with no particular smell. Macroscopic analysis of the samples revealed no texture change, phase separation or change in odor during this three-month period. The color of the samples became slightly darker and went from off-white to light yellow. The freshly prepared gel samples were light, foamy and bright white formulations with a characteristic smell. Macroscopic analysis of the samples revealed texture change and possible phase separation during the three-month testing period. The gel samples went from being light and smooth to irregular with visible transparent Carbopol® pieces, which disappeared upon mixing, in the formulation. The color of the samples became slightly darker and went from bright white to off-white, however the odor remained unchanged. Finally, visible water droplets on the lids of the cream and gel containers proved that water evaporated from the formulations and that there was water-loss from the final products.

Microscopic analysis (10 X magnification) did not reveal any substantial changes regarding the structure of the cream emulsion. A 40 X magnification showed no visible flocculation of the droplets, but slight crystal formation after three months. Microscopic analysis (10 X magnification) of the gel revealed significant changes. Visible air bubbles, which had completely disappeared during the three-month testing period, suggested that the degassing method was unsuccessful. The 40 X magnification revealed crystal formation and suggested that carnosine crystallized during the testing period.

Finally, after considering all the stability parameters (see Tables 4, 5, 6 and 7), the two products could not be considered stable and therefore not suitable for manufacturing.

2.9 Diffusion studies

2.9.1 Membrane release studies

The aim of the membrane release experiments was to determine if carnosine was released from the preparations. Fig. 3 indicates the flux values of the preparations and includes the

average, median and the minimum and maximum flux values for each preparation. The niosomes had the best release with a median flux of 1 139.1 µg/cm^2.h. The proniosomes and the gel followed with median fluxes of 1 096.2 µg/cm^2.h and 796.4 µg/cm^2.h, respectively, while the cream revealed only a small amount of carnosine was released (median flux of 378.6 µg/cm^2.h). The median values were slightly lower than the average values in all the cases, except for the niosomes, where it was slightly higher. This was preferred, as it was a more accurate representation of the skewed data (Dawson & Trapp, 2001; Gerber *et al.*, 2008). A much bigger difference between the pre-formulations and the semi-solid formulations were observed than between proniosomes and niosomes or the gel and the cream. This suggested that the formulations (cream and gel) had a negative influence on the carnosine release from the pre-formulations (niosomes and proniosomes). Regardless of the amounts, all the preparations revealed successful carnosine release, and release of the active could be ruled out if poor topical/transdermal diffusion was revealed.

2.9.2 Transdermal diffusion studies

The aim of the diffusion studies was to determine whether transdermal and/or topical delivery were reached and to investigate the differences between the preparations. None of the four preparations reached the receptor phase. Due to the focus of this study, being topical and not transdermal delivery, the preferred outcome was reached and no carnosine was delivered in the blood stream (receptor phase).

2.9.3 Tape stripping

Fig. 4 indicates the results obtained from the tape strip experiments. Carnosine was targeted in the stratum corneum-epidermis (SCE) and the epidermis-dermis (ED) by all four preparations. Twelve hours after application, the gel delivered the highest median concentration carnosine (2.5 µg/ml) to the SCE, followed by the niosomes (1.7 µg/ml), proniosomes (1.2 µg/ml) and the cream (1.1 µg/ml). The niosomes delivered the highest median concentration carnosine (2.5 µg/ml) to the ED, followed by the gel (1.4 µg/ml), the proniosomes (1.0 µg/ml) and lastly the cream (0.9 µg/ml). The niosomes delivered a much higher concentration to the ED than to the SCE, while the gel delivered a much higher

concentration to the SCE than to the ED; the other 2 preparations (proniosomes and cream) delivered more or less the same amount of carnosine to both the SCE and the ED. Fig. 5 indicates the concentrations of carnosine delivered to the SCE and ED and includes the median, average, as well as minimum and maximum values. One outlier in the data obtained after application of the niosomes could be observed.

The niosomes and the gel have proven to be the best pre-formulation and semi-solid formulation for targeting topical delivery, respectively. Better topical delivery of these two preparations could be attributed to better release of the active from them. When taking the release into consideration (Section 2.9.1), no substantial difference between the niosomes and proniosomes was noticed, but the gel revealed much higher release than the cream. Better release of carnosine resulted in a higher concentration delivered to the stratum corneum and since diffusion is proportional to the concentration gradient, a higher concentration at the stratum corneum provided for a higher concentration gradient, which finally resulted in improved diffusion (Barry 2007). The aqueous nature of the gel also possibly contributed to better skin permeation. An increased hydration status of the stratum corneum will result in increased skin permeability and water is a natural skin hydrator (Benson and Watkinson 2012). The aqueous nature of the gel hydrated the stratum corneum and increased topical delivery.

Furthermore, the better topical delivery of the niosomes compared to the proniosomes could be attributed to the characteristics of the vesicles (Table 1). Firstly, the EE% of the niosomes was slightly better than the proniosomes (See 2.5) and better transdermal diffusion was expected. Secondly, the niosome dispersion consisted of a vast amount of small droplets, compared to the small amount of large droplets in the proniosome dispersion (Figs. 1 and 2, respectively). According to Williams (2003), the size of molecules and transdermal flux are inversely proportional and consequently smaller droplets will be expected to penetrate the skin better than larger droplets. The generous amount of small niosome droplets provided for a big membrane surface of entrapped carnosine, enabling many of the droplets to reach the stratum corneum at once and release carnosine.

307 **2.10 Data analysis**

308 Non-parametric tests were performed on the flux data obtained from the membrane release
309 studies after 6 h. Significant differences were revealed with the Kruskal-Wallis test,
310 consequently, a multiple comparisons test was performed to determine where these
311 significant differences lay. There was no significant statistical difference between the gel
312 and the cream ($p = 0.419$), or between the niosomes and the proniosomes ($p = 1.000$).
313 Statistical significant differences ($p < 0.05$) were found between a) the cream and the
314 proniosomes ($p = 0.000$), b) the cream and the niosomes ($p = 0.000$), c) the gel and the
315 proniosomes ($p = 0.007$) and d) the gel and the niosomes ($p = 0.048$).

316 Non-parametric tests were performed on the data obtained from the SCE and ED after 12 h
317 diffusion studies. The Kruskal-Wallis test revealed no significant statistical differences in the
318 ED ($p = 0.156$), but significant statistical differences in the SCE ($p = 0.042$). Furthermore,
319 the Mann-Whitney test indicated there were no statistical differences between the SCE and
320 ED for the niosomes ($p = 0.676$), the cream ($p = 0.791$) and the gel ($p = 0.345$), but the
321 proniosomes had a significant statistical difference between the SCE and ED ($p = 0.023$).

322 **3 Experimental**

323 **3.1 Analytical method for the analysis of carnosine in study samples**

324 An Agilent® 1200 Series HPLC system (Agilent Technologies, United States of America),
325 equipped with an Agilent® 1200 pump, diode array detector, autosampler injection system
326 and a vacuum degasser was used to perform the HPLC analysis. The system is equipped
327 with ChemStation Rev. A.10.02 software for data analysis. A Venusil® ASB C_8 column (250
328 x 4.6 mm) was used as stationary phase. The mobile phase consisted of acetonitrile and
329 0.075 M octane sulphonic acid at pH 3.45 (20/80%). A flow rate of 1.0 ml/min, detection
330 wavelength of 210 nm and injection volume of 10 µl were maintained.

331 **3.2 Aqueous solubility determination of carnosine**

332 A test tube containing a magnetic stirrer and 5 ml of the chosen buffer (PBS at pH 7.4) was
333 placed in a preheated water bath (25 °C). An excess amount of carnosine was then added
334 to the test tube and stirred continuously for 24 h at 25 °C. After this period, the samples

were removed and filtered through 0.45 µm polytetrafluoroethylene (PTFE) filters. The solution was diluted in a ratio of 1 ml to 100 ml PBS (pH 7.4) before HPLC analysis. The experiment was done in triplicate.

3.3 Octanol-buffer distribution coefficient determination of carnosine

Co-saturation of the *n*-octanol phase and the PBS phase occurred after equilibration of equal volumes of *n*-octanol and PBS (pH 7.4) for 24 h. The pre-saturated PBS (pH 7.4) phase was used to prepare a carnosine solution by adding approximately 60 mg carnosine to 3 ml PBS (pH 7.4), to which was added 3 ml pre-saturated *n*-octanol phase. The solution was placed in a shaker bath at 32 °C for 12 h and centrifuged. The PBS (pH 7.4) and *n*-octanol solutions were diluted 1 ml to 20 ml with PBS (pH 7.4) and methanol, respectively, before HPLC analysis. The experiment was done in triplicate.

3.4 Preparation of niosomes and proniosomes

The oil phase of the niosomes was prepared by dissolving cholesterol and Span® 60 with chloroform in a glass beaker. The solution was then placed on a magnetic stirrer in a hood. A controlled temperature of 60 °C was maintained for the chloroform to evaporate. The water phase of the niosomes was then prepared by dissolving carnosine in HPLC grade water. Once all the chloroform had evaporated from the oil phase, the mixture formed a lipid layer on the bottom of the beaker. The water phase was then added and stirred for approximately 2 min at a low temperature. The final mixture was allowed to cool down before being sonicated, on ice, for 1 min. The oil phase of the proniosomes was prepared by dissolving the cholesterol and Span® 60 in chloroform. The sorbitol was weighed into a 50 ml beaker and placed on a magnetic stirrer, in a hood, as a dry powder. The chloroform solution was added drop wise to the sorbitol to prevent over wetting and to ensure proper evaporation of the chloroform. The formulation was placed in a desiccator overnight to ensure complete dryness. Upon usage, the water phase was prepared by dissolving the carnosine in HPLC grade water. The dry powder obtained previously was placed on the magnetic stirrer and the water phase was added and allowed to stir for approximately 5 min.

362 The final mixture was allowed to cool down well before being sonicated on ice for 1 min.
363 The final niosome and proniosome formulas are indicated in Table 1.

364 **3.5 Characteristics of niosomes and proniosomes**

365 **3.5.1 Morphology of the vesicles**

366 A TEM FEI Tecnai G2 (FEI, Holland) at 120 Kv was used to prove the formation of the
367 vesicles and to analyze the morphology. One drop of the dispersion was diluted with 3 ml
368 HPLC grade water, where after a drop of the diluted solution was placed on a copper grid
369 (carbon-coated 300 mesh). Osmium was used to oxidize the lipids while the fluid phase
370 evaporated. After complete evaporation, the sample was colored with two heavy metals,
371 uranyl acetate and lead citrate. The first heavy metal was used to induce binding sites for
372 the second heavy metal to bind and color the sample. The excess fluid was removed by
373 filter paper and the grid was allowed to air dry completely. Only the pre-formulations
374 containing no carnosine were used to determine the morphology of the vesicles, as there
375 was a risk the carnosine might crystallize, which could possibly damage the TEM.

376 **3.5.2 Vesicle size and polydispersity index (PdI)**

377 A Malvern Zetasizer Nano (Nano SZ) (Malvern Instruments, United Kingdom) was used to
378 determine the droplet size and PdI of the pre-formulations at 25 °C. The samples were
379 diluted (0.5 ml/20.0 ml) with purified water to reduce particle interactions. The experiment
380 was done in triplicate.

381 **3.5.3 Zeta-Potential**

382 A Malvern Zetasizer Nano (Nano SZ) (Malvern Instruments, United Kingdom) was used to
383 determine the zeta potential of the pre-formulations at 25 °C. The samples were diluted (0.5
384 ml/20.0 ml) with purified water to reduce particle interactions. The experiment was done in
385 triplicate on each formula.

386 **3.5.4 Entrapment efficiency**

387 The difference between the un-entrapped free carnosine and the total amount of carnosine
388 in the vesicle systems were determined by using the HPLC. The niosome dispersion was
389 diluted to 20 ml and placed in Eppendorf® tubes. A Beckman Coulter Optimal L-100XP

Ultracentrifuge (Beckman Coulture, South Africa) was used to centrifuge the samples, at 25 °C for 30 min at 25 000 rpm. The proniosome dispersion was diluted to 40 ml and placed in Eppendorf® tubes and centrifuged for 30 min at 25 000 rpm. The clear supernatant was then extracted for HPLC analysis. The following equation adapted from Mali *et al*. (2013) was used to determine the percentage entrapment efficiency:

EE (%) = Cr / Ct x 100 **Equation 1**

Where Cr is the amount of carnosine entrapped and Ct is the total amount of carnosine used.

3.5.5 pH

A Mettler Toledo pH meter with a Mettler Toledo InLab 410 electrode (Mettler Toledo, Switzerland) was used to determine the pH of the pre-formulations. The pH meter was calibrated with buffer solutions before the experiment. The experiment was done in triplicate.

3.5.6 Viscosity

A Brookfield® Viscometer model DV-III Ultra (Brookfield Engineering Laboratories, United States of America) was used to determine the viscosity of the pre-formulations. The samples were placed in a water bath to reach 25 °C. For this experiment, 6.7 ml of each dispersion was placed into a flow jacket connected to the waterbath to measure the viscosity. A SC4-18 spindle was used to measure the viscosity 8 times in intervals of 10 seconds at a speed of 200 rpm.

3.6 Formulation of semi-solids containing carnosine encapsulated in niosomes

The final formulas used to prepare the niosome cream and gels are indicated in Table 3. The niosome formula was manipulated to contain 4.152% carnosine to formulate the cream. The final cream formula contained 72.25% niosomes and therefore 3% carnosine. The cream was formulated by preparing the water **(A)** and the oil **(B)** phase separately. Phase A was prepared by heating the niosomes and Span® 20 to 60 °C, while adding the Veegum® to the mixture while stirring continuously; Phase B was prepared by heating all the ingredients to 60 °C. As soon as both phases reached temperature, they were stirred together for

418 preliminary emulsification to take place. The process was finalized with continuous stirring
419 at 1 000 rpm until the formulation had completely cooled down. It is evident in the
420 concentration assays that the heating process did not influence the niosomes.
421 The niosome formula, for the purpose of formulation of the gel, was manipulated to contain
422 3.158% carnosine; the final gel formula consisted of 95% niosomes and therefore 3%
423 carnosine. The gel was formulated by dissolving the parabens in propylene glycol and
424 adding it to the niosomes. Carbopol® was then sprinkled over the mixture and allowed to
425 hydrate for 20 min. The mixture was then homogenized at 1 000 rpm for 30 min until
426 uniform. The formulation was degassed in a sonication bath for 10 min.

427 **3.7 Analytical method for assays of the active ingredient and excipients in the topical**
428 **formulations**

429 The same HPLC system and column (Section 3.1) was used for the assays of the excipients.
430 Mobile phase A was acetonitrile and mobile phase B was 0.075 M octane sulphonic acid at
431 pH 3.45. The initial ratio up to 3 min was 20/80%, followed by a linear gradient to reach
432 70/30% after 7 min, and then it changed back to 20/80%. A flow rate of 1.0 ml/min,
433 detection wavelength of 210 nm and injection volume of 10 µL was maintained.

434 **3.8 Stability testing of semi-solid formulations**

435 The two semi-solid formulations underwent accelerated stability tests for three months.
436 Guidelines by The International Conference on Harmonisation (ICH) were followed and
437 formulation changes were assessed during accelerated (40 ± 2 °C/75 ± 5% RH),
438 intermediate (30 ± 2 °C/60 ± 5% RH) and long-term (25 ± 2 °C/60 ± 5% RH) storage
439 conditions (ICH 2003). Tests were performed in triplicate monthly on the same day of every
440 month. Baseline readings were taken for all the tests performed.

441 **3.8.1 Concentration assay of active ingredient and excipients**

442 Approximately 0.5 g of both formulations, representative of each storing condition, was
443 accurately weighed and dissolved in methanol in a 100 ml volumetric flask. The samples
444 were transferred to HPLC auto-sampler vials for analysis.

445

3.8.2 pH

A Mettler Toledo pH meter with a Mettler Toledo InLab 410 electrode (Mettler Toledo, Switzerland) was used to determine the pH of the samples in triplicate. The pH meter was calibrated with buffer solutions before the experiment.

3.8.3 Conductivity

A Mettler Toledo SevenMulti™ pH/conductivity meter, equipped with a glass Mettler Toledo InLab 731 electrode (Mettler Toledo, Switzerland) was used to determine the conductivity of the samples in triplicate. The conductivity meter was calibrated with standard solutions before the experiment. The conductivity was measured in micro Siemens per centimeter (µS/cm).

3.8.4 Viscosity

A Brookfield® Viscometer model DV-III Ultra (Brookfield Engineering Laboratories, United States of America) was used to measure the viscosity of the samples. The samples were placed in a water bath to reach 25 °C. Approximately 15 ml of each sample, representative of each storage condition, was transferred into a cylindrical sample chamber and placesd in a flow jacket connected to the water bath. A SC25- spindle was used to measure the viscosity at a speed of 1 rpm and 2 rpm for the cream and the gel, respectively. The percentage torque was approximately 50%. The average viscosity was determined from 32 data points collected at intervals of 10 sec.

3.8.5 Zeta potential

A Malvern Zetasizer Nano (Nano SZ) (Malvern Instruments, United Kingdom) was used to determine the zeta-potential of the samples at 25 °C. Each of the samples was diluted (0.1 g/20.0 ml) with purified water to reduce particle interactions.

3.8.6 Mass loss

The mass loss of the formulations from the packaging was measured by weighing the exact same samples, representative of each storage condition, at all the time intervals. The results were compared to the baseline readings.

3.8.7 Macroscopic analysis

A macroscopic analysis of the samples, representative of each storage condition, was performed. Images were taken at all the time intervals for comparison with an iPhone 6s 12-megapixel camera (Apple Inc., United States of America).

3.8.8 Microscopic analysis

A microscopic analysis of the samples, representative of each storage condition, was performed. A Motic microscope (Motic, Hong Kong), equipped with a Moticam 3 camera (Motic, Hong Kong), using Motic Images Plus software was used during this experiment. The samples were placed on a glass slide, spread out and covered with a glass slide, which was then mounted on the microscope and images taken at all the time intervals for comparison.

3.9 Diffusion studies

3.9.1 Membrane release studies

Membrane release experiments were conducted on all four preparations to determine if carnosine was successfully released. A magnetic stirrer plate (Variomag) was placed inside a Grant JB series water bath and the bath was preheated to 37 °C. Vertical Franz cells were used to perform the experiments. Each cell consisted of a donor and receptor compartment with a diffusion area of approximately 1.075 cm^2 and a receptor capacity of about 2 ml. PBS (pH 7.4) was preheated to 37 °C (normal body temperature) before the experiment started. Magnetic stirrers were placed in the receptor compartments of the cells and small circles of lipophilic polyvinylidene difluoride (PVDF) membrane filters (pore size: 0.45 µm) were placed on the receptor compartment before the donor compartment was positioned carefully on top. The Franz cells were sealed with Dow Corning® high vacuum grease to prevent leakage before attaching the clamps. The receptor compartment of the cells was carefully filled with 2.0 ml PBS (pH 7.4) and the donor compartment was filled with 1.0 ml of the preparation. Parafilm® was used to seal the donor compartment to prevent loss of any active. The cells were finally placed in the water bath at a controlled temperature of 37 °C and the receptor phase was extracted and replaced every hour up to 6 h to ensure sink conditions throughout

the experiment. The extracted samples were analyzed with HPLC to determine the concentration active that released from the preparation through the membrane into the receptor phase.

3.9.2 Skin preparation

The North-West University Ethics Committee granted ethical approval for the use of biological material obtained from human subjects in the experiments (NWU-00114-11-A5). The laboratory (Biosafety level 2) is approved and fully equipped for the intended experiments. Patient information and written consent were handled confidentially. Female, abdominal Caucasian skin was used to perform the transdermal diffusion studies. A Zimmer™ electric dermatome model 8821 was used to obtain the preferred thickness (400 µm) of the skin, which contained the stratum corneum, viable epidermis and upper dermis. The dermatomed skin was then allowed to air dry on Whatman® filter paper with the stratum corneum facing upwards. Small circles were cut out and the prepared skin was wrapped in aluminum foil and stored at – 20 °C until needed. The prescribed methods for disposal of biological waste were followed.

3.9.3 Transdermal diffusion studies

The same method described in membrane release studies was used in skin diffusion studies, except that dermatomed skin instead of PVDF membrane filters was used. The prepared small circles of the skin were placed between the two compartments of the Franz cells with the stratum corneum facing upwards. The receptor compartments were then extracted and replaced every 20 min up to 2 h, and every 2 h up to 12 h. The samples were then analyzed using HPLC.

3.9.4 Tape stripping

The tape strip method was obtained from Pellet *et al*. (1997). After the diffusion studies, the skin was carefully removed from the Franz cells, pinned to the Parafilm® on the solid surface and dabbed dry with towel paper. The first tape strip was discarded due to possible contamination from the formulation. The next 15 tape strips were placed in 5 ml PBS (suitable extraction solution) (pH 7.4) and kept overnight in the refrigerator at 4 °C. The

diffusion areas were then cut into small pieces and also placed in 5 ml PBS (pH 7.4) in the refrigerator at 4 °C overnight; both solutions were filtered and analyzed using HPLC.

3.10 Statistical analysis

The data obtained from the membrane release studies and skin diffusion studies were used to determine if carnosine was released from the four different preparations and to calculate the %carnosine which had diffused into the SCE and ED, respectively. The topical delivery of carnosine from the preparations was compared and investigated. Statistica (StatSoft Inc. 2016) was used for data analysis. Non-parametric tests were performed, as the p-p-plot obtained from this study did not indicate normality of data. The non-parametric tests involved a Kruskal-Wallis rank test to determine a statistical significant effect by the four preparations, followed by multiple comparisons between the different pairs of preparations. The non-parametric Mann-Whitney rank sum test was performed for comparison of the concentration active delivered to the ED and SCE. A p-value of less than 0.05 ($p < 0.05$) indicates a significant statistical difference.

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660

661 **Tables**

662 **Table 1:** The final niosome and proniosome formulas

	Niosomes	Proniosomes
Cholesterol	600 mg	300 mg
Chloroform	10 ml	10 ml
Span® 60	300 mg	600 mg
Carnosine	300 mg	300 mg
Milli-Q® water	10 ml	10 ml
Sorbitol		2 g

663

664

665 **Table 2:** The characteristics of the niosomes and proniosomes

	Niosomes	**Proniosomes**
Average vesicle size	360.2 ± 15.5 nm	391.77 ± 33.91 nm
Average PDI	0.722 ± 0.031	0.590 ± 0.044
Average zeta-potential	- 59.80 ± 0.51 mV	- 45.73 ± 1.68 mV
Average pH	8.077 ± 0.004	8.110 ± 0.009
Average viscosity	3.00 ± 0.026 cP	4.31 ± 0.033 cP
Average EE%	46.72 ± 1.02%	44.50 ± 0.33%

666

667

668 **Table 3:** The final semi-solid formulation formulas

	Ingredient	%m/m	Purpose
Final cream formula			
A	Niosomes	72.25	Vesicles containing carnosine
	Veegum®	2.50	Thickening agent
	Span® 20	1.00	Emulsifying agent
B	Liquid paraffin	10.00	Oil phase
	Propylene glycol	3.00	Solvent for preservatives
	Methylparaben	0.20	Preservative
	Propylparaben	0.05	Preservative
	Cetyl alcohol	10.00	Thickening agent
	Tween® 20	1.00	Emulsifying agent
Final gel formula			
Niosomes		95.00	Vesicles containing carnosine
Propylene glycol		5.00	Solvent for preservatives
Propylparaben		0.05	Preservative
Methylparaben		0.20	Preservative
Carbopol®		0.50	Gelling agent

669

670

671 **Table 4:** The concentration assay results the cosmeceutical cream

	Initial (T0)	Month 1 (T1)	Month 2 (T2)	Month 3 (T3)
25 ± 2 °C/60 ± 5% RH				
%Carnosine	113.12	107.93	119.93	109.88
%Methylparaben	124.56	114.51	120.90	112.27
%Propylparaben	110.88	113.17	127.21	112.35
30 ± 2 °C/60 ± 5% RH				
%Carnosine	113.12	106.56	121.92	112.52
%Methylparaben	124.56	114.86	119.34	110.62
%Propylparaben	110.88	126.50	128.97	113.67
40 ± 2 °C/75 ± 5% RH				
%Carnosine	113.12	109.23	117.59	110.02
%Methylparaben	124.56	114.14	114.60	105.20
%Propylparaben	110.88	114.49	128.67	113.94

672

673

674 **Table 5:** The concentration assay results of the cosmeceutical gel

	Initial (T0)	Month 1 (T1)	Month 2 (T2)	Month 3 (T3)
25 ± 2 °C/60 ± 5% RH				
%Carnosine	110.87	95.66	114.75	106.54
%Methylparaben	119.57	112.51	120.51	113.14
%Propylparaben	109.33	79.67	127.31	113.54
30 ± 2 °C/60 ± 5% RH				
%Carnosine	110.87	102.82	116.88	109.38
%Methylparaben	119.57	112.08	112.65	108.24
%Propylparaben	109.33	111.08	117.69	106.89
40 ± 2 °C/75 ± 5% RH				
%Carnosine	110.87	99.31	116.19	104.94
%Methylparaben	119.57	108.21	107.87	97.99
%Propylparaben	109.33	115.58	121.29	101.93

675

676

677 **Table 6:** The stability parameter results of the cosmeceutical cream

	Initial (T0)	Month 1 (T1)	Month 2 (T2)	Month 3 (T3)
25 ± 2 °C/60 ± 5% RH				
pH	8.20	7.99	7.82	7.62
Conductivity (µs/cm)	214.00	153.27	173.60	113.20
Viscosity (cP)	227 146.9	98 863.8	128 383.8	334 121.9
Zeta-potential (mv)	- 52.91	- 60.58	- 60.72	- 59.79
Mass (g)	34.799 g	34.799 g	34.587 g	34.526 g
30 ± 2 °C/60 ± 5% RH				
pH	8.20	7.91	7.84	7.53
Conductivity (µs/cm)	214.00	123.70	111.27	83.43
Viscosity (cP)	227 146.9	180 300.0	316 340.6	264 059.4
Zeta-potential (mv)	- 52.91	- 58.17	- 63.98	- 54.02
Mass (g)	39.217 g	39.191 g	39.148 g	39.142 g
40 ± 2 °C/75 ± 5% RH				
pH	8.20	7.71	7.61	7.42
Conductivity (µs/cm)	214.00	86.03	77.33	67.77
Viscosity (cP)	227 146.9	295 581.3	247 393.8	273 428.1
Zeta-potential (mv)	- 52.91	- 64.26	- 62.33	- 62.74
Mass (g)	38.517 g	38.325 g	38.142 g	37.998 g

678

679

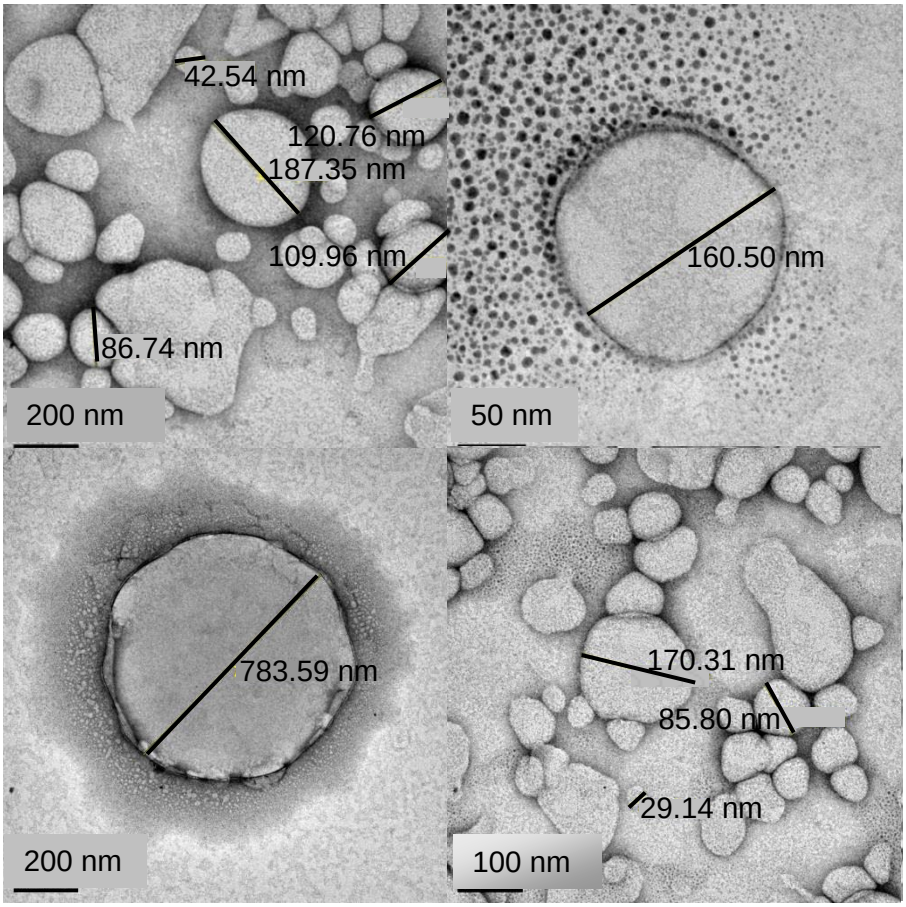
680 **Table 7:** The stability parameter results of the cosmeceutical gel

	Initial (T0)	Month 1 (T1)	Month 2 (T2)	Month 3 (T3)
25 ± 2 °C/60 ± 5% RH				
pH	7.65	7.58	7.64	7.57
Conductivity (µs/cm)	153.13	495.33	541.33	566.33
Viscosity (cP)	77 301.7	114 571.9	91 654.2	130 631.3
Zeta-potential (mv)	- 64.53	- 62.08	- 59.03	- 64.34
Mass (g)	20.616 g	20.605 g	20.574 g	20.571 g
30 ± 2 °C/60 ± 5% RH				
pH	7.65	7.49	7.55	7.56
Conductivity (µs/cm)	153.13	1 259.00	755.67	595.33
Viscosity (cP)	77 301.7	104 266.9	87 786.3	107 350.0
Zeta-potential (mv)	- 64.53	- 69.89	- 56.53	- 60.36
Mass (g)	19.031 g	18.995 g	18.949 g	18.937 g
40 ± 2 °C/75 ± 5% RH				
pH	7.65	7.59	7.59	7.58
Conductivity (µs/cm)	153.13	567.00	618.67	557.33
Viscosity (cP)	77 301.7	104 266.9	87 786.3	106 406.5
Zeta-potential (mv)	- 64.53	- 69.47	- 53.58	- 81.27
Mass (g)	21.109 g	20.975 g	20.931 g	20.912 g

681

682

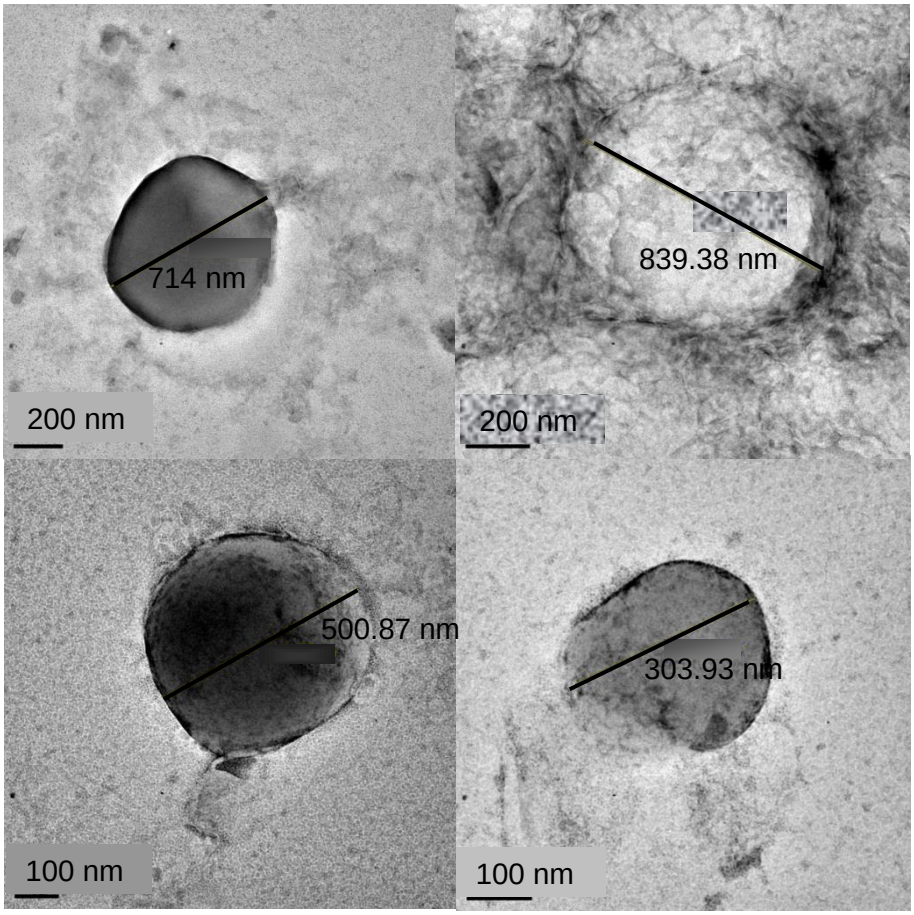
683 **Figures**



684

685 **Figure 1:** Micrographs to illustrate the niosomes using the TEM

686



687

688 **Figure 2:** Micrographs to illustrate the proniosomes using the TEM

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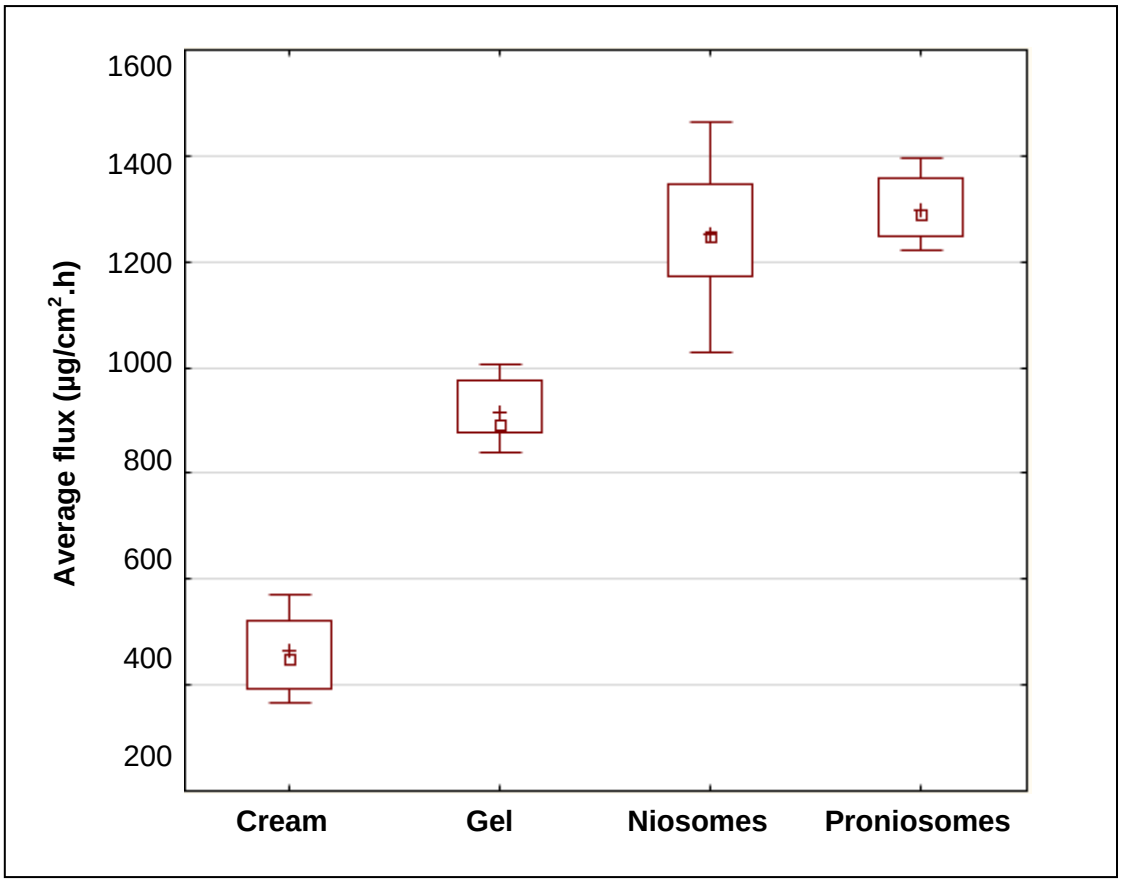
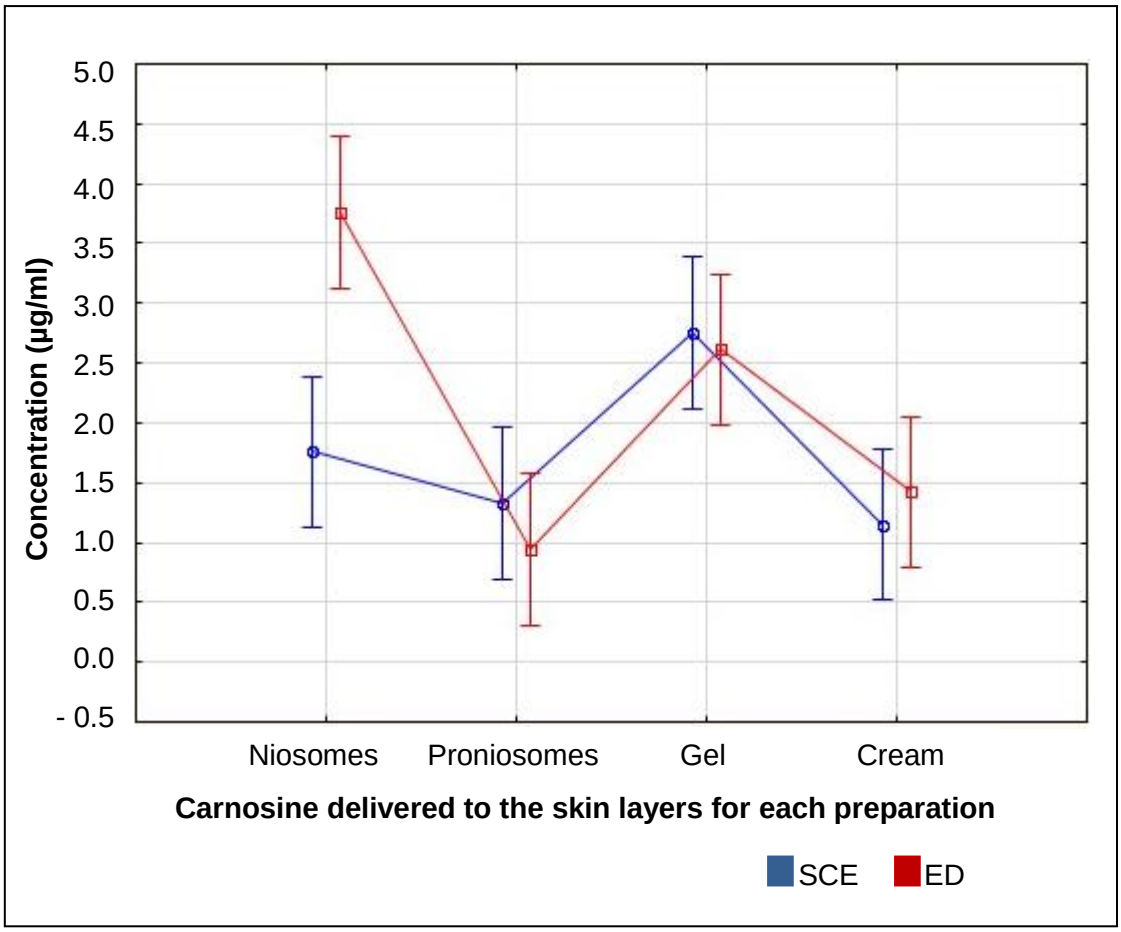


690

691 **Figure 3:** Box-plots of the flux values of the four preparations illustrating the average (+)

692 and median (□), as well as minimum and maximum values.

693



694

695 **Figure 4:** The concentration carnosine (µg/cm^3) in the SCE and ED for each of the four

696 preparations

697

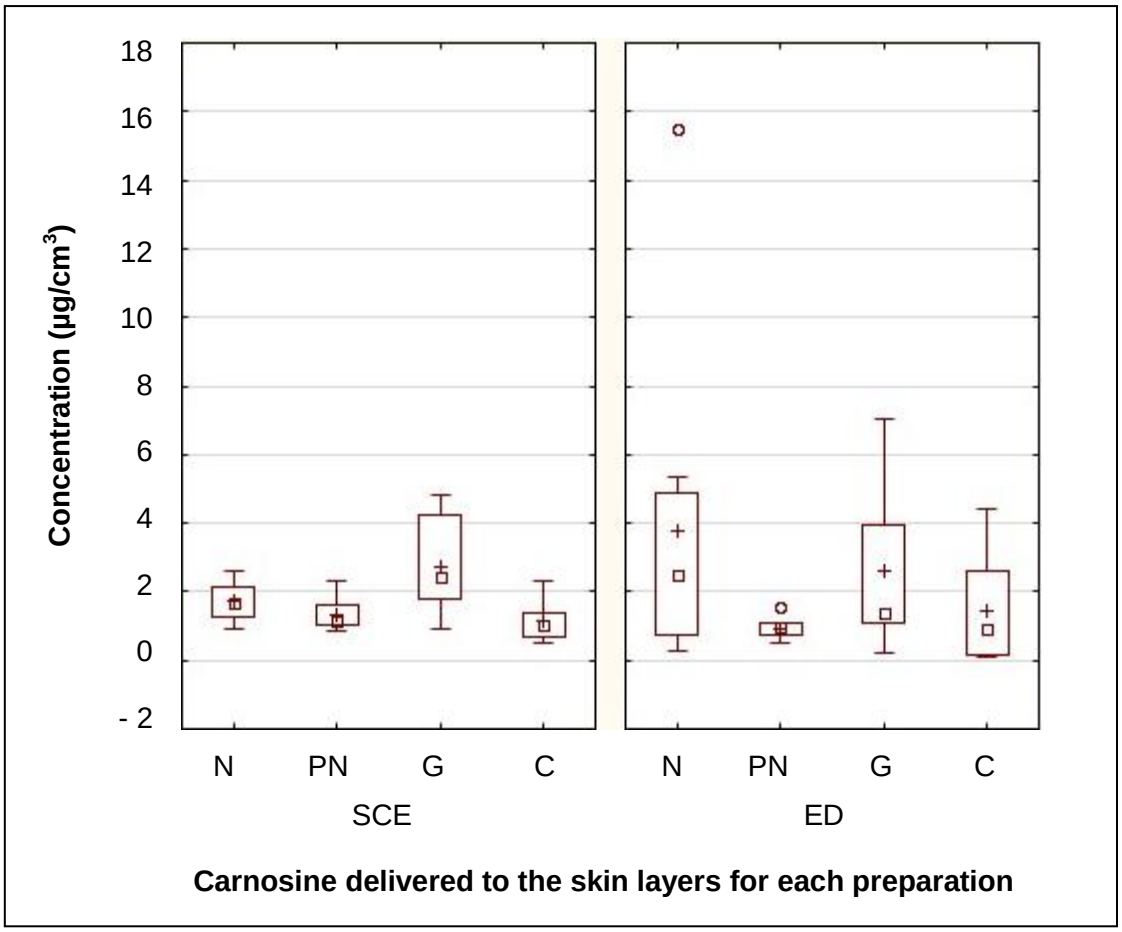


Figure 5: Box-plots of the SCE and ED values of the four preparations illustrating the average (+) and median (□), as well as the minimum and maximum values and outliers (o). The following key can be used for the different preparations: N: Niosomes, PN: Proniosomes, G: Gel and C: Cream

CHAPTER 4

FINAL CONCLUSIONS AND FUTURE PROSPECTS

The extended history of cosmetic products is depicted by the Greek description *kosm tikos*; meaning “having the power to arrange” (Milstein *et al.,* 2001:5-6). Cosmetics have been used to intensify personal beauty, to camouflage blemishes and to promote the original for many years. Both the intrinsic activity and the delivery of the cosmetic active determine the efficacy of the product (Wiechers, 2008:1-18). Carnosine is a compound with many anti-ageing benefits, but has unfavourable physicochemical properties for topical delivery (Goebel *et al.,* 2012:282; Kyriazis, 2010:45-49). Successful delivery is therefore necessary to benefit from the anti-ageing properties.

The aim of this study was to successfully deliver carnosine topically. Carnosine was incorporated into two pre-formulations, niosomes and proniosomes, in order to promote skin permeation. Two topical semi-solid formulations were formulated from the niosomes. The topical delivery from the four different preparations was investigated and compared. Furthermore, the quality of the semi-solid formulations was investigated with accelerated stability tests over a three month testing period.

The objectives of this study were as follow:

- Development and validation of a HPLC method to quantitatively determine the concentration of carnosine at different stages during the study;
- determine the aqueous solubility and log D of carnosine;
- formulation of niosomes and proniosomes as vesicles to entrap carnosine effectively for topical delivery;
- determine the characteristics of the vesicles;
- perform membrane studies to determine the release of carnosine from the vesicles;
- perform diffusion studies with skin, followed by tape stripping to determine if carnosine diffuses through and/or into the skin, respectively;
- formulation of semi-solid dosage forms;
- perform stability tests where the semi-solid formulations will be stored in long-term conditions (25 ± 2 °C/60 ± 5% RH), intermediate storing conditions (30 ± 2 °C/60 ± 5% RH) and accelerated storage conditions (40 ± 2 °C/75 ± 5% RH). HPLC methods were developed and validated to determine the concentrations of all the excipients together with the active ingredient used in the formulations. Evaluations,

including concentration assays, pH, conductivity, viscosity, zeta-potential, mass loss, microscopic analysis and macroscopic analysis, were conducted at zero, one, two and three months;

- perform membrane studies to determine the release of carnosine from the semi-solid formulations; and
- perform diffusion studies with skin followed by tape stripping to determine if carnosine diffused through and/or into the skin, respectively.

An HPLC method was successfully developed and validated in terms of linearity, accuracy, precision, ruggedness, robustness, specificity and limit of detection (LOD; 0.08675 µg/ml) and lower limit of quantification (LLOQ; 0.17350 µg/ml), in co-operation with Prof Jan du Preez at the Analytical Technology Laboratory, North-West University, Potchefstroom Campus, South Africa; the method proved to be suitable for the determination of carnosine in study samples. HPLC analysis was used for the aqueous solubility determination, log D determination, entrapment efficiency experiments and diffusion studies.

The aqueous solubility of carnosine was determined in phosphate buffer solution (PBS; pH 7.4) at 25 °C. An aqueous solubility of at least 1 mg/ml is essential for passive skin diffusion of molecules (Naik *et al.,* 2000:319). The experimental aqueous solubility was determined as 122.8 ± 0.7 mg/ml. The aqueous solubilities obtained from Singh *et al.* (2009:734) were 205.9 mg/ml at 25 °C with pH 7.0 and 52.0 mg/ml at pH 8.0. The slight difference in solubility could be attributed to the pH differences. With an adequate aqueous solubility, carnosine proved to be a good candidate for topical delivery. Unfortunately, one single parameter cannot be utilised to predict topical delivery. The log P of a compound is also important to consider for skin permeation. The ideal log P for skin diffusion is 1 to 3 (Khalid *et al.*, 2016:129). The experimental log D of carnosine determined, - 2.89 ± 0.01, correlated well with literature as the log P of carnosine obtained from Goebel *et al.* (2012:282) was - 2.97 ± 0.46. This value was not ideal, and vesicle systems were used to promote skin permeation.

Different niosome formulas were prepared to determine which formula had the most favourable characteristics and to decide on the optimal concentration of carnosine. The niosomes containing 3% carnosine was the most favourable formula, since it had the best overall results and a higher concentration carnosine was preferred to enhance *in vitro* performance. This niosome formula was then used to prepare the proniosomes. Different proniosome formulas were also prepared to determine the optimal amount of sorbitol to include in the formula. The formula containing 2 g sorbitol was chosen as it had the best overall results and was easier and quicker to formulate. The average size and polydispersity index (PdI) of the niosomes were 360.2 ± 15.5 nm and 0.722 ± 0.031, and for the proniosomes, it was 391.77 ± 33.91 nm and

0.590 ± 0.044, respectively. Although the sizes seemed large, Bolzinger *et al.* (2012:163) explained that particles smaller than 3 µm are expected to easily penetrate the stratum corneum through the intercellular pathway. With nanometre being a 1000 times smaller than micrometre, it can be predicted that these sizes will not affect skin penetration negatively. The average zeta-potential of the niosomes was - 59.80 ± 0.51 mV and for the proniosomes, - 45.73 ± 1.68 mV. Both pre-formulations had highly negative zeta-potentials, lower than - 30 mV, which revealed excellent stability (Marianecci *et al.,* 2013:75). With pH values between 5 and 9, the preparations proved to be suitable for topical use (Naik *et al*., 2000:319). The viscosities of the dispersions were remarkably low, and the incorporation into semi-solid formulations was necessary to alter the viscosity and increase the stability (Roland *et al.,* 2003:93). Both of the pre-formulations also revealed acceptable entrapment efficiencies (EE%).

Two semi-solid formulations, a cream and a gel containing carnosine encapsulated in niosomes, were formulated. The niosomes were the preferred pre-formulation, since they had overall good characteristics and were quicker and easier to prepare compared to the proniosomes. The formulations were visually attractive. The cream had a homogenous texture, off-white colour, no particular smell and applied easily to the skin. The gel was foamy, had a bright white colour, a characteristic odour and applied easily, leaving the skin feeling moist and light as the excess fluid evaporated quickly. Accelerated stability tests were performed on the semi-solid formulations and formulation changes were assessed during accelerated (40 ± 2° C/75 ± 5% RH), intermediate (30 ± 2 °C/60 ± 5% RH) and long-term (25 ± 2 °C/60 ± 5% RH) storage conditions (ICH, 2003:3). The tests involved all relevant ingredients, as well as the exact packaging to be used for marketing and storage (Barnes, 2007:663; York, 2007:12-13). According to the ICH (2003:9), a significant change is defined as a 5% change in stability parameter from the initial value. Furthermore, the degradation of ingredients, appearance changes (colour, cracking and phase separation) and pH changes were assessed (ICH, 2003:3, 9).

A second HPLC method was successfully developed and validated in terms of linearity, accuracy, precision, ruggedness, robustness and specificity, in co-operation with Prof Jan du Preez at the Analytical Technology Laboratory, North-West University, Potchefstroom Campus, South Africa. The method proved to be suitable for the determination of the active ingredient (carnosine) and the preservatives (methylparaben and propylparaben) during the concentration assays during stability tests.

Significant changes, for both of the formulations, had occurred with regards to the concentration assays, conductivity, viscosity and zeta-potential. The gel had good stability, whilst the cream revealed significant changes regarding the pH. The gel revealed a disappearance of air bubbles, whilst crystal formation was noticed in both formulations during the microscopic

assessments. During the macroscopic assessments, colour changes and condensation for both formulations were noticed, whilst the smell remained unchanged. Visible phase separation was also noticed in the gel formulation. In summary, the two products did not meet the ICH requirements for stability and could not be considered stable or suitable for manufacturing.

Membrane release experiments were performed on all four preparations to determine if carnosine was successfully released. The results obtained indicated that, regardless of the amounts, all four preparations successfully released carnosine. With the highest median flux (1 139.1 µg/cm^2), the niosomes revealed the best release, followed by the proniosomes (1 096.2 µg/cm^2), the gel (796.4 µg/cm^2) and lastly, the cream (378.64 µg/cm^2). Although the average flux and median values were more or less the same, the median values were a better representation of the data as it was not influenced by the outliers in the data (Dawson & Trapp, 2001:30; Gerber *et al*., 2008:190). A much bigger difference between the vesicular systems and the semi-solid formulations than between proniosomes and niosomes, and the gel and the cream were noticed. This suggested the formulations (cream and gel) had a negative effect on the release from the pre-formulations (niosomes and proniosomes).

Transdermal diffusion studies, followed by tape strip experiments were performed after the membrane release studies. None of the four preparations reached the receptor phase and carnosine was delivered topically. Tape strip analysis revealed the gel delivered the highest median concentration carnosine (2.46 µg/ml) to the SCE, followed by the niosomes (1.66 µg/ml), proniosomes (1.16 µg/ml) and the cream (1.05 µg/ml). Furthermore, the niosomes delivered the highest median concentration carnosine (2.47 µg/ml) to the ED, followed by the gel (1.38 µg/ml), the proniosomes (0.96 µg/ml) and lastly, the cream (0.92 µg/ml). The median and average values were more or less the same, but the median was preferred as it is a more accurate representation of skewed data (Dawson & Trapp, 2001:30; Gerber *et al*., 2008:190).

Future prospects for this study include:

- Perform dermal toxicity tests to determine dermal irritation and dermal corrosion of carnosine and/or the formulations;
- perform dermal metabolism tests with a carnosine solution to determine the activity of cutaneous enzymes that can have an effect on carnosine concentrations;
- prepare different vesicle systems to entrap carnosine and investigate the release accordingly;
- optimise the semi-solid formulations with regards to stability;
- optimise the semi-solid formulations with regards to topical delivery of carnosine;
- formulate different semi-solids and investigate the release of carnosine accordingly;

- perform clinical sensitivity tests to discover any possible adverse effects of the formulation and/or carnosine on the skin; and
- perform clinical efficacy tests to evaluate the anti-ageing claims of carnosine due to its anti-oxidation and anti-glycation properties.

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APPENDIX A

VALIDATION OF AN HPLC ANALYTICAL METHOD FOR ANALYSIS OF CARNOSINE IN STUDY SAMPLES

A.1 Purpose of the validation

The purpose of the development and validation of an HPLC method for the analysis of carnosine (Sigma-Aldrich, Aston Manor, SA) is to ensure sensitivity and reliability during the quantitative determination of the amount of carnosine in study samples (Smith, 2013:63). This method was developed and validated in co-operation with Prof Jan du Preez at the Analytical Technology Laboratory, North-West University, Potchefstroom Campus, South Africa.

A.2 Chromatographic conditions

The following chromatographic conditions formed part of the HPLC method for the analysis of carnosine in study samples:

Analytical instrument: The HPLC analysis was performed with an Agilent® 1200 Series HPLC system (Agilent Technologies, United States of America), which is equipped with ChemStation Rev. A.10.02 software for data analysis. The system is equipped with an Agilent® 1200 pump, diode array detector, autosampler injection system and a vacuum degasser.

Column: A Venusil® ASB C_8 column (250 x 4.6 mm) (Agela Technologies, Newark, DE) was used.

Mobile phase: Acetonitrile/0.075 M octane sulphonic acid (20/80%) at pH 3.45.

Solvent: Phosphate buffer solution (PBS) at pH 7.4.

Flow rate: 1 ml/min

Injection volume: 10 µl

Retention time: Approximately 5.5 min

Run time: 7.0 min

Detection wavelength: 210 nm

Figures A.1 to A.3 indicate instruments used during HPLC analysis.

Figure A.1: The Agilent® 1200 Series HPLC system

Figure A.2: The Venusil® ASB C_8 column (250 x 4.6 mm)

Figure A.3: An HPLC sample vial

A.3 Preparation of the mobile phase

The octane sulphonic acid solution was prepared by adding 1.5 g octane sulphonic acid (Merck Millipore, Modderfontein, SA) to 900 ml Milli-Q® HPLC grade water, prepared in-house with a Milli-Q purification system, (Merck Millipore, Modderfontein, SA). After using the magnetic stirrer to ensure complete dissolution, 2 ml ammonia was added to increase the buffer capacity of the solution; 2 M Phosphoric acid was added in small amounts until a pH of 3.45 was reached. Milli-Q® HPLC grade water was used to fill the volume to 1000 ml.

A.4 Preparation of the solvent

PBS was prepared by adding approximately 6.5 g potassium dihydrogen orthophosphate (Merck Millipore, Modderfontein, SA) and 1.5 g sodium hydroxide (Merck Millipore,

Modderfontein, SA) to 250 ml and 400 ml Milli-Q® HPLC grade water, respectively (BP, 2015b). After complete dissolution, the two mixtures were combined and depending on the pH, either phosphoric acid or ammonia was added in small amounts until a pH of 7.4 was reached. Milli-Q® HPLC grade water was then used to fill the volume to 1000 ml.

A.5 Preparation of samples and standard solutions

The standard solutions were prepared by dissolving an accurately weighed amount of carnosine in PBS (pH 7.4) in a 100 ml volumetric flask. The solutions were then either injected in different volumes or diluted, depending on the validation parameters.

A.6 Calculations

A Microsoft Excel spread sheet was used to calculate linearity from the concentration and peak areas of the standard solutions. The concentrations of different samples were then calculated by using the slope, y-intercept and peak areas. Mean percentage recovery (%), standard deviation (SD) and relative standard deviation (%RSD) were important parameters during the method development and validation.

A.7 Validation parameters

A.7.1 Linearity

The linearity is a validation parameter to measure whether the test results and the concentration of carnosine in the test samples are directly proportional (ICH, 2005:5). Linear regression was used to determine the linearity of carnosine when the peak area versus the concentration obtained from HPLC analysis is plotted on a graph. A minimum of five different concentrations is essential to confirm linearity (ICH, 2005:8). The data is best described by a linear equation:

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

In this equation, y is the peak area, m is the slope, x the concentration and c the y-intercept. The regression coefficient (R^2) describes a straight line and should be at least 0.997 (Shabir, 2005:7).

A.7.1.1 Method

A sample was prepared by weighing 14.1 mg carnosine accurately and dissolving it in PBS (pH 7.4) in a 100 ml volumetric flask. The solution was diluted twice to form a range of 1.41 – 141.00 µg/ml. Each solution was then injected in different injection volumes and in duplicate for HPLC analysis. The linearity of carnosine was determined by plotting the mean peak areas against concentration and performing linear regression analysis.

A.7.1.2 Results and discussion

Carnosine demonstrated a good linear correlation coefficient (R^2) value of 0.999. This value indicates that the HPLC system was stable and that carnosine expressed linearity within the concentration range of 0.03 – 141.00 µg/ml. The linearity results and the regression curve of linearity follow in Table A.1 and Figure A.4, respectively.

Table A.1: The linearity results of carnosine

Standard concentration (µg/ml)	Peak area 1	Peak area 2	Mean peak area
0.03	3.2	3.1	3.2
0.07	6.3	6.5	6.4
0.14	13.3	12.3	12.8
0.28	24.2	22.0	23.1
0.56	42.4	42.7	42.6
0.85	99.0	100.8	99.9
1.13	118.5	119.4	119.0
1.41	143.2	133.6	138.4
2.82	306.5	306.5	306.5
5.64	611.4	619.8	615.6
8.46	912.7	908.2	910.5
11.28	1191.8	1194.3	1193.1
14.10	1421.4	1164.9	1293.2
28.20	3063.7	3063.3	3063.5
56.40	6032.9	6047.3	6040.1
84.60	8928.3	8920.4	8924.4
112.80	11673.0	11685.9	11679.5
141.00	15351.3	15372.4	15361.9
		Slope	**106.8**
		y-intercept	**- 18.834**
		R^2	**0.9992**

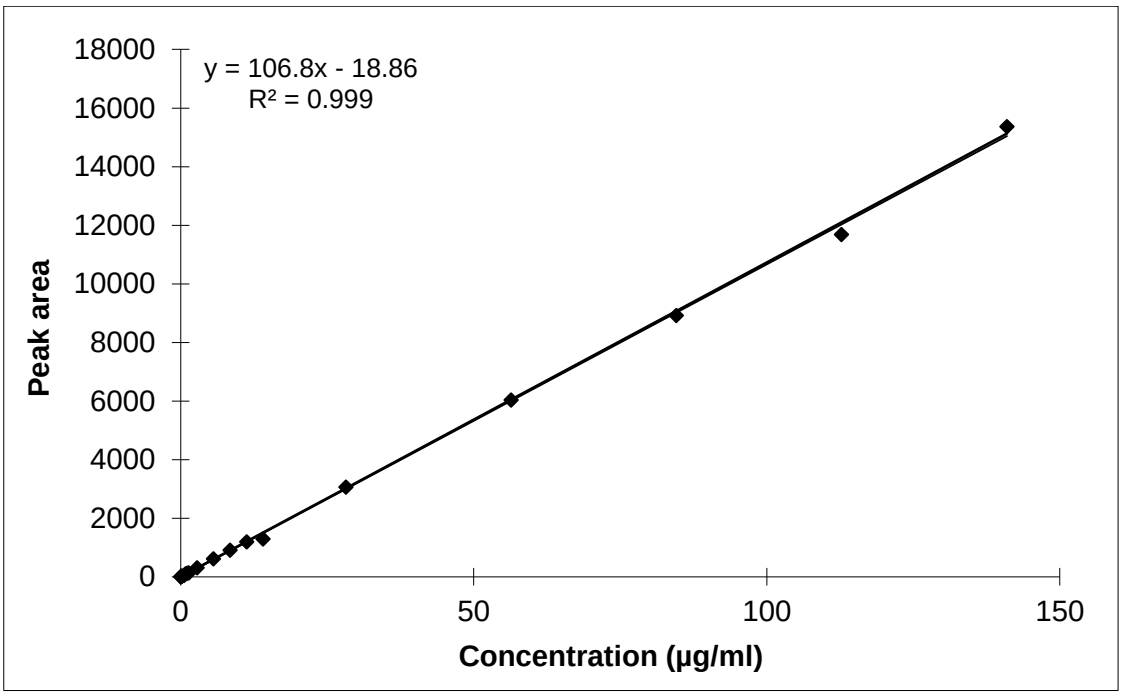

Figure A.4: The linear regression curve of carnosine standard solutions

A.7.2 Accuracy

The accuracy is a validation parameter used to express how close the experimental value is to the true theoretical value (ICH, 2005:4). %Recovery is used to indicate accuracy; the acceptable range for the mean percentage recovery is within 15% of the actual true value (CDER, 2001:5). The accuracy can only be confirmed with at least nine determinations of three concentrations (ICH, 2005:10).

A.7.2.1 Method

A standard solution was prepared by weighing 10.29 mg carnosine and dissolving it in PBS (pH 7.4) in a 100 ml volumetric flask. This solution was injected in duplicate in injection volumes of 2, 4, 6, 8 and 10 µl. The samples were prepared by dissolving 8.76, 8.40 and 8.78 mg carnosine in PBS (pH 7.4) in a 100 ml volumetric flask, respectively. Next, 5 ml of each solution was diluted to 10 ml in a volumetric flask and thereafter 2 ml of each solution was diluted to 10 ml in a volumetric flask. All three of the sample solutions were injected in duplicate, where after the recovery was calculated in concentration and percentage.

A.7.2.2 Results and discussion

The average recovery of carnosine was determined as 101.6% with a %RSD of 2.31%, which is acceptable. All the recovery values were within 15% of the actual true value and the accuracy results were acceptable. The accuracy results are indicated in Table A.2.

Table A.2: The accuracy results of carnosine determination

Concentration (µg/ml)	Peak area 1	Peak area 2	Mean peak area	Recovery	
				(µg/ml)	%
8.76	181.7	182.2	182.0	8.92	101.8
8.40	178.2	178.0	178.1	8.73	104.0
8.78	179.4	181.1	180.3	8.84	100.7
43.75	900.0	909.5	904.8	44.36	101.4
42.00	883.5	882.4	883.0	43.29	103.1
43.75	884.1	886.3	885.2	43.40	99.2
87.60	1806.9	1815.1	1811.0	88.79	101.4
84.00	1810.3	1810.0	1810.2	88.75	105.7
87.80	1740.0	1743.5	1742.8	85.40	97.3
				Mean	**101.6**
				SD	**2.35**
				%RSD	**2.31**

A.7.3 Precision

The precision is a validation parameter that measures how close the agreement between the test results is if a series of measurements are done on the same sample (ICH, 2005:4). Precision can be divided in intra-day repeatability and inter-day repeatability. For the precision to be accepted, the %RSD must be ≤ 2% (Shabir, 2005:9).

A.7.3.1 Intra-day repeatability

A.7.3.1.1 Method

The standard solution was prepared and injected as described in Section A.7.2.1. The samples were prepared by dissolving 8.76, 8.40 and 8.78 mg carnosine in PBS (pH 7.4) in a 100 ml volumetric flask, respectively. Each solution was diluted to a ratio of 5 ml to 10 ml in a volumetric flask and all three samples were injected in duplicate. The recovery was calculated in concentration and percentage.

A.7.3.1.2 Results and discussion

The intra-day repeatability results are indicated in Table A.3. It had a %RSD of 1.57%, which is within the acceptable range of ≤ 2%.

Table A.3: The intra-day repeatability results of carnosine determination

Concentration (µg/ml)	Peak area 1	Peak area 2	Mean peak area	Recovery	
				(µg/ml)	%
43.75	900.0	909.5	905.0	44.36	101.4
42.00	883.5	882.4	883.0	43.29	103.1
43.75	884.1	886.3	885.0	43.40	99.2
				Mean	**101.2**
				SD	**1.58**
				%RSD	**1.57**

A.7.3.2 Inter-day repeatability

The inter-day repeatability was performed over three days.

A.7.3.2.1 Method

The following procedures were followed over the three different days:

Day 1: The standard solution and samples were prepared as described in Section A.7.3.1.1.

Day 2: A standard solution was prepared by weighing 10.16 mg carnosine and dissolving it in PBS (pH 7.4) in a 100 ml volumetric flask. This solution was injected in duplicate in injection volumes of 2, 4, 6, 8 and 10 µl. The samples were prepared by dissolving 9.48, 9.10 and 9.12 mg carnosine in PBS (pH 7.4) in a 100 ml volumetric flask, respectively. Next, 5 ml of each solution was diluted to 10 ml in a volumetric flask.

Day 3: A standard solution was prepared by weighing 10.08 mg carnosine and dissolving it in PBS (pH 7.4) in a 100 ml volumetric flask. This solution was injected in duplicate in injection volumes of 2, 4, 6, 8 and 10 µl. The samples were prepared by dissolving 9.25, 9.04 and 9.10 mg carnosine in PBS in a 100 ml volumetric flask, respectively. Next, 5 ml of each solution was diluted to 10 ml in a volumetric flask.

A.7.3.2.2 Results and discussion

The inter-day repeatability %RSD of carnosine was calculated as 1.84%. The variance between intra-day and inter-day repeatability did not differ significantly. The repeatability was therefore within acceptable limits and when executed by different personnel in different laboratories, the assay will perform well. The results are indicated in Table A.4.

Table A.4: The inter-day repeatability results of carnosine determination

	Day 1	Day 2	Day 3	Between days
	101.40	98.00	102.90	
	103.10	102.30	101.40	
	99.20	100.00	104.10	
Mean	**101.22**	**100.09**	**102.79**	**101.36**
SD	**1.58**	**1.75**	**1.10**	**1.87**
%RSD	**1.57**	**1.75**	**1.07**	**1.84**

A.7.4 Ruggedness

Ruggedness can be divided into sample stability and system repeatability. The ruggedness is a validation parameter to measure whether the test results will be reproducible when the samples are analysed under different conditions (ICH, 2005:5). The test conditions can include doing the analysis over different time periods, in different laboratories with different machines and at different temperatures. The %RSD must be ≤ 2 for the results to be acceptable (Shabir, 2005:9-10).

A.7.4.1 Sample stability

A.7.4.1.1 Method

A standard sample was prepared by dissolving 5.0 mg carnosine in PBS (pH 7.4) in a 100 ml volumetric flask. The sample was injected for analysis every hour for 24 h to measure the stability. The injection volume was 20 µl.

A.7.4.1.2 Results and discussion

The mean recovery was 100.00% and the %RSD was calculated as 0.05%. These values, indicated in Table A.5, confirm stability of the sample over 24 h.

Table A.5: The sample stability of carnosine over 24 h

Time (h)	Peak area	%Recovery
0	2409.7	100.0
1	2409.9	100.0
2	2410.0	100.0
3	2410.0	100.0
4	2410.7	100.0
5	2411.1	100.1
6	2409.3	100.0
7	2410.2	100.0
8	2409.6	100.0
9	2413.0	100.1
10	2412.9	100.1
11	2411.2	100.1
12	2410.2	100.0
13	2410.8	100.0
14	2412.9	100.1
15	2411.1	100.1
16	2411.9	100.1
17	2408.9	100.0
18	2411.9	100.1
19	2409.3	100.0
20	2408.8	100.0
21	2408.7	100.0
22	2410.9	100.0
23	2410.8	100.0
24	2409.3	100.0
Mean	**2410.5**	**100.0**
SD	**1.25**	**0.05**
%RSD	**0.05**	**0.05**

A.7.4.2 System repeatability

A.7.4.2.1 Method

A standard sample was prepared by dissolving 5.0 mg carnosine in water in a 100 ml volumetric flask. The sample was injected seven times.

A.7.4.2.2 Results and discussion

The %RSD of the retention times was calculated as 0.154% and the %RSD for peak area was 0.060%. Both these values are less than 2.000% and are therefore acceptable. The repeatibility results are indicated in Table A.6.

Table A.6: The systems repeatability results of carnosine

Injection number	Peak area	Retention time (min)
1	2412.0	5.574
2	2411.0	5.583
3	2408.7	5.582
4	2408.4	5.596
5	2407.0	5.595
6	2409.0	5.591
Mean	**2409.00**	**5.587**
SD	**1.67**	**0.008**
%RSD	**0.07**	**0.141**

A.7.6 Robustness

The robustness is a validation parameter to measure if the analysis will be unaffected when small variations in method parameters are made deliberately (ICH, 2005:5). This parameter expresses the reliability of the HPLC method during normal usage. The method must prove to tolerate small changes in chromatographic conditions.

A.7.6.1 Method

A standard sample was prepared by dissolving 10.0 mg carnosine in 100 ml Milli-Q® HPLC grade water in a volumetric flask. The following changes were made to the chromatographic conditions:

Change 1:

Mobile phase: Acetonitrile/0.075 M octane sulphonic acid (22/78%) at pH 3.45

Flow rate: 0.8 ml/min

Injection volume: 18 µl

Detection wavelength: 208 nm

Change 2:

Mobile phase: Acetonitrile/0.075 M octane sulphonic acid (18/82%) at pH 3.45

Flow rate: 1.2 ml/min

Injection volume: 22 µl

Detection wavelength: 212 nm

Change 3:

Mobile phase: Acetonitrile/0.075 M octane sulphonic acid (20/80%) at pH 3.45

Flow rate: 1 ml/min

Injection volume: 10 µl

Detection wavelength: 210 nm

A.7.6.2 Results and discussion

The HPLC method tolerated the small changes in chromatographic conditions and the carnosine peak was easily identifiable, as seen in Figure A.5. The method will therefore perform well when used.

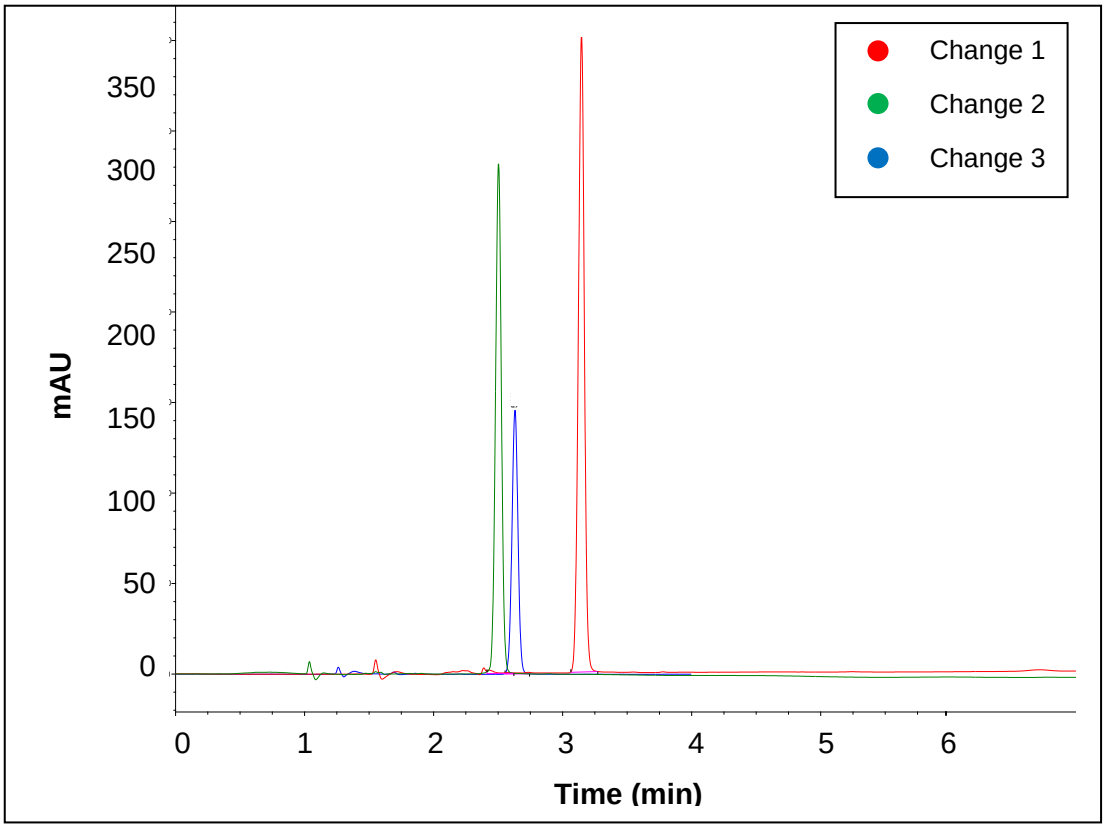

Figure A.5: The chromatogram obtained from the robustness analysis

A.7.7 Specificity

The specificity is a validation parameter to analyse carnosine in the presence of components such as impurities or degradants which are expected to be present (ICH, 2005:4). The degraded samples must not interfere with the determination of carnosine (ICH, 2005:4).

A.7.7.1 Method

A standard sample was prepared by weighing 10.16 mg carnosine and dissolving it in Milli-Q® HPLC grade water in a 100 ml volumetric flask; 5 ml of this solution was diluted to 10 ml in a volumetric flask with Milli-Q® HPLC grade water. The analytical samples were prepared by adding 1 ml of the standard sample to 200 µl hydrochloric acid (HCl), sodium hydroxide (NaOH) and hydrogen peroxide (H_2O_2), respectively. These samples were vortexed for 2 min and injected in duplicate for HPLC analysis.

A.7.7.2 Results and discussion

The carnosine peak was easily identifiable in the presence of the degradants. The degradation products did not interfere with carnosine determination. Figures A.6 to A.9 represent the chromatograms obtained from the specificity analysis.

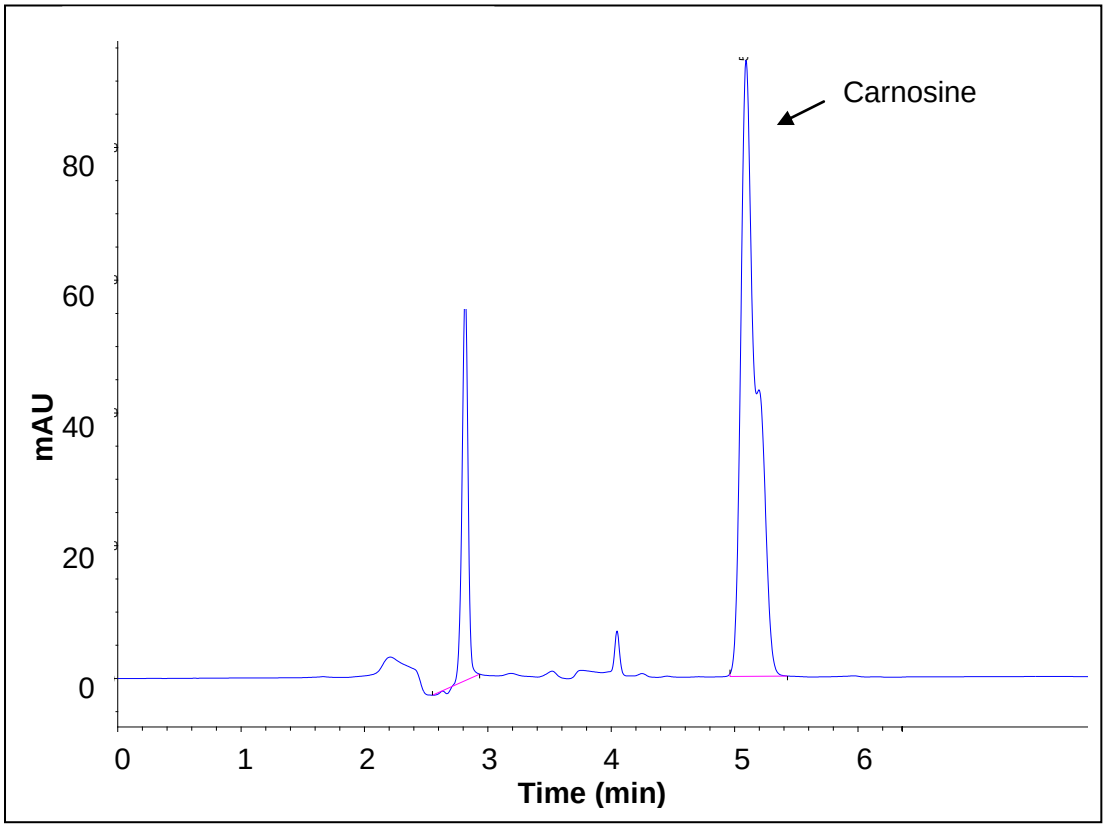

Figure A.6: The chromatogram obtained from the sample mixed with HCl

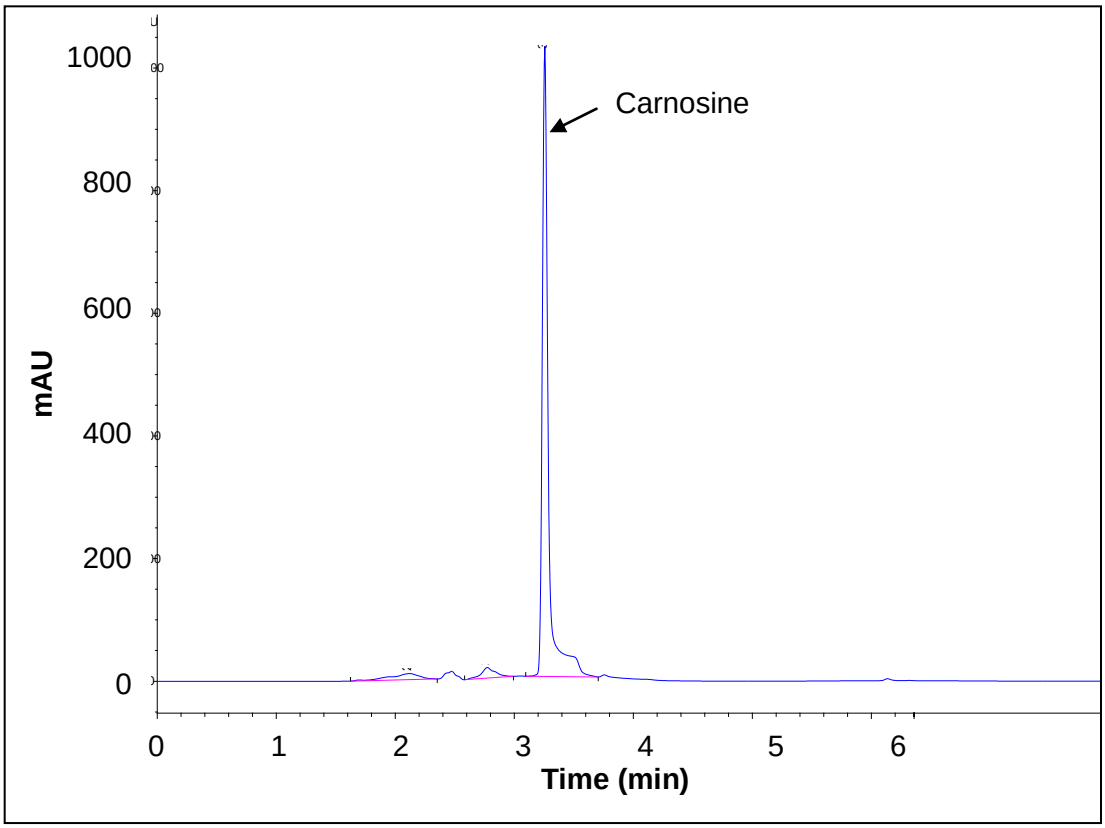

Figure A.7: The chromatogram obtained from the sample mixed with NaOH

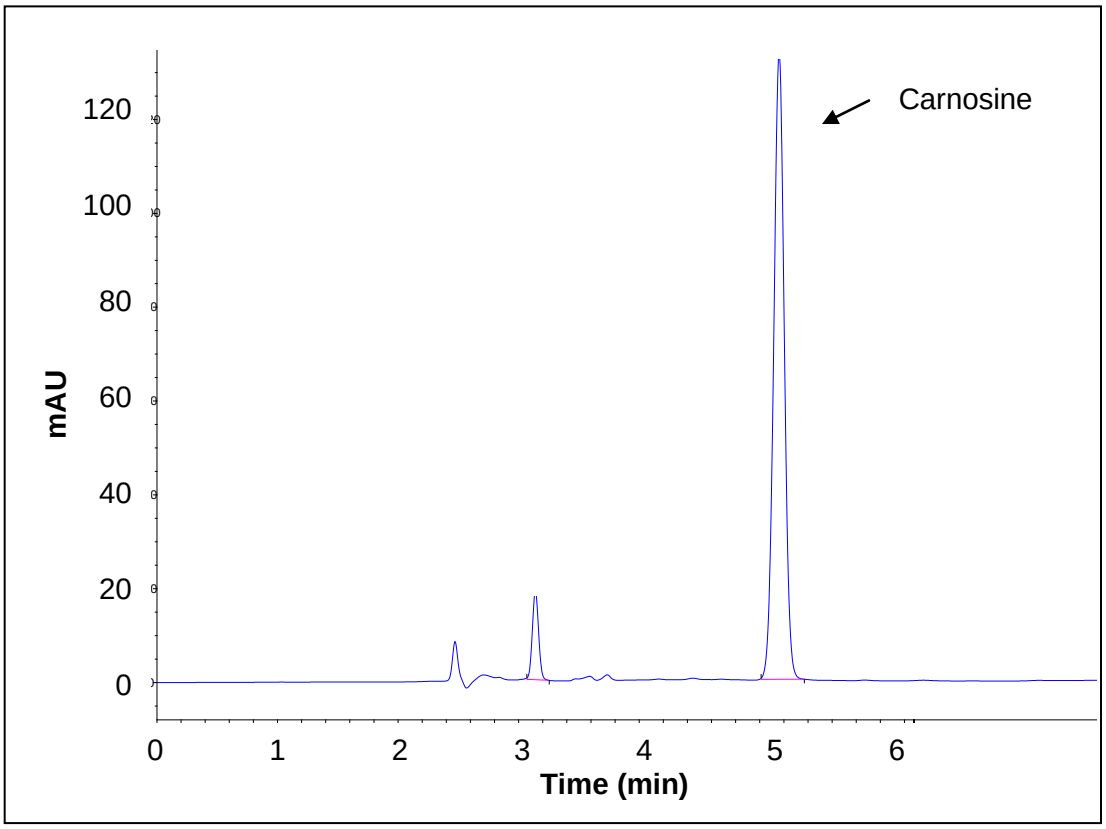

Figure A.8: The chromatogram obtained from the sample mixed with H_2O_2

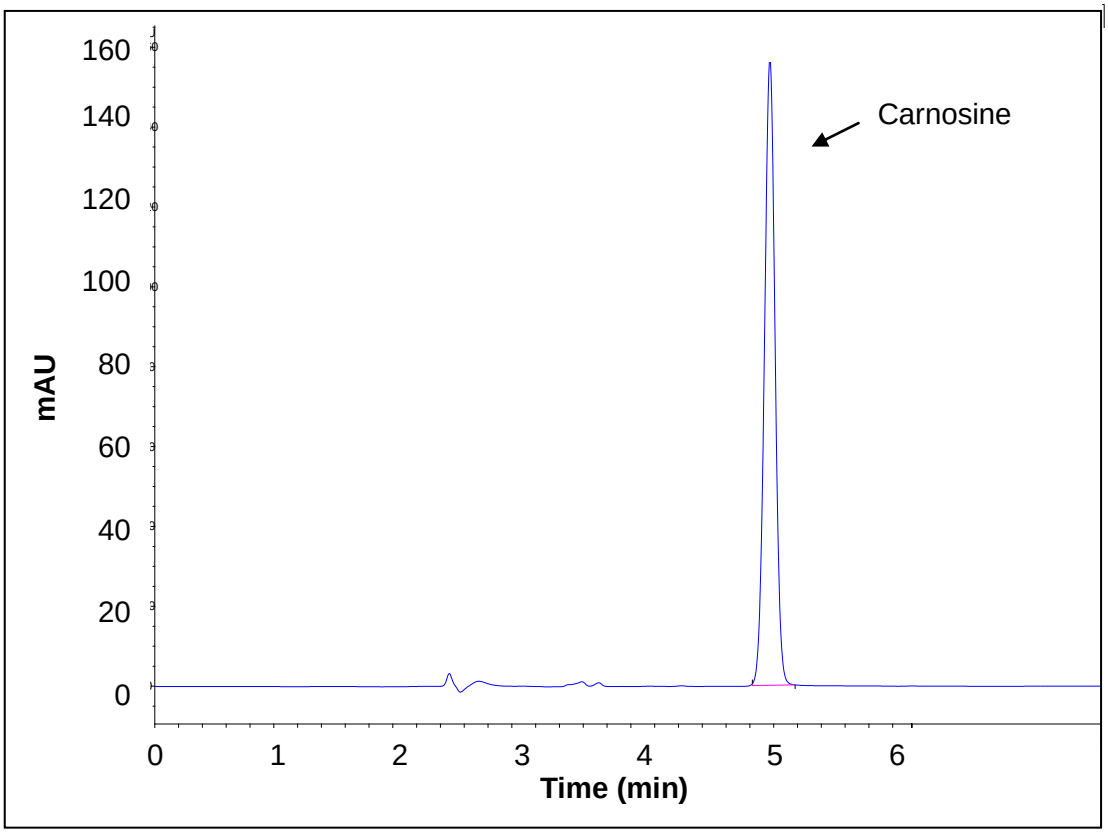

Figure A.9: The chromatogram obtained from the sample mixed with water

A.7.8 Limit of detection and limit of quantification

The limit of detection (LOD) is described as the lowest concentration of the analyte that the procedure can reliably detect (CDER, 2001:6). The lower limit of quantification (LLOQ) is the lowest concentration of the analyte that can be quantitatively determined with acceptable precision and accuracy (CDER, 2001:6). The LLOQ is acceptable if the analyte peak is discrete, identifiable and reproducible with a mean value within 20% of the actual value (CDER, 2001:6).

A.7.8.1 Method

A standard sample was prepared by weighing 34.70 mg of carnosine and dissolving it in Milli-Q® HPLC grade water in a 100 ml volumetric flask. The solution was diluted to a concentration of 0.1735 µg/ml and injected in different volumes for both the LOD and LLOQ tests.

A.7.8.2 Results and discussion

Tables A.7 and A.8 represent the results obtained from the LOD and LLOQ analyses. The LOD was 0.08675 µg/ml and the peak was identifyable but not quantifiable with a %RSD of 12.01. The LLOQ was 0.17350 µg/ml, the peak was quantifiable and the %RSD (6.14%) ensures minimal variation amongst samples.

Table A.7: The LOD data for carnosine

Concentration (µg/ml)	0.08675	0.17350	0.26025
Area	2.2	4.7	7.1
	2.2	6.0	7.5
	2.8	5.1	7.4
	2.9	5.9	6.8
	2.4	4.9	7.8
	2.3	5.3	6.4
	2.1	5.1	6.9
Mean	**2.41**	**5.29**	**7.13**
SD	**0.29**	**0.45**	**0.44**
%RSD	**12.01**	**8.61**	**6.17**

Table A.8: The LLOQ data for carnosine

Concentration (µg/ml)	0.08675	0.17350	0.26025	0.34600
Area	2.60	3.90	6.20	7.80
	1.50	4.10	6.10	7.70
	1.80	4.00	6.20	8.20
	2.00	4.40	5.80	7.60
	2.00	4.60	6.40	7.70
	1.40	4.00	6.70	7.50
	1.90	4.50	5.80	7.70
Mean	**1.89**	**4.21**	**6.17**	**7.74**
SD	**0.36**	**0.26**	**0.30**	**0.21**
%RSD	**19.31**	**6.14**	**4.80**	**2.66**

A.8 Conclusion

The HPLC method was successfully validated. It was adequately reliable and sensitive for the determination of the active ingredient, carnosine, in different study samples.

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CDER ***see*** Center for Drug Evaluation and Research.

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APPENDIX B

FORMULATION OF VESICULAR AND PROVESICULAR SYSTEMS ENTRAPPING CARNOSINE

B.1 Introduction

Carnosine is a hydrophilic active ingredient, which does not have the ideal characteristics to enable it to diffuse through the stratum corneum. One way to promote diffusion is to incorporate the active into a vesicular system. Vesicular systems offer a number of advantages such as non-toxicity, biodegradability, modulation of bioavailability for systemic actives and the amphiphilic nature (Muzzalupo *et al.*, 2011:28). Niosomes is a vesicular system often used to increase the permeation of especially hydrophilic actives across the skin (Marianecci *et al.,* 2013:65; Muzzalupo *et al.*, 2011:28).

Niosomes can be formulated using a number of methods, which include, but are not limited to, reverse phase evaporation, the bubble method, ethanol injection method, heating method and microfluidisation (Nasir *et al.,* 2012:481). The method of preparation must be chosen carefully, for it can have an effect on important characteristics, such as size distribution, number of bilayers, EE% and the stability of the niosomes (Tangri & Khurana, 2011:50).

During this study, niosomes were prepared by both the thin-film hydration method and by hydrating proniosomes. According to Agarwal *et al.* (2001:45), the thin-film hydration method involves dissolving the chosen surfactants in an appropriate organic solvent for evaporation to occur. After evaporation, a thin lipidic film, visible inside the flask, is hydrated with an aqueous phase. Depending on the hydrophilic and lipophilic properties of the active, it is either incorporated into the lipophilic or aqueous phase. According to Marianecci *et al.* (2013:71), the proniosome method produces a dry dispersion by coating water-soluble carriers, which include sugars such as sorbitol, lactose and glucose, with the surfactants. A thin film then covers each particle and a dry powder is obtained after evaporation of the organic phase. The dry powder can be stored and hydrated before use. Upon hydration, the sugar dissolves into the aqueous phase to entrap the active in the niosomes.

To ensure high quality of the final product, the vesicular systems must be characterised after preparation. Parameters such as morphology, size and PdI, zeta-potential and EE% can affect the stability and *in vivo* performance and are critical to evaluate (Marianecci *et al.,* 2013:74).

B.2 Ingredients used to formulate vesicular and provesicular systems

B.2.1 Carnosine

Carnosine (Sigma-Aldrich, Aston Manor, SA) is the active ingredient in this study, having various clinical benefits due to its antioxidative and antiglycating properties (Hipkiss *et al.,* 2002:285-294; Kyriazis, 2010:45). It is water-soluble, and will be entrapped in the hydrophilic layer of the vesicle system. The formulation involves dissolving carnosine in the aqueous phase and hydrating the lipidic film for entrapment to occur.

B.2.2 Purified water

Milli-Q® HPLC grade, prepared in-house with a Milli-Q purification system, (Merck Millipore, Modderfontein, SA) water will be used as the aqueous phase to hydrate the lipidic film. Carnosine is soluble in water, and purified water will be used as the aqueous phase throughout the study (MSDS, 2013).

B.2.3 Cholesterol

According to Mahale *et al.* (2012:48-49), cholesterol (Sigma-Aldrich, Aston Manor, SA) has an influence on the structure and physical properties of niosomes due to the biological interaction with the non-ionic surfactant. The addition of cholesterol will ensure rigidity and fluidity of the vesicles, and regulate cohesion and water permeability of the membrane (Marianecci *et al.,* 2013:67). Cholesterol forms part of the lipophilic phase of the vesicles.

B.2.4 Non-ionic surfactants

Non-ionic surfactants, which consist of a hydrophilic head and a hydrophobic tail (Marianecci *et al.,* 2013:65), are often used as surface active agents in vesicles due to advantages in respect to toxicity, stability and compatibility when compared to other surfactants (Kumar & Rajeshwarrao, 2011:209). Span® 60 (Merck Millipore, Modderfontein, SA) is the non-ionic surfactant used as a wetting agent, solubiliser and permeability enhancer for the formulation of the vesicular systems (Kumar & Rajeshwarrao, 2011:209). Together with cholesterol, it forms part of the lipophilic phase of the vesicles. Span® 60 produces vesicles with the highest EE% (entrapment efficiency) when compared to other surfactants (Mali *et al.,* 2013:590).

B.2.5 Organic solvent

Chloroform (Merck Millipore, Modderfontein, SA) is used as the organic solvent for the cholesterol and Span® 60 when the thin-film hydration method is used to formulate niosomes (Agarwal *et al.*, 2001:45). The chloroform will evaporate while the lipidic film is prepared.

B.2.6 Water soluble carriers

Sorbitol (Merck Millipore, Modderfontein, SA) is the water-soluble carrier chosen for the preparation of proniosomes. It is coated with a thin film of dry surfactant to result in proniosomes, and this dry powder has to be hydrated with the aqueous phase before use (Mahale *et al.,* 2012:50-51). Upon hydration, the sorbitol particles will dissolve into the hydration medium, resulting in entrapment of the active ingredient (Marianecci *et al.,* 2013:71).

B.3 Preparation of vesicular systems

Four different niosome dispersions were prepared in order to determine which formula had the most favourable characteristics. The ratio of cholesterol, Span® 60 and carnosine was approximately 2:1:1. Three different samples containing 1%, 2% and 3% carnosine were prepared and characterised. The fourth sample was a placebo i.e. did not contain the active (carnosine).

Six different proniosome dispersions were prepared from the niosome formula that represented the best characteristics. These samples contained 2 g, 3 g and 4 g sorbitol, respectively. Three of the six proniosome dispersions were placebos and three were prepared including carnosine as active. These samples were characterised after hydration to determine the optimal amount of sorbitol needed.

B.4 Characteristics of the vesicular systems

Vesicle morphology, size and PdI, zeta-potential and EE% were determined for all the named preparations. The final niosome and proniosome formulas, with the most favourable characteristics, were chosen from the different formulas and used for diffusion studies and formulation of semi-solid dosage forms.

B.5 Methods

B.5.1 General method used to prepare niosomes

The niosomes were prepared by dissolving the cholesterol and Span® 60 in chloroform and placing the solution on a magnetic stirrer in a hood. The temperature was controlled at 60 °C for the chloroform to evaporate; this is slightly above the transition temperature of Span® 60 (56-58 °C) (Kumar & Rajeshwarrao, 2011:210). As soon as all the chloroform evaporated, the mixture formed a lipid layer on the bottom of the beaker. The aqueous phase, which was either purified water or purified water containing carnosine, depending on the formula, was added and the mixture was stirred. The mixture was allowed to cool down on ice before sonication. Figures B.1 and B.2 indicate important apparatus used during preparation of the niosomes.

Figure B.1: The heating plate and magnetic stirrer

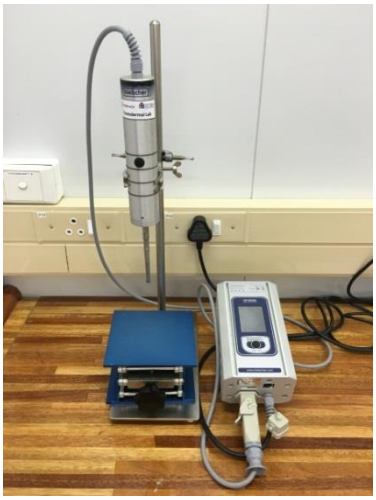

Figure B.2: The sonicator

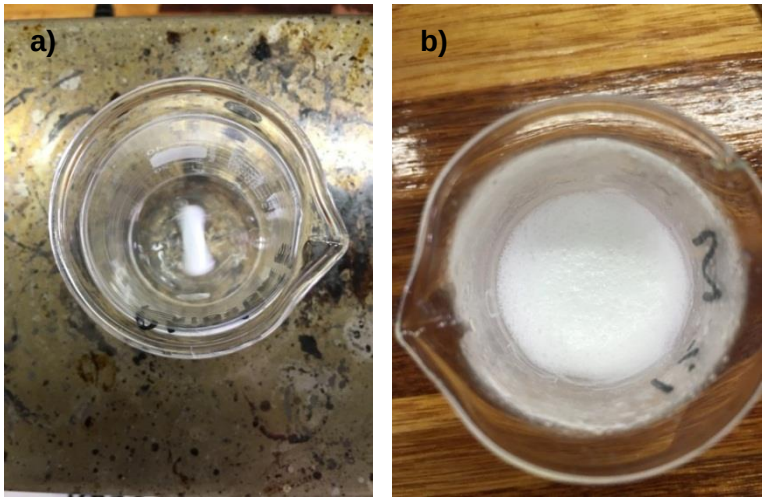

Figure B.3: The formulation process of niosomes: a) Span® 60 and cholesterol dissolved in chloroform on the magnetic stirrer to form the lipidic film and b) the final niosome dispersion obtained

B.5.2 General method used to prepare proniosomes

The proniosomes were prepared by dissolving the cholesterol and Span® 60 in chloroform. The amount of sorbitol used for the formula was weighed into a 50 ml beaker and placed on a magnetic stirrer in a hood. The chloroform solution was added drop wise to the sorbitol to prevent over wetting and ensure proper evaporation of the chloroform. The proniosomes was put in a dessicator overnight to ensure complete dryness. The dry powder obtained previously was placed on the magnetic stirrer and the aqueous phase, which was either purified water or purified water containing carnosine, depending on the formula, was added while stirring. The mixture was allowed to cool down on ice before sonication. Figure B.4 indicates the preparation process of proniosomes, whereas Figure B.5 indicates the dessicator used.

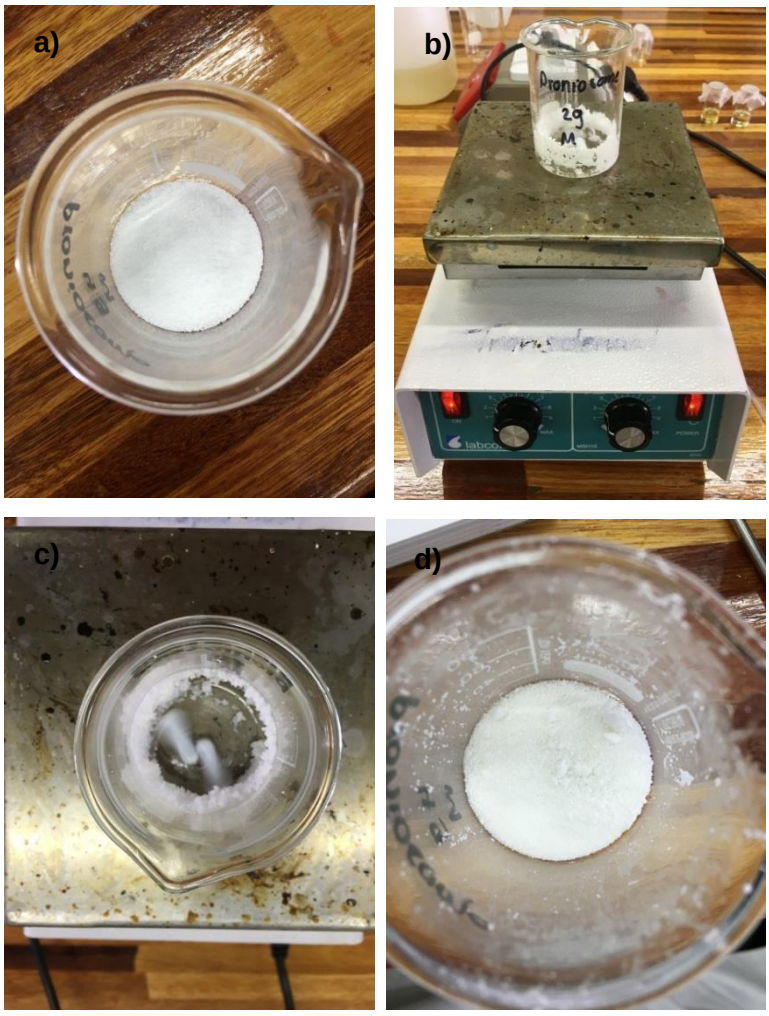

Figure B.4: The formulation process of proniosomes: a) sorbitol powder, b) sorbitol wetted with the chloroform mixture on the heating plate, c) mixing process for evaporation and d) the dry powder obtained

Figure B.5: The dessicator

B.5.3 Morphology

The morphology of the vesicular systems was determined in conjunction with Dr A Jordaan, Laboratory for Electron Microscopy, North-West University, Potchefstroom Campus. A transmission electron microscope (TEM), FEI Tecnai G2 (FEI, Holland), at 120 Kv was used to prove the formation of the vesicles and to analyse the morphology. One drop of the vesicle system was diluted with 3 ml of water. A drop of the diluted solution was placed on a copper grid (carbon-coated 300 mesh). Osmium was used to oxidise the lipids, while the fluid phase evaporated. After complete evaporation, the sample was coloured with two heavy metals, uranyl acetate and lead citrate. The first heavy metal was used to induce binding sites for the second heavy metal to bind and colour the sample. The excess fluid was removed by filter paper and the grid was allowed to air dry completely for about 10 min. Only the placebo niosome and proniosome dispersions were used to determine the morphology of the vesicles, as no active ingredients may be included when working on the TEM.

B.5.4 Vesicle size and polydispersity index

The vesicle size and PdI were determined by using a Malvern Zetasizer Nano (Nano SZ) (Malvern Instruments, United Kingdom) at a temperature of 25 °C. The samples were diluted (0.5 ml/20.0 ml) with purified water to reduce particle interactions. This experiment was done in triplicate on each formula.

B.5.5 Zeta-potential

The zeta-potential of the dispersions was determined using a Malvern Zetasizer Nano (Nano SZ) (Malvern Instruments, United Kingdom) at 25 °C. The samples were diluted

(0.5 ml/20.0 ml) with purified water to reduce particle interactions. This experiment was done in triplicate on each formula. The zetasizer and zetasel used are indicated in Figure B.6.

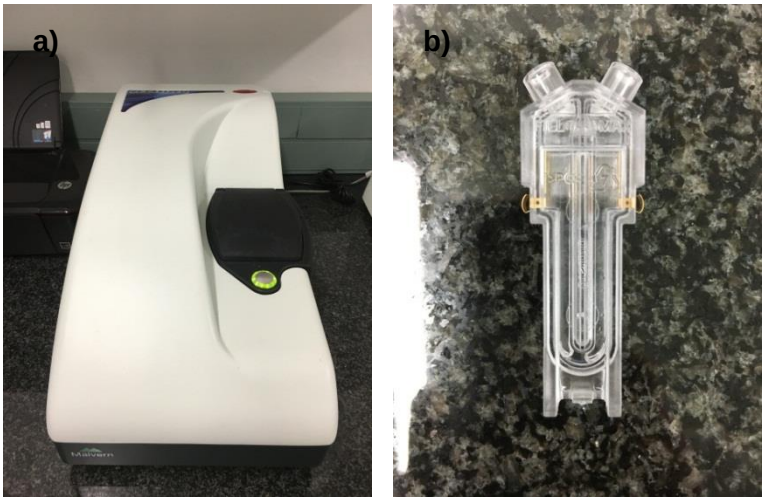

Figure B.6: a) The Zetasizer and b) a cell used in the Zetasizer

B.5.6 Entrapment efficiency

The difference between the un-entrapped free active and the total amount of active in the vesicle system was determined by HPLC. A calibration curve was created using solutions of carnosine in Milli-Q® (HPLC grade) water. The niosome dispersions were diluted to 20 ml and placed in Eppendorf® tubes. A Beckman Coulter Optima™ L-100XP Ultracentrifuge (Beckman Coulter, South Africa) was used to centrifuge the samples at 25 °C for 30 min at 25 000 rpm. The proniosome dispersions were diluted to 40 ml and placed in Eppendorf® tubes and centrifuged for 30 min at 25 000 rpm. The clear supernatant was then extracted for HPLC analysis. The following equation, adapted from Mali *et al.* (2013:588), was used to determine the percentage EE%:

$$EE\% = Cr / Ct \times 100 \qquad \textbf{Equation B.1}$$

Where Cr is the amount of carnosine entrapped and Ct is the total amount of carnosine used.

B.6 Formulating and testing for the optimised vesicle preparation

B.6.1 Preparation of the niosomes

Four niosome samples were formulated and characterised. The samples contained 1% **(1)**, 2% **(2)** and 3% **(3)** active ingredient respectively; the fourth sample **(4)** was a placebo. The formulas are indicated in Tables B.1 and B.2. The active ingredient was incorporated during formulation by dissolving it in the water phase. The ratio of cholesterol, Span® 60 and carnosine

remained 2:1:1 throughout the formulation process. After characterisation of the vesicles, the ideal concentration of the active for the final formula was chosen.

Table B.1: The placebo niosome formula **(4)**

Vesicle component	Amount
Cholesterol	600 mg
Chloroform	10 ml
Span® 60	300 mg
Milli-Q® water	10 ml

Table B.2: The niosome formulas containing carnosine

Vesicle component	(1)	(2)	(3)
Cholesterol	200 mg	400 mg	600 mg
Chloroform	10 ml	10 ml	10 ml
Span® 60	100 mg	200 mg	300 mg
Carnosine	100 mg	200 mg	300 mg
Milli-Q® water	10 ml	10 ml	10 ml

B.6.1.1 Results and discussion

B.6.1.1.1 Transmission electron microscope

The morphology of the vesicular systems includes the shape of the vesicles as well as the composition between the aqueous and lipid phases in the formula. The micrographs obtained from the TEM are shown in Figure B.7. The vesicles formed and were in the nanometric scale. The light regions visible are the vesicle membranes and osmium fixed to the lipid layers of the droplets. The shape of the vesicles varied, but was mostly spherical. Only formula **(4)** was used on the TEM, as no active ingredients may be included in this experiment as they could crystallise and damage the TEM.

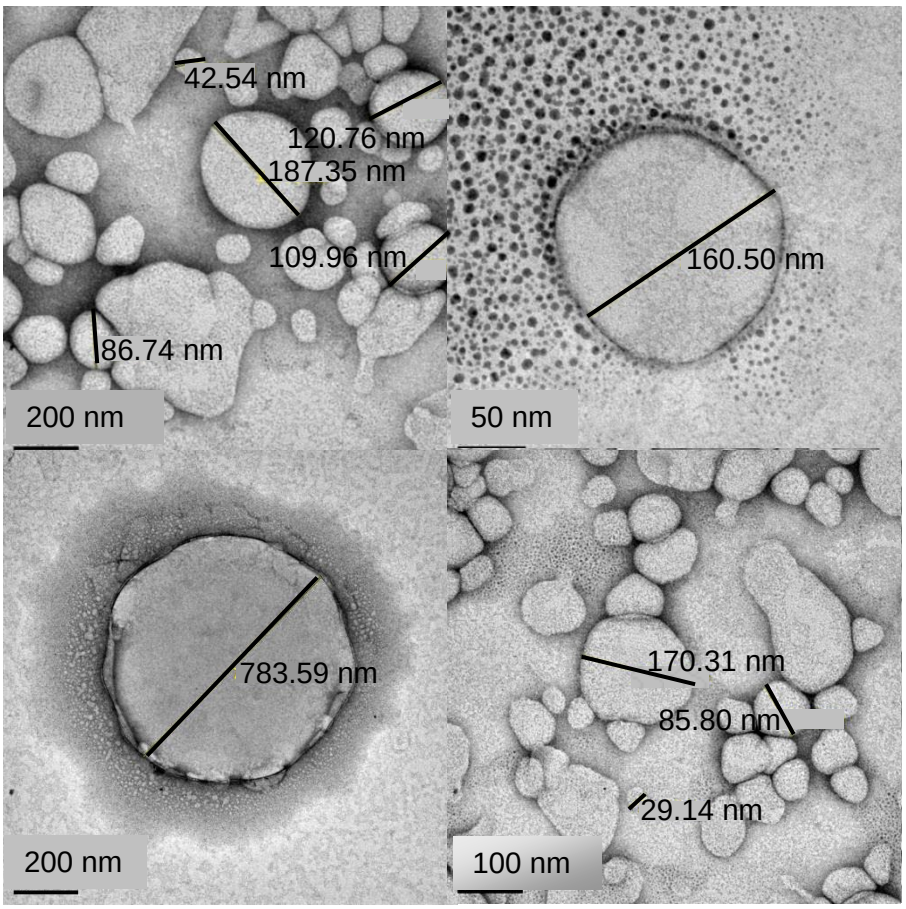

Figure B.7: Micrographs to illustrate the niosomes using the TEM

B.6.1.1.2 Vesicle size and PdI

Vesicle size and PdI can have an effect on both the physical properties and stability of the formula (Kumar & Rajeshwarrao, 2011:212; Marianecci *et al.,* 2013:74). The size of niosomes can differ according to the method used for formulation, but are usually in the nanometric scale (Tangri & Khurana, 2011:50). The PdI indicates size distribution and describes the uniformity of the dispersion; it should be as close to zero as possible, but a PdI of less than 0.400 is acceptable (Marianecci *et al.,* 2013:74; Nobbmann, 2014).

The average vesicle size and PdI of the formulas are indicated in Figures B.8 and B.9, as well as in Tables B.3 and B.4, respectively. Three sets of readings were obtained in triplicate (n=9) on each formula. Multi-lamellar vesicles containing active ingredient with a size of 100 - 400 nm formed. The average size of formula **(4)** was 566.66 ± 28.67 nm. The average size formulas **(1)**, **(2)** and **(3)** were 280.92 ± 4.44 nm, 268.56 ± 24.16 nm and 373.38 ± 13.78 nm, respectively. Formula **(2)** had the smallest droplets. All the vesicles that formed were in the nanometric scale.

The area of the peaks, in Figures B.8 and B.9, indicates the volume of a certain population and therefore the size distribution. Formula **(1)** had the best PdI results of 0.520 ± 0.033, formulas

(2) and **(3)** had PdI's of 0.655 ± 0.030 and 0.761 ± 0.049 respectively and formula **(4)** had an average PdI of 0.767 ± 0.005. The size distribution of the vesicular systems was poor, being not below 0.400 (Marianecci *et al.,* 2013:75). Despite these poor results, no linear correlation between the PdI value and the true monodispersity of a formula could be drawn by Gaumet *et al.* (2008:5).

Table B.3: The average vesicle size of the niosome formulas

	(4)	**(1)**	**(2)**	**(3)**
Vesicle size (nm)	607.10 544.00 548.87	281.80 275.10 285.87	254.63 302.53 248.50	360.20 392.40 367.53
Mean	566.66	280.92	268.55	373.38
SD	28.67	4.44	24.16	13.78
%RSD	5.06	1.58	8.99	3.69

Table B.4: The average PdI of the niosome formulas

	(4)	**(1)**	**(2)**	**(3)**
PdI	0.769 0.760 0.771	0.527 0.556 0.476	0.653 0.693 0.619	0.725 0.728 0.831
Mean	0.767	0.520	0.655	0.761
SD	0.005	0.033	0.030	0.049
%RSD	0.624	6.363	4.617	6.472

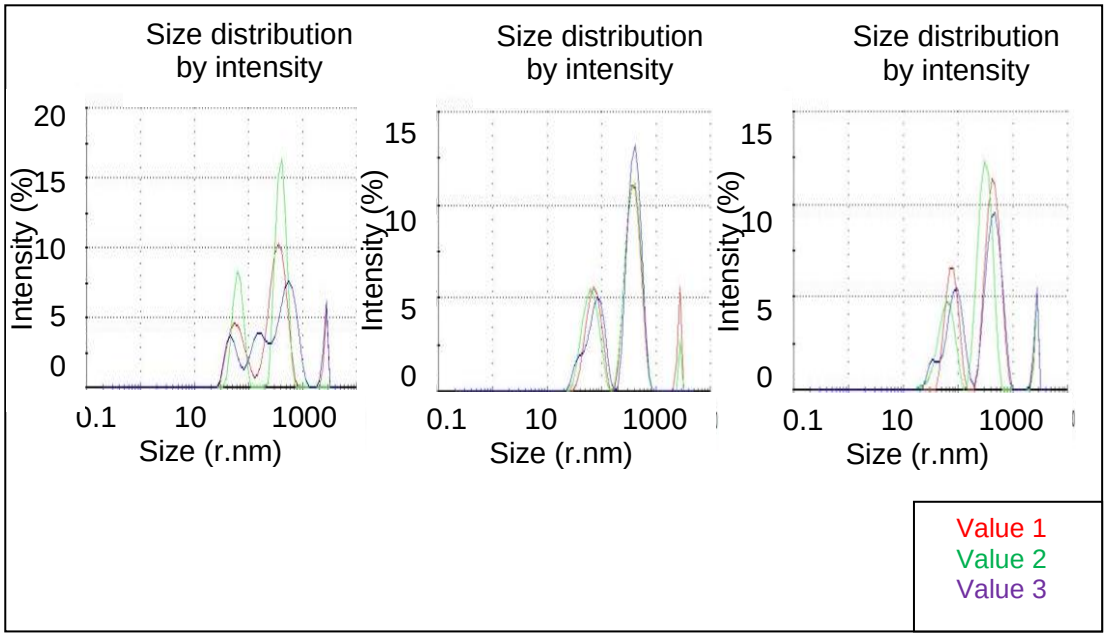

Figure B.8: The droplet size distribution of placebo niosome formula **(4)**

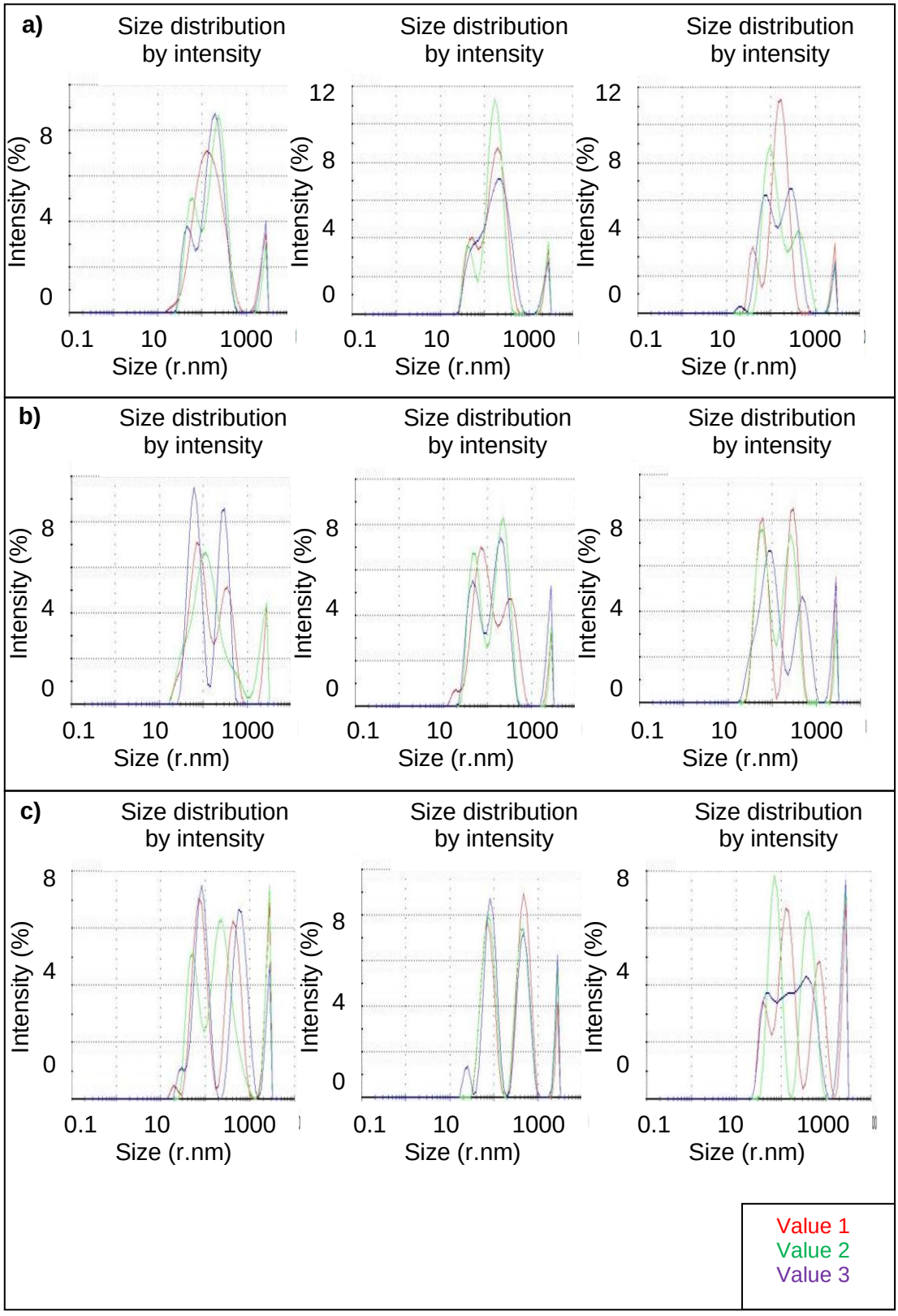

Figure B.9: The droplet size distribution of the niosome formulas containing carnosine: a) size distribution of formula **(1)**, b) size distribution of formula **(2)** and c) size distribution of formula **(3)**

B.6.1.1.3 Zeta-potential

The zeta-potential was measured to determine the surface charge of the droplets in the vesicular systems. The charge influences the system's stability and should be lower than - 30 mV or higher than + 30 mV to result in good stability (Malvern Instruments, 2015:3; Marianecci *et al.,* 2013:75).

The average zeta-potential of the niosome formulas are shown in Figure B.10. Formula **(4)** had the lowest average zeta-potential (- 69.63 ± 1.88 mV), whilst the average zeta-potential of formulas **(1)**, **(2)** and **(3)** were - 48.64 ± 0.89 mV, - 47.74 ± 0.54 mV and - 57.66 ± 1.59 mV, respectively. The zeta-potential of all the formulas was highly negative and indicated excellent stability. When omitting the placebo formula's **(4)** results, the niosomes containing 3% carnosine **(3)** had the highest zeta-potential and therefore the most stable of them all.

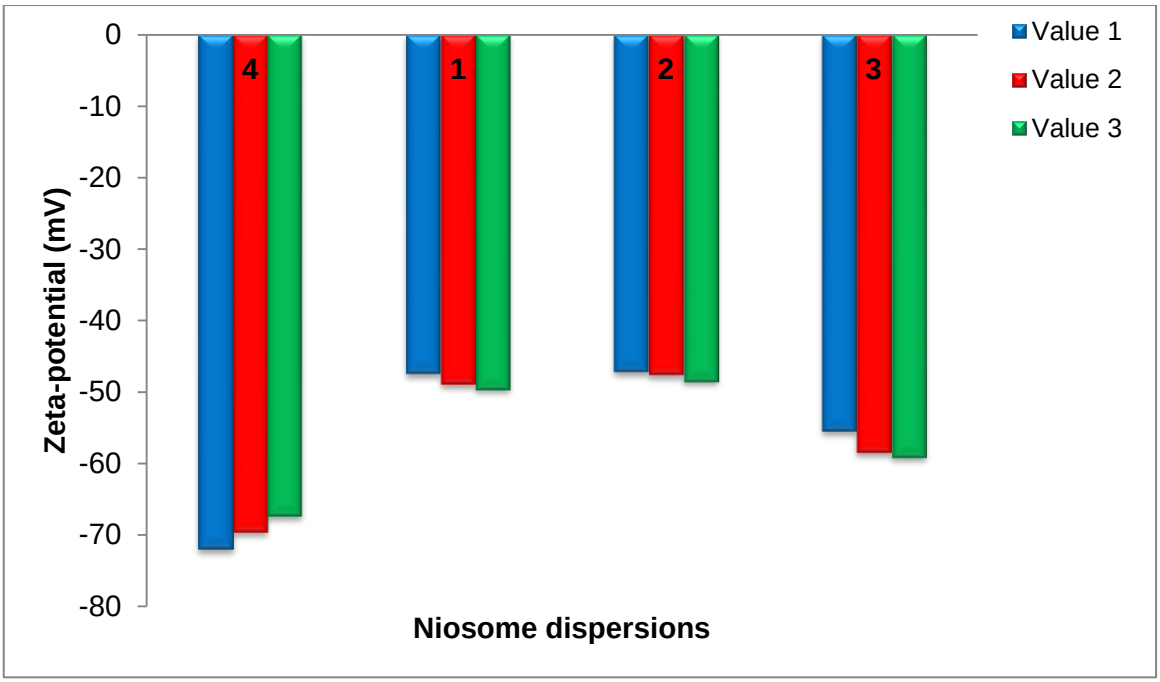

Figure B.10: The zeta-potential of the niosome formulas

B.6.1.1.4 Entrapment efficiency

The EE% refers to the amount of active ingredient that is effectively incorporated in the vesicle system and was one of the most important considerations to finalise the formula (Marianecci *et al.,* 2013:77).

The EE% of the formulas is indicated in Table B.5. Formula **(3)** had the best entrapment (46.73% ± 1.02), whilst formulas **(1)** and **(2)** had EE% values of 32.53 ± 0.83% and 39.14 ± 1.07%, respectively.

Table B.5: The entrapment efficiency of the niosome formulas

	(1)	(2)	(3)
%EE	31.60 32.38 33.62	40.65 38.33 38.45	45.49 46.71 47.99
Mean	32.53	39.14	46.72
SD	0.83	1.07	1.02
%RSD	2.55	2.73	2.19

B.6.1.2 Conclusion

The final niosome formula was chosen according to the results obtained from various tests. The tests conducted on the formulas included TEM, vesicle size and PdI, zeta-potential and EE%. The formula containing 3% carnosine was chosen as it had excellent stability, acceptable size and size distribution results, plus the best EE%. A higher concentration formula is also preferred to enhance the *in vitro* performance. The 3% concentration was used to prepare the proniosome formulas.

B.6.2 Preparation of the proniosomes

Six provesicular systems were prepared and characterised. Three formulas contained no active ingredient and 2 g **(5)**, 3 g **(6)** and 4 g **(7)** sorbitol, respectively; the other three formulas contained active ingredient and 2 g **(8)**, 3 g **(9)** and 4 g **(10)** sorbitol, respectively. The formulas are indicated in Tables B.6 and B.7. The ideal amount of sorbitol was determined according to the characteristic results of the formulas.

Table B.6: The placebo proniosome formulas

Vesicle component	(5)	(6)	(7)
Sorbitol	2 g	3 g	4 g
Chloroform	10 ml	10 ml	10 ml
Cholesterol	600 mg	600 mg	600 mg
Span® 60	300 mg	300 mg	300 mg
Milli-Q® water	10 ml	10 ml	10 ml

Table B.7: The proniosome formulas containing carnosine

Vesicle component	(8)	(9)	(10)
Carnosine	300 mg	300 mg	300 mg
Sorbitol	2 g	3 g	4 g
Chloroform	10 ml	10 ml	10 ml
Cholesterol	600 mg	600 mg	600 mg
Span® 60	300 mg	300 mg	300 mg
Milli-Q® water	10 ml	10 ml	10 ml

B.6.2.1 Results and discussion

B.6.2.1.1 Transmission electron microscopy

The morphology of the vesicles obtained from the TEM is shown in Figure B.11. The morphology includes the formation of the vesicles, as well as the vesicles' shape and sizes. The provesicles formed are larger than the vesicles, but are still in the nanometric scale. The dark regions visible are the vesicle membranes and osmium fixed to the lipid layers of the droplets. The shape of the vesicles is mostly spherical. Only formula **(5)** was used on the TEM as no active ingredients may be included in this experiment.

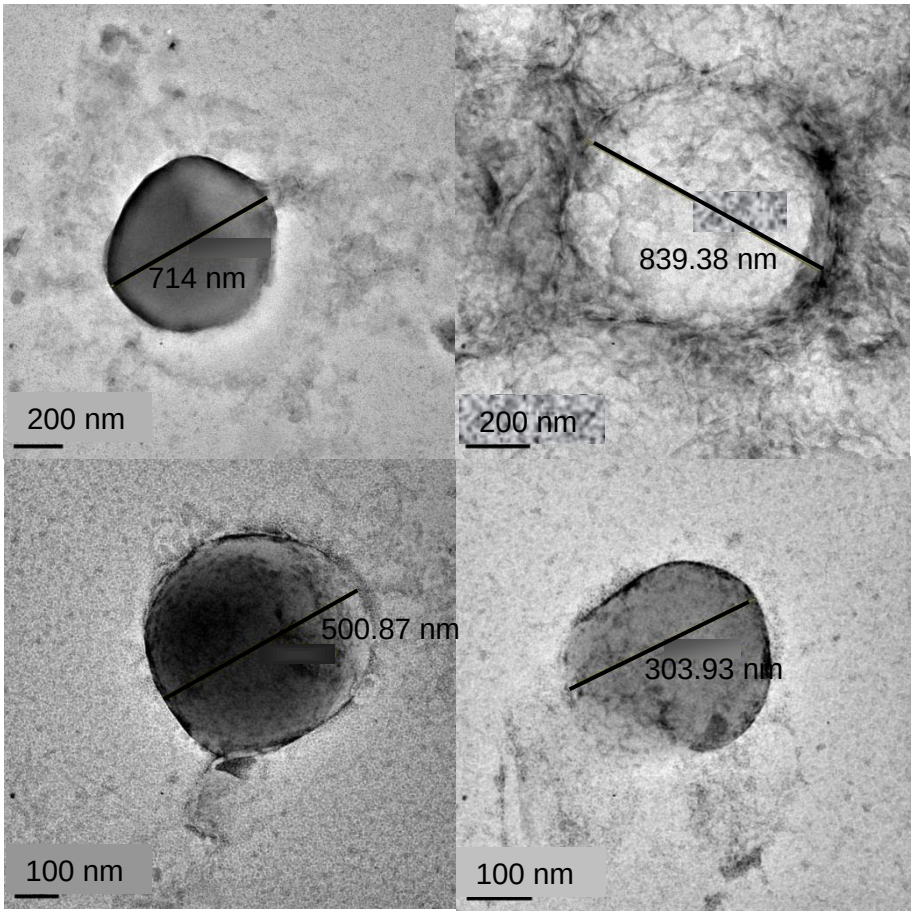

Figure B.11: Micrographs to illustrate the proniosomes using the TEM

B.6.2.1.2 Vesicle size and PdI

As mentioned in Section B.6.1.1.2, the vesicle size and PdI affect both the physical properties and stability of the formula (Kumar & Rajeshwarrao, 2011:212; Marianecci *et al.,* 2013:74). The size of proniosomes can differ in respect to the method of preparation, but are generally in the nanometric scale (Tangri & Khurana, 2011:50). The PdI is indicative of the size distribution. Dispersions with a PdI of less than 0.400 are usually uniform (Marianecci *et al.,* 2013:74; Nobbmann, 2014).

The average vesicle size and PdI of the formulas are indicated in Figures B.12 and B.13, and Tables B.8 and B.9 respectively. Three sets of readings were obtained in triplicate (n=9) on each formula. Multi-lamellar provesicles formed and were slightly larger than the vesicles. The average sizes of formulas **(5)**, **(6)** and **(7)** were 469.94 ± 94.26 nm, 512.233 ± 9.43 nm and 453.24 ± 14.65 nm, respectively and formulas **(8)**, **(9)** and **(10)**, 417.00 ± 32.49 nm, 463.62 ± 12.30 nm and 453.79 ± 9.89 nm, respectively. All the vesicles that formed were in the nanometric scale.

The area of the peaks in Figures B.12 and B.13 indicates the size distribution of the provesicular systems. Both the placebo and the active proniosome formula containing 2 g sorbitol had the best PdI results. The PdI of formula **(5)** was 0.682 ± 0.071 and of formula **(8)**, 626 ± 0.029. When considering the placebo formulas, the size distribution of formula **(6)** was the most unfavourable (0.831 ± 0.022). Conversely, among the proniosomes containing 3% carnosine, formula **(10)** had the most unfavourable results with an average PdI of 0.798 ± 0.038. Overall, the size distribution of the provesicular systems was poor and not below 0.400, but as mentioned previously, Gaumet *et al.* (2008:5) could not have drawn a linear correlation between the PdI value and the true monodispersity of the formula.

Table B.8: The average vesicle size of the proniosome formulas

	(5)	**(6)**	**(7)**	**(8)**	**(9)**	**(10)**
Vesicle size (nm)	600.80 382.50 426.53	504.23 507.00 525.47	460.73 466.23 432.77	462.87 396.37 391.77	480.70 457.93 452.23	442.37 452.50 466.50
Mean	469.94	512.23	453.24	417.00	463.62	453.79
SD	94.26	9.43	14.65	32.49	12.30	9.89
%RSD	20.06	1.84	3.23	7.79	2.65	2.18

Table B.9: The average PdI of the proniosome formulas

	(5)	(6)	(7)	(8)	(9)	(10)
PdI	0.773 0.600 0.674	0.853 0.839 0.802	0.790 0.760 0.769	0.590 0.660 0.627	0.749 0.719 0.678	0.850 0.781 0.762
Mean	0.682	0.831	0.773	0.626	0.715	0.798
SD	0.071	0.023	0.013	0.029	0.029	0.038
%RSD	10.387	2.588	1.626	4.570	4.068	4.740

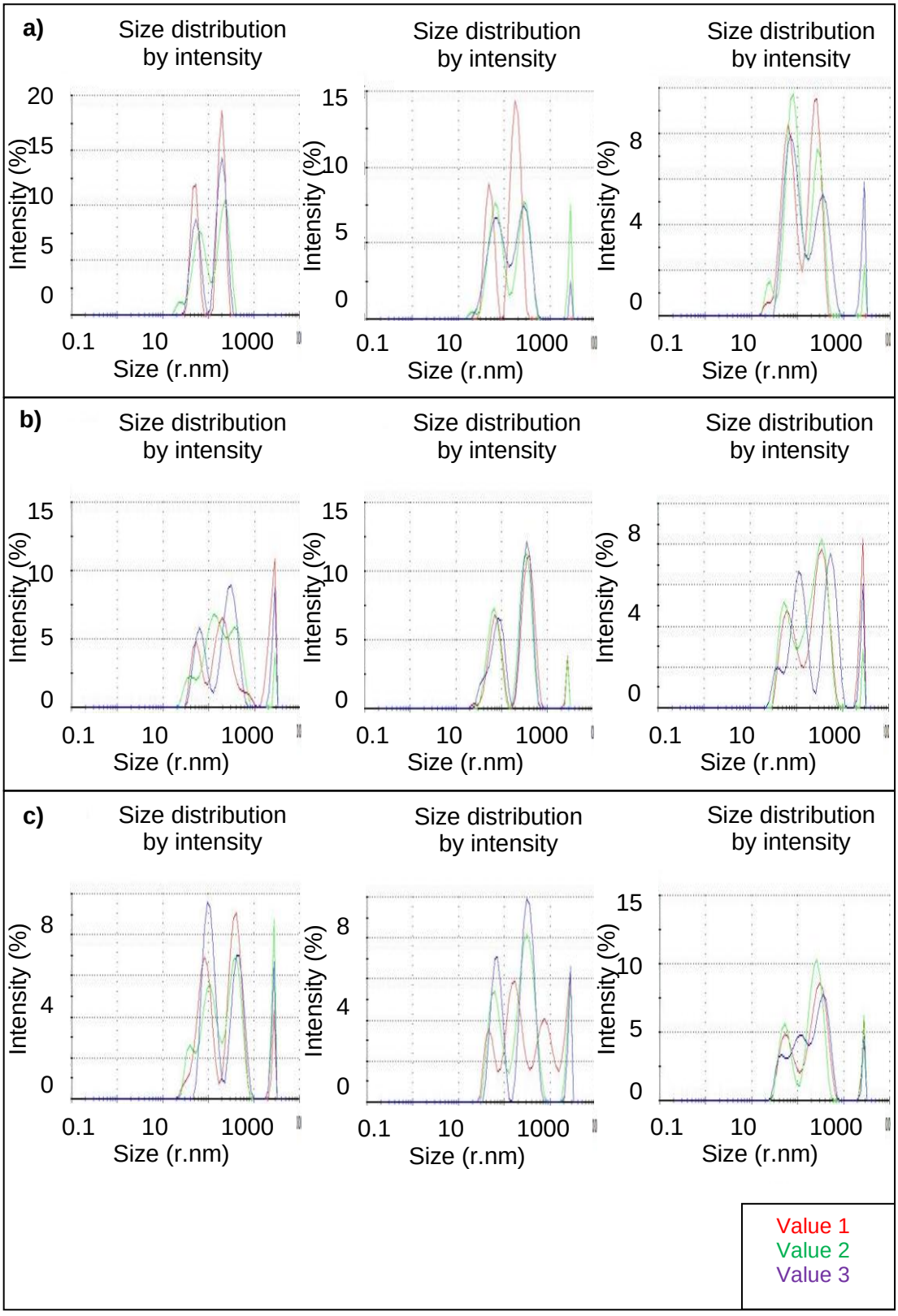

Figure B.12: The droplet size distribution of the placebo proniosome formulas: a) size distribution of formula **(5)**, b) size distribution of formula **(6)** and c) size distribution of formula **(7)**

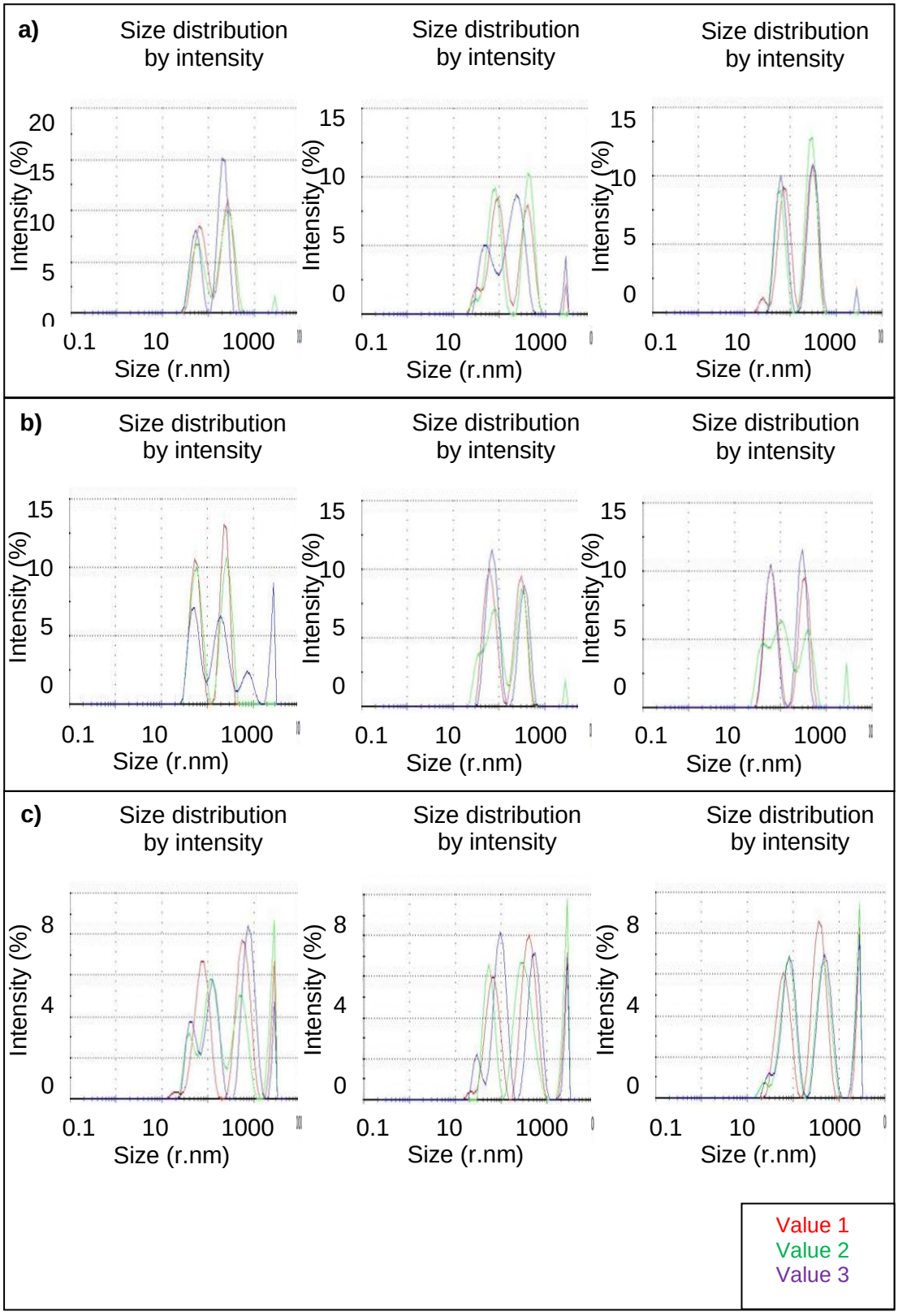

Figure B.13: The droplet size distribution of the proniosomes containing 3% carnosine: a) size distribution of formula **(8)**, b) size distribution of formula **(9)** and c) size distribution of formula **(10)**

B.6.2.1.3 Zeta-potential

The zeta-potential was measured to determine the surface charge of the droplets in the vesicular systems. The charge influences the system's stability and should be lower than - 30 mV or higher than + 30 mV to obtain good stability (Malvern Instruments, 2015:3; Marianecci *et al.,* 2013:75).

The average zeta-potential of the proniosome formulas are shown in Figure B.14. The average zeta-potential of formulas **(5)**, **(6)** and **(7)** were - 55.47 ± 0.34 mV, - 45.93 ± 1.36 mV and - 47.46 ± 0.55 mV. The average zeta-potential of formulas **(8)**, **(9)** and **(10)** were - 46.64 ± 1.48 mV, - 47.94 ± 0.79 mV and - 50.34 ± 0.53 mV. The zeta-potential of all the formulas was lower than - 30 mV and highly negative, which indicates good stability.

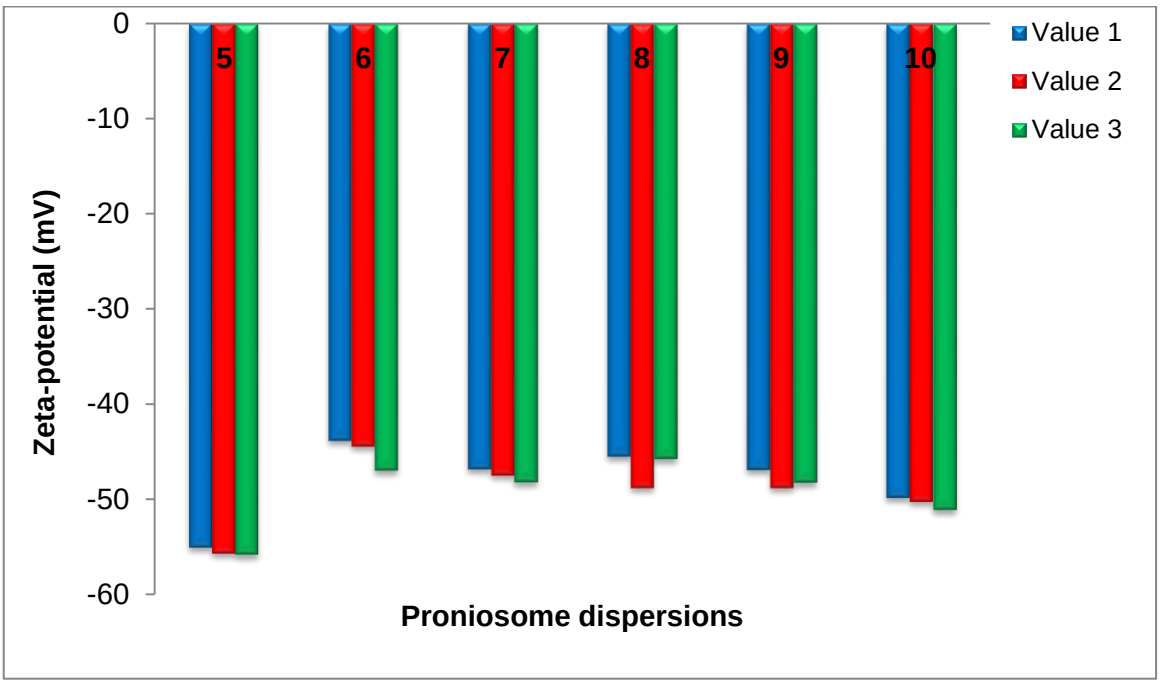

Figure B.14: The average zeta-potential of the proniosome formulas

B.6.2.1.4 Entrapment efficiency

The EE% was one of the most important considerations to finalise the formula. The EE% refers to the amount active ingredient effectively incorporated in the vesicle system (Marianecci *et al.,* 2013:77). The EE% of the formulas is indicated in Table B.10. Although the results did not differ significantly, formula **(8)** had the best entrapment (44.50 ± 0.33%), with formulas **(9)** and **(10)** being slightly lower, with an EE% of 41.86 ± 0.32% and 41.98 ± 3.28%, respectively**.**

Table B.10: The entrapment efficiency of the proniosome formulas

	(8)	(9)	(10)
%EE	44.05 44.63 44.82	42.21 41.92 41.44	44.39 44.22 37.34
Mean	44.50	41.86	41.98
SD	0.33	0.32	3.28
%RSD	0.73	0.76	7.82

B.6.2.2 Conclusion

The final proniosome formula was chosen according to the results obtained from various tests. The tests conducted on the formulas included TEM, vesicle size and PdI, zeta-potential and EE%. The proniosome formula with 2 g sorbitol **(8)** was selected, since it had good stability, acceptable size and size distribution results and the highest EE%. The proniosome formula with 2 g sorbitol was also the quickest to prepare.

B.7 Final formula of vesicular and provesicular systems

The niosome and proniosome formulas with good EE% results, as well as good appearance in vesicle size and PdI and zeta-potential, were selected as the final formulas for vesicle and provesicle preparation.

B.7.1 Final formulation of the vesicular systems

The final formula, as determined according to the aforementioned results, was the niosome formula containing 3% carnosine **(3)**.

B.7.1.1 Method of preparation

The final noisome dispersion was prepared as described in Section B.5.1. In the final method, the water phase contained carnosine. The amount of carnosine, for a dispersion containing 3% active, was accurately weighed and dissolved in the purified water, then added to the lipidic film and stirred for approximately 2 min at a low temperature. The mixture was allowed to cool down, on ice, before being sonicated on ice for 1 min.

B.7.1.2 Outcome

The niosome formula formed a visually attractive, slightly oily, white milky suspension without any larger particles or sediment visible.

B.7.2 Final formulation of the provesicular systems

The final formula, as determined according to the aforementioned results, was the proniosome formula containing 3% carnosine and prepared with 2 g sorbitol.

B.7.2.1 Method of preparation

The final proniosome dispersion was prepared as described in Section B.5.2. In the final method, the water phase contained carnosine. The amount of carnosine, for a dispersion containing 3% active, was accurately weighed and dissolved in the purified water. This water phase was then added to the dry powder and stirred for approximately 5 min at a low temperature. The mixture was allowed to cool down, on ice, before being sonicated on ice for 1 min.

B.7.2.2 Outcome

The proniosome formula formed a white milky suspension that was slightly thicker than the niosome formula. It was visually attractive without any larger particles or sediment visible.

B.8 Final conclusion

The final formulas for the niosomes and proniosomes were selected according to the results obtained from various physical characteristics. These physical characteristics included TEM, vesicle size and PdI, zeta-potential and EE%. Overall, formulas **(3)** and **(8)** had the best results, having the highest EE% as well as acceptable results in stability, size and size distribution. These two formulas will be used for skin diffusion studies and the niosome formula will be used to formulate the topical niosome containing gel and the topical niosome containing cream.

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MSDS ***see*** Material Safety Data Sheet.

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APPENDIX C

CHARACTERISTICS OF VESICULAR AND PROVESICULAR SYTEMS

C.1 Introduction

The quality of the prepared niosomes and proniosomes primarily depends on the characteristics of the dispersions. The characteristics to determine include morphology, size and size distribution, zeta-potential, pH, viscosity and entrapment efficiency of the vesicles. These characteristics can have a substantial effect on the stability and the *in vivo* performance of the vesicle systems (Marianecci *et al.*, 2013:74). Furthermore, the characteristics are important to consider when drawing conclusions regarding the vesicle systems and the results obtained from skin diffusion studies.

The physical properties and stability of the dispersions are mainly determined by the vesicle size and size distribution of the vesicle systems (Marianecci *et al.*, 2013:74). The size of the vesicles can differ depending on the ingredients used and the method of preparation, but are usually on the nanometric scale (Tangri & Khurana, 2011:50). PdI is a parameter to describe the size distribution of the droplets, or in other words, the uniformity of the dispersion; a perfectly uniform sample would therefore have a PdI of 0 (Nobbmann, 2014). Although a PdI of 0.4 generally indicates a monodispersed dispersion, no linear correlation could previously be drawn between the PdI value and the true monodispersity of a dispersion (Gaumet *et al.,* 2008:5; Marianecci *et al.,* 2013:75).

Another important indicator of the stability of a formulation is the zeta-potential, which is a measurement of the surface charge of each dispersed particle in the dispersion (Lamba *et al.*, 2015:719; Marianecci *et al.,* 2013:75). If the particles in a formulation have a highly positive or negative zeta-potential, flocculation will be prevented and the particles will repel each other (Malvern Instruments, 2015:3; Marianecci *et al.,* 2013:75). However, if the zeta-potential of the particles is low, the tendency to aggregate will increase and the particles will cluster together (Malvern Instruments, 2015:3; Marianecci *et al.,* 2013:75). Flocculation refers to two droplets that are attached and separated by a liquid film, whilst aggregation involves more droplets, clustering together but still retaining the liquid film between them (Roland *et al.,* 2003:85). Coalescence can occur when the liquid film is removed, to finally result in the formation of large droplets and visible phase separation (Roland *et al.,* 2003:86). A zeta-potential higher than + 30 mV or lower than - 30 mV generally indicates a stable formulation (Marianecci *et al.,* 2013:75).

The pH was measured to determine if the dispersions would be suitable for topical use. A pH between 5 and 9 is considered ideal for topical preparations because the stratum corneum is exceptionally resistant to pH alterations (Barry, 2007:576; Naik *et al*., 2000:319). Measurements of pH are also important for quality assurance, since variations may explain differences between the preparations (Roland *et. al.,* 2003:93).

Stoke's law explains that the rate of phase separation of a dispersion depends on gravity, the radius of the droplets and the viscosity of the medium (Roland *et al.,* 2003:88). Viscosity and rate of phase separation are inversely proportional, meaning a lower viscosity will result in faster phase separation. The viscosity of the dispersion is important for quality assurance as variations may indicate differences between preparations (Roland *et al.,* 2003:93). Viscosity measurements must be done on fresh preparations at a constant temperature since operational changes may cause considerable changes in results (Roland *et al.,* 2003:88).

The entrapment efficiency refers to the amount of active ingredient effectively incorporated in the vesicle system (Marianecci *et al.,* 2013:77).

C.2 Methods

C.2.1 Vesicle size and polydispersity index

The same method as described in Section B.5.4 was used to determine the vesicle size and PdI of the final pre-formulations. This experiment was performed in triplicate.

C.2.2 Zeta-potential

The same method as described in Section B.5.5 was used to determine the zeta-potential of the final pre-formulations. This experiment was performed in triplicate.

C.2.3 pH

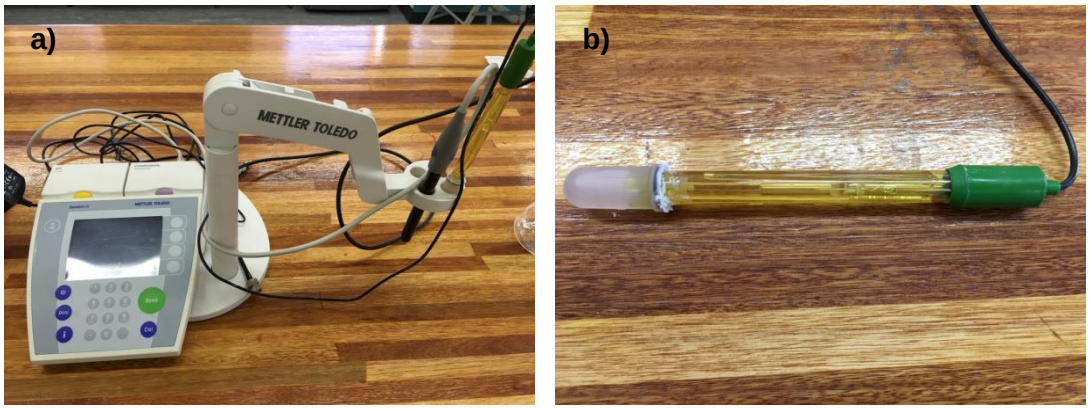

Figure C.1: a) The Mettler Toledo pH meter and b) Mettler Toledo InLab 410 electrode

The pH of the dispersions was determined using a Mettler Toledo pH meter with a Mettler Toledo InLab 410 electrode (Mettler Toledo, Switzerland). The pH meter was calibrated at a pH of 4, 7 and 10, with buffer solutions before the experiment. The pH of the dispersions was determined by placing the probe in 5 ml samples. The pH of each formula was measured in triplicate. The pH meter and electrode are indicated in Figure C.1.

C.2.4 Viscosity

The viscosity of the dispersions was determined using a Brookfield® Viscometer model DV-III Ultra (Brookfield Engineering Laboratories, United States of America). The samples were placed in a water bath to reach 25 °C. For this experiment, 6.7 ml of each formula was placed into a sample adapter in a flow jacket connected to a water bath. A SC4-18 spindle was used to measure the viscosity at a speed of 200 rpm. The viscosity of the dispersions was measured eight times in intervals of 10 sec. Figure C.2 illustrates the Brookfield viscosity meter.

Figure C.2: The Brookfield® Viscometer model DV-III Ultra

C.2.5 Entrapment efficiency

The difference between the un-entrapped free active and the total amount of active in the vesicle system was determined using HPLC methods. The same method as described in Section B.5.6 was used.

The following equation, adapted from Mali *et al.* (2013:588), was used to determine the percentage entrapment efficiency:

$$EE\ (\%) = Cr / Ct \times 100 \qquad \textbf{Equation C.1}$$

Where EE is the entrapment efficiency in percentage, Cr is the amount of carnosine entrapped and Ct is the total amount of carnosine used.

C.3 Characteristics of vesicular and provesicular systems with entrapped carnosine

Two final dispersions, representing the formulas used for diffusion studies, were chosen after a trial-and-error approach in preparing the vesicular systems. One vesicular and one provesicular dispersion were prepared and characterised to determine the zeta-potential, vesicle size and PdI, pH, viscosity and entrapment efficiency results. The niosomes contained 3% carnosine **(3)** and the proniosomes were prepared with 2 g sorbitol, which also contained 3% carnosine **(8)**.

C.4 Results and discussion

C.4.1 Vesicle size and polydispersity index

As mentioned in Section B.6.1.1.2, the vesicle size and PdI can have an effect on both the physical properties and stability of the dispersion (Kumar & Rajeshwarrao, 2011:212; Marianecci *et al.,* 2013:74). Despite the influence of the method of preparation on the noisome sizes, it is usually in the nanometric scale (Tangri & Khurana, 2011:50). The PdI is indicative of the size distribution and a dispersion with a PdI value of less than 0.400 is usually regarded as uniform (Marianecci *et al.,* 2013:74; Nobbmann, 2014). The average size and PdI of the final pre-formulations are indicated in Table C.1.

Table C.1: The average vesicle size and PdI of the final pre-formulations

	Niosomes	**Proniosomes**
Vesicle size (nm)	354.80 381.30 344.50	352.30 435.10 387.90
Mean	**360.20**	**391.77**
SD	**15.50**	**33.91**
%RSD	**4.30**	**8.66**
PdI	0.757 0.682 0.726	0.537 0.589 0.644
Mean	**0.722**	**0.590**
SD	**0.031**	**0.044**
%RSD	**4.26**	**7.40**

The average vesicle size and PdI of the dispersions are indicated in Figure C.3 and Table C.1, respectively. Multi-lamellar vesicles formed. The provesicles were slightly bigger than the vesicles, but still on the nanometric scale. The average size of the niosomes was 360.20 ± 15.50 nm and for the proniosomes, it was 391.77 ± 33.91 nm. Particles smaller than 3000 nm can penetrate the stratum corneum through the intercellular pathway (Bolzinger *et al*., 2012:163).

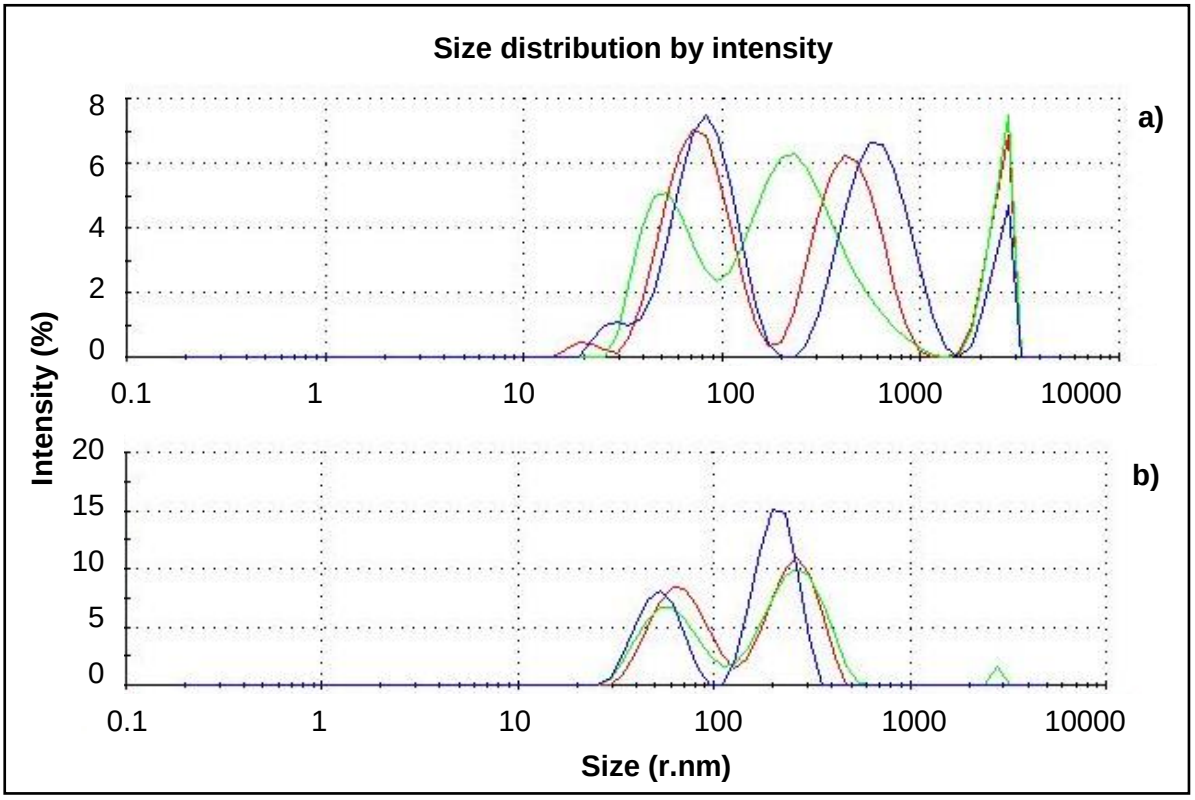

Figure C.3: The droplet size distribution of the final dispersions: a) size distribution of the niosomes and b) size distribution of the proniosomes

The area of the peaks in Figure C.3 indicates the volume of a certain population, in other words, the size distribution of the vesicular and provesicular systems. The niosomes had an average PdI of 0.722 ± 0.031. The average PdI of the proniosomes was 0.590 ± 0.044 and therefore slightly lower than the niosomes. The size distribution of both vesicle systems was poor and not below 0.400. Despite these poor results, Gaumet *et al.* (2008:5) found no linear correlation between the PdI value and the true monodispersity of a formulation could be drawn. A possible suggestion to improve these results is to increase the sonication time.

C.4.2 Zeta-potential

The average zeta-potential of the final vesicle systems is shown in Figure C.4. The zeta-potential is an indication of the surface charge of the droplets in the dispersions. The surface charge affects the dispersions' stability and in general, a zeta-potential of lower than - 30 mV or higher than + 30 mV is indicative of good stability (Malvern Instruments, 2015:3; Marianecci *et al.,* 2013:75).

The average zeta-potential of the niosomes was - 59.80 ± 0.51 mV and the average of the proniosomes was slightly lower (- 45.73 ± 1.68 mV); both these values were highly negative and indicate excellent stability.

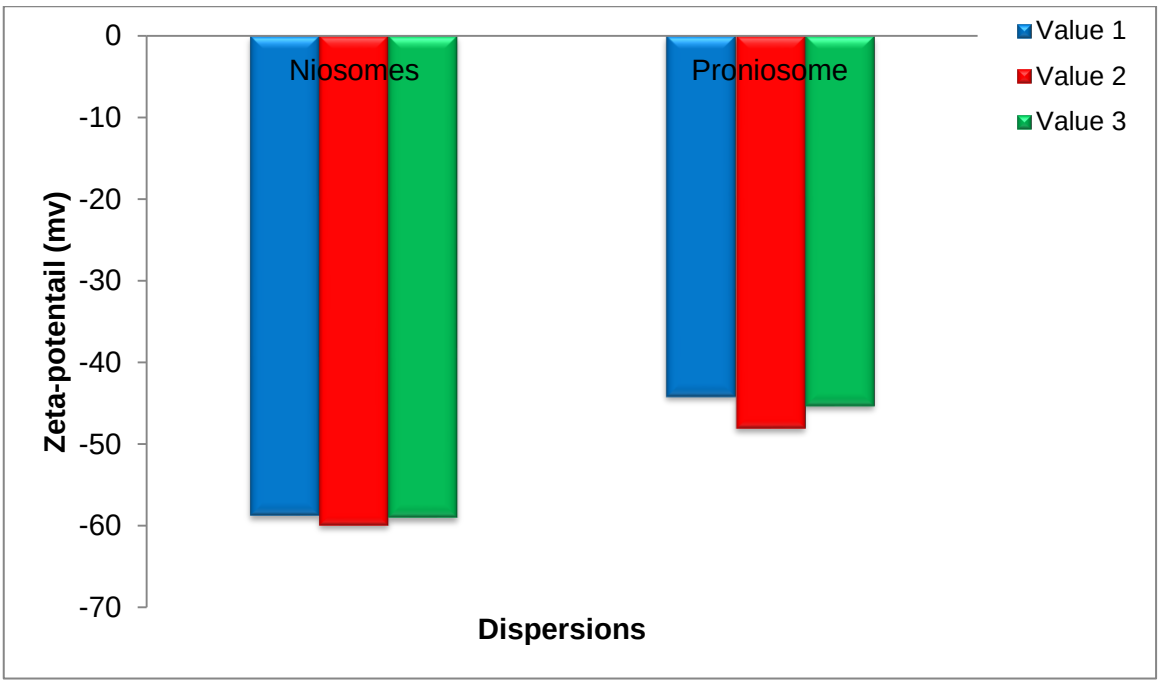

Figure C.4: The zeta-potential of the final vesicle systems used

C.4.3 pH

The niosomes and proniosomes had an average pH of 8.077 ± 0.004 and 8.110 ±0.009 respectively. The pH measurements were slightly below 9 and the dispersions will be suitable for topical use (Naik *et al*., 2000:319). There were also no variations between the dispersions prepared in triplicate, which indicated good quality of the vesicle systems (Roland *et al.,* 2003:93).

C.4.4 Viscosity

The vesicle systems had a very low, but constant viscosity. The viscosity of the niosomes was 3.000 ± 0.026 cP and the proniosomes, 4.31 ± 0.033 cP. The proniosomes' viscosity was slightly higher due to the addition of sorbitol. Both the vesicle systems' viscosities were low, but constant. These constant values indicate good quality of the vesicle systems (Roland *et al.,* 2003:93). Phase separation may possibly occur due to the low viscosities but, these two preparations are pre-formulations and will be incorporated into a semi-solid formulation to alter the viscosity and consequently increase the stability.

C.4.5 Entrapment efficiency

The EE% of the dispersions is indicated in Table C.2. The niosomes had an EE% of 46.72 ± 1.02%, whereas the proniosomes had an EE% of 44.50 ± 0.33%. The niosomes prepared by Bayindir and Yuksel (2010:2055) and proniosomes prepared by Ammar *et al.* (2011:145-146) had EE% which ranged from 12.10% to 96.60% and 22.35% to 91.95%, respectively. This is evidence that the EE% varies among studies and that the niosomes and proniosomes prepared in this study had acceptable EE%.

Table C.2: The entrapment efficiency of the final vesicle systems

	Niosomes	**Proniosomes**
%EE	45.49 46.71 47.99	44.05 44.63 44.82
Mean	**46.72**	**44.50**
SD	**1.02**	**0.33**
%RSD	**2.19**	**0.73**

C.5 Conclusion

The characteristics of the vesicles have a significant effect on the quality, stability and *in vivo* performance of the vesicles (Marianecci *et al.*, 2013:74). From the aforementioned results, the vesicles formed with sizes on the nanometric scale and poor, but acceptable, size distributions. The niosomes' size was slightly smaller than the proniosomes, but the proniosomes' size was more evenly distributed. Both of the vesicle systems were stable with high negative zeta-potential values, which results in minimal aggregation and flocculation of droplets. The pH of both vesicle systems was within range for topical delivery, without any variances amongst different preparations, to indicate good quality. Both dispersions also had a constant viscosity amongst different preparations, indicating good quality. The EE% of both dispersions was good with values slightly below 50%. These characteristics will assist when drawing conclusions in the experimental results of this study.

Membrane release studies and skin diffusion studies will be performed on these vesicle systems, and the niosomes will be used to formulate topical niosome based semi-solid formulations, i.e. a cream and a gel. The niosome dispersion was chosen since it had better overall characteristics and the preparation process was quicker and easier.

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APPENDIX D

FORMULATION OF A CREAM AND GEL CONTAINING CARNOSINE ENCAPSULATED IN NIOSOMES

D.1 Introduction

According to Milstein *et al.* (2001:5-6), the Greek description *kosm tikos* meaning “having the power to arrange” explains the extended history of cosmetic products. Despite these early Greek descriptions, some recommend the true origin of cosmetics lies even further in antiquity. Over the years, cosmetics have been used with three main goals in mind, namely to intensify personal appeal with decoration, to camouflage blemishes in the skin and to alter or promote the original. In 1938, the Federal Food, Drug and Cosmetic Act (FD&C Act) defined cosmetics as products developed and designed to be applied to the body for beautifying, cleansing, enhancing attractiveness or changing appearances, as well as articles used in these products, excluding soaps.

Niosomes have been studied by cosmetic researchers for their potential to act as drug carriers in cosmetic products (Uchegbu & Vyas, 1998:33-70; Nasir *et al.,* 2012:479-487). It provides advantages such as increased skin penetration, prolonged retention time of the active ingredient in the skin, as well as reduced systemic absorption (Mali *et al.,* 2013:587; Nasir *et al.,* 2012:484). Unfortunately, due to factors such as the low viscosity, visual appearance and instability, it cannot be used as a dosage form in this form. A proper semi-solid dosage form containing the niosomes must therefore be formulated in order to benefit from the advantages and to improve patient compliance.

According to Stahl (2015:209-218), semi-solid dosage forms can be classified into gels, creams, ointments, powders and pastes. These dosage forms do not flow and will stay on the skin for a prolonged time. This will increase the chance of diffusion of the active ingredient through the formulation into the skin. Patient preference is one of the main factors that are considered when determining the type of formulation to prepare (Kurian & Barankin, 2011:4).

Mitsui (1997:341) describes creams as a type of emulsion in which two phases are made into a dispersion; these are generally more stable than lotions and oils. Conversely, gels are referred to as a type of base with a uniform appearance and a light feeling. The wide variety of cosmetic products available today is a result of extensive scientific progress in the cosmetic industry.

In this study, both a conventional cream and a less conventional gel will be formulated. The effects of these formulations on the release of carnosine from the niosomes will then be

investigated. The niosomes were chosen for it had a higher entrapment percentage, smaller droplet sizes, increased stability and was easier and faster to prepare compared to the proniosomes. In order to determine quality and stability of the final preparations, the cream and gel will undergo stability tests. A programme according to the International Conference on Harmonisation (ICH) was followed. The important parameters used during stability testing included concentration assays, pH, conductivity, viscosity, microscopic analysis, visual analysis and mass loss.

D.2 Ingredients used to formulate semi-solid dosage forms

D.2.1 Niosomes containing carnosine

The niosome dispersion is a white, milky liquid containing the active ingredient, carnosine (Sigma-Aldrich, Aston Manor, SA). It is slightly oily due to the cholesterol content. Niosomes are generally used as pre-formulations to be incorporated into dosage forms. The niosomes containing the active ingredient, carnosine, were prepared according to the method described in Section B.7.1.1. The formula was slightly manipulated to ensure a semi-solid dosage form containing 3.00% of the active ingredient.

For the cream, 4.152% carnosine was used during pre-formulation of the niosomes since only 72.25% niosomes were used in the final cream formula. This has resulted in a final cream formulation containing 3.000% carnosine.

For the gel, 3.158% carnosine was used during pre-formulation of the niosomes, since only 95% niosomes were used in the final gel formula. This has resulted in a final gel formulation containing 3.000% carnosine.

D.2.2 Propylene glycol

Propylene glycol (Merck Millipore, Modderfontein, SA) is a clear colourless liquid, looking and feeling like glycerine. Propylene glycol is used as a solvent for the parabens (Weller, 2009:592-593). It is also a well-known humectant and can be used to maintain skin moisture, as well as the moisture content and stability of the product (Mitsui, 1997:134). The general concentration used in topical formulations is 5.0 to 80.0% (Weller, 2009:592-593).

D.2.3 Preservatives

Preservation of cosmetics is important to prevent contamination of products which may unexpectedly result in infection due to slight illness, broken skin or certain drug therapies (Harry, 1973:656). Although the ideal preservative has not yet been discovered, the essential requirement for preservatives are non-toxicity, stability when heating or storing and compatibility

with other ingredients in the formula (Harry, 1973:663). According to Haley (2009:441-445, 596-598), parabens are widely used in cosmetics as antimicrobial preservatives. It can be used either alone or in combination with other antimicrobial agents. Parabens have a broad spectrum antimicrobial activity and are effective over a wide pH range. The efficacy is generally increased by adding propylene glycol to the formulation and by combining two or more parabens. Methylparaben and propylparaben (Merck Millipore, Modderfontein, SA) are generally combined in pharmaceutical formulations. Methylparaben is the least effective of all parabens. The concentration ranges of methylparaben and propylparaben used in topical formulation are 0.02 to 0.30% and 0.01 to 0.60%, respectively. Parabens have been used as preservatives for over 80 years and, despite the controversy about the use of parabens in cosmetics, they are still considered safe and well tolerated (Kirchhof & de Gannes, 2013:6).

D.2.4 Mineral oil

Mineral oil is a colourless oily liquid also known as liquid paraffin (Merck Millipore, Modderfontein, SA) (Sheng, 2009:445-447). It is generally used in topical formulations for the emollient properties, to enhance the feeling on use and to control moisture loss from the skin (Mitsui, 1997:124; Sheng, 2009:445-447). The concentration used in topical emulsions and lotions is 1.0 to 32.0%. It forms part of the oil phase of the cream.

D.2.5 Cetyl alcohol

According to Unvala (2009:155-156), cetyl alcohol (Merck Millipore, Modderfontein, SA) is a white waxy substance with a melting point of 49 °C. It is used in semi-solid formulations as an emulsifying and a stiffening agent. It will also improve the stability and texture of the formulation. Cetyl alcohol has emollient properties and due to its retention in the epidermis it will soften and lubricate the skin.

D.2.6 Surface active agents

According to Harry (1973:609-613), surface active agents are used to alter the surface energy of a surface or solvent with which the product comes into contact. The lowering of surface energy can be observed as improved spreading of a liquid on a solid. The use of surfactants can be divided into three main areas depending on the properties required, namely detergents, wetting agents and emulsifying agents. Emulsifying agents are mainly used in emulsions such as creams. Furthermore, surfactants are grouped into cationic, non-ionic and anionic, from which non-ionic surfactants mainly serve as emulsifying agents. Tween® 20 and Span® 20 (Merck Millipore, Modderfontein, SA) are examples of non-ionic surfactants with emulsifying properties (Zhang, 2009:549-553). It is generally used in cosmetic formulations and regarded as non-toxic and safe.

D.2.7 Magnesium aluminium silicate

Magnesium aluminium silicate is generally referred to as Veegum® (Palmieri, 2009:393). According to Knowlton and Pearce (1993:20), Veegum®) RT Vanderbilt Co Inc, Norwalk, CT) is a clay, derived from naturally occurring minerals. It is insoluble, but will swell in the presence of water to alter the thickness of formulations. The necessary concentration to increase viscosity of a formulation is 2 to 10% (Palmieri, 2009:393).

D.2.8 Carbomer

Carbomer is also known as Carbopol® (Lubrizol Advanced Materials, Cleveland, OH). According to Draganoiu *et al.* (2009:110-113), it is a fine, white, acidic powder. It is used as a rheology modifier and will increase the viscosity of semi-solid formulations. Highly viscous gels are produced when it is dispersed in an aqueous phase. The general concentration for Carbopol® as a gelling agent is 0.5 to 2.0%. Carbopol is less prone to microbial contamination in comparison to natural thickeners (Knowlton & Pearce, 1993:20).

D.3 Formulation of a cosmeceutical cream

D.3.1 Purpose and function of a cream

According to Mitsui (1997:342), maintaining the moisture balance of the skin and keeping the skin supple and moist through oil, humectants and water supply are the main functions of creams. In addition to the moisturising functions, other functions include make-up removal, cleansing the skin and stimulating circulation. A wide variety of creams to suite the different purposes can be formulated by altering the formulas or ingredients used.

D.3.2 General method for formulation of a cream

The general method to formulate a cream involves the following:

- adding the humectants and water phase ingredients to purified water and heating it to 70 °C;
- preparing the oil phase by making a solution of oils, preservatives and anti-oxidants and heating it to 70 °C;
- stirring the perfume into the oil phase just before emulsification;
- stirring the oil phase into the water phase for preliminary emulsification to take place;
- finalise the emulsification with the correct apparatus to ensure uniform particles; and
- degas, filter and cool down the formulation (Mitsui, 1997:343).

D.4 Formulation of a cosmeceutical gel

D.4.1 Purpose and function of a gel

According to Mitsui (1997:351), the main functions of gels are to moisturise and to supply water to the skin. For oily skin, a water base gel will leave the skin feeling light and moist and for dry skin, an oil base gel will provide complete moisture. Other functions of gels include make-up removal, cleansing of the skin and stimulating circulation. A wide variety of gels can be formulated to suite the different purposes.

D.4.2 General method for formulation of a gel

According to Mitsui (1997:353), the methods of mixing, degassing, filtering and cooling of gels are important during the formulation process. Due to the high viscosity of gels, the necessary equipment must be available to ensure quality.

D.5 Formulation of a topical niosome cream containing carnosine

D.5.1 Pre-formulation of a topical niosome cream

During the pre-formulation of the cream, a trial-and-error approach was used. Existing formulas were used and manipulated in order formulate the cream. Different thickening agents were used and the formulations where examined visually. The final formulation was chosen according to visual aspects, such as creaminess, viscosity and colour.

D.5.2 Final niosome cream formulation containing carnosine

The final formula of the cream formulation is indicated in Table D.1.

Table D.1: The final formula of the niosome cream containing carnosine

Ingredient	%m/m	Purpose
A		
Niosomes	72.25	Vesicles containing carnosine
Veegum	2.50	Thickening agent
Span® 20	1.00	Emulsifying agent
B		
Liquid paraffin	10.00	Oil phase
Propylene glycol	3.00	Solvent for preservatives
Methylparaben	0.20	Preservative
Propylparaben	0.05	Preservative
Cetyl alcohol	10.00	Thickening agent
Tween® 20	1.00	Emulsifying agent

D.5.2.1 Method

First, the water phase (**A**) was prepared by heating the niosomes and Span® 20 to 60 °C and slowly adding the Veegum® to the mixture with continuous stirring. The concentration assays (Section F.4.1) are proof that carnosine was stable and did not degrade during the heating process. Next, the preservatives were dissolved in the propylene glycol on a magnetic stirrer. The oil phase (**B**) was prepared by adding all the oil ingredients to the liquid paraffin and heating it to 60 °C. When both phases reached temperature, Phase **B** was added to Phase **A** while stirring for preliminary emulsification to take place. The emulsion was finalised with continuous stirring at 1000 rpm until the formulation completely cooled down.

D.5.2.2 Outcome

The cream had a homogenous texture and an off-white colour. It applied easily and was not too oily. Despite the non-greasy feeling, it needed some rubbing for complete disappearance. The topical cream had no particular smell.

D.6 Formulation of a topical niosome gel containing carnosine

D.6.1 Pre-formulation of a topical niosome gel

During the pre-formulation of the gel, a trial-and-error approach was used. Different gelling agents were used and the formulations were examined visually. Two different degassing methods were tested to reduce the air bubbles in the gel. The method involving the vacuum oven was unsuccessful as an unwanted reaction, with the niosomes occurred, as seen in Figure D.1. The method involving the sonicator bath was less effective than the vacuum oven, but was chosen as no unwanted reactions occurred. The final formulation was chosen according to visual aspects, such as viscosity and colour.

Figure D.1: The outcome using the vacuum oven for degassing the gel

D.6.2 Final niosome gel formulation containing carnosine

The final formula of the gel formulation is indicated in Table D.2.

Table D.2: The final formula of the niosome gel containing carnosine

Ingredient	%m/m	Purpose
Niosomes	95.00	Vesicles containing carnosine
Propylene glycol	5.00	Solvent for preservatives
Propylparaben	0.05	Preservative
Methylparaben	0.20	Preservative
Carbopol®	0.50	Gelling agent

D.6.2.1 Method

First, the preservatives were dissolved in the propylene glycol in a magnetic stirrer. After complete dissolution, it was added to the niosomes. The Carbopol® was then sprinkled over the mixture and allowed to hydrate for 20 min. Thereafter, the mixture was homogenised at 1000 rpm for 30 min to ensure uniformity of the formulation. Finally, the formulation was degassed in a sonicator bath for 10 min.

D.6.2.2 Outcome

The gel had a bright white colour and applied easily. It was foamy and left the skin feeling moist and light as the excess fluid evaporated quickly. Almost no rubbing was necessary for complete absorption. The gel had a characteristic odour, possibly due to the addition of Carbopol®.

D.7 Summary

Carnosine was used as active ingredient for the formulation of a cosmeceutical cream and a cosmeceutical gel. The active ingredient was firstly incorporated into niosomes. The niosomes were then used to formulate the cream and the gel. Both of the formulations contained 3% carnosine. Sufficient quantities of the formulations were prepared and stored to perform stability tests at different storage conditions. The visual appearance, texture and smell of the final formulations were satisfactory before storage. The stability tests performed will be discussed in Appendix F. Membrane release studies and skin diffusion studies have also been performed on the cream and the gel. These studies will be discussed in Appendix G.

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APPENDIX E

VALIDATION OF AN HPLC ANALYTICAL METHOD FOR THE COMBINED ANALYSIS OF CARNOSINE, METHYLPARABEN AND PROPYLPARABEN

E.1 Purpose of the validation

The purpose of the development and validation of an HPLC method for the analysis of the active and the excipients is to ensure sensitivity and reliability during concentration assay analysis. The active ingredient is carnosine (Sigma-Aldrich, Aston Manor, SA) and the excipients are methylparaben and propylparaben. This method was developed and validated in co-operation with Prof Jan du Preez at the Analytical Technology Laboratory, North-West University, Potchefstroom Campus, South Africa.

E.2 Chromatographic conditions

The following chromatographic conditions formed part of the HPLC method for assay analysis of excipients:

Analytical instrument: An Agilent® 1200 Series HPLC system (Agilent Technologies, United States of America) was used to perform the HPLC analysis. The system is equipped with ChemStation Rev. A.06.02 software for data analysis. The system consists of an Agilent® 1200 pump, diode array detector, autosampler injection and a vacuum degasser.

Column: A Venusil® ASB C8 column (250 x 4.6 mm) (Agela Technologies, Newark, DE) was used

Mobile phase A: Acetonitrile

Mobile phase B: 0.075 M octane sulphonic acid (pH 3.45)

Gradient table: The gradient table are indicated in Table E.1.

Table E.1: The gradient table of the analytical method

Time (min)	%Mobile phase A	%Mobile phase B
0.0	20	80
1.0	20	80
3.0	70	30
7.0	70	30
7.1	20	80

Solvent: Methanol

Flow rate: 1 ml/min

Injection volume: 10 µl

Retention time: Carnosine eluted first at 5 min, followed by methylparaben (6.8 min) and propylparaben (7.5 min)

Run time: 12 min

Detection wavelength: 210 nm

The chromatogram of active ingredients is seen in Figure E.1. The active ingredient and the two excipients, with their retention times, are indicated.

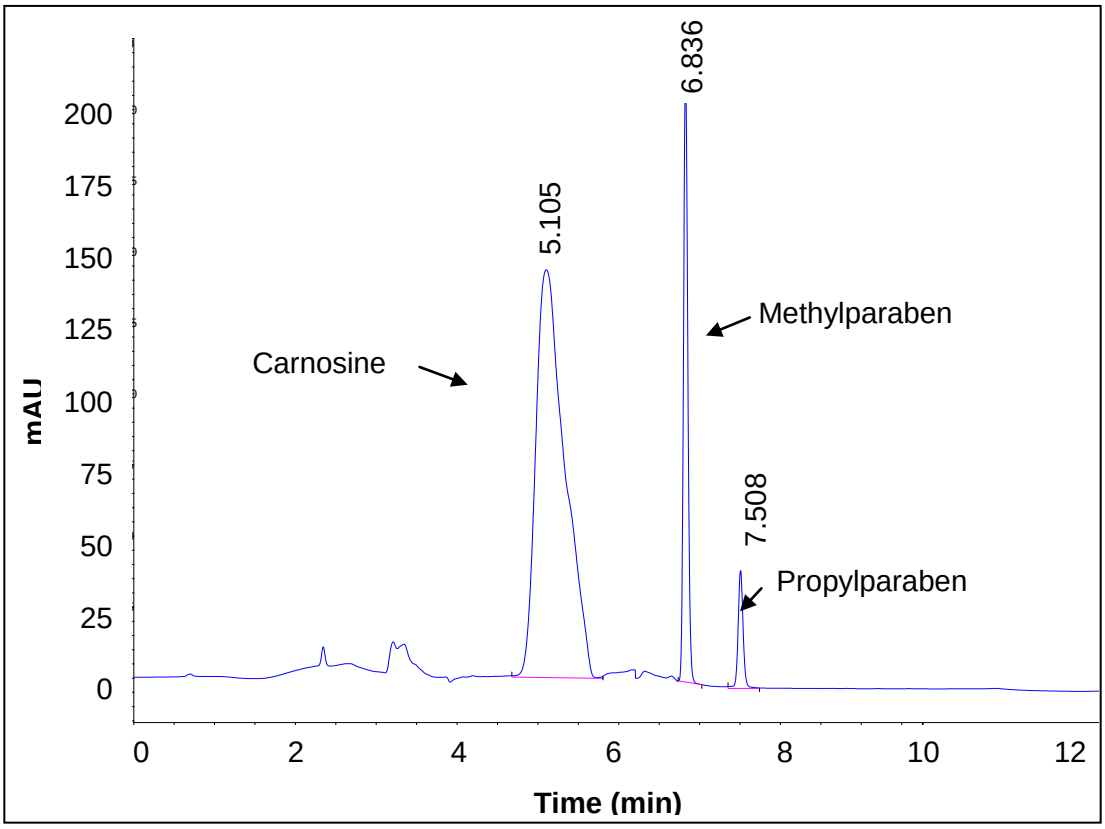


Figure E.1: Chromatogram of the active ingredient and the excipients

E.3 Preparation of mobile phase

The octane sulphonic acid solution was prepared as described in Section A.3.

E.4 Preparation of standard solutions

The standard solutions were prepared by dissolving accurately weighed amounts of carnosine, methylparaben and propylparaben in an 80:20 methanol (HPLC grade) and water (HPLC grade) mixture in a 200 ml volumetric flask. First, approximately 35 mg carnosine was dissolved in 40 ml water in a 200 ml volumetric flask, then 40 ml methanol was added to the solution. Approximately 10.0 mg methylparaben and 3.2 mg propylparaben were then dissolved in 20 ml methanol in a volumetric flask; 5 ml of this solution was transferred to the 200 ml volumetric flask containing the carnosine solution. The volumetric flask was then filled to volume with methanol (HPLC grade).

E.5 Calculations

The calculations were done as described in Section A.6.

E.6 Validation parameters

E.6.1 Linearity

The linearity is a validation parameter to measure whether the test results and the concentrations of the active ingredient and the excipients in the test samples are directly proportional (ICH, 2005:5). The linearity was determined with linear regression when the concentrations versus the peak areas obtained were plotted on a graph. A minimum of five concentrations is required to establish linearity (ICH, 2005:8). The straight line is described by the regression coefficient (R^2) and should be at least 0.0997 (Shabir, 2005:7).

E.6.1.1 Method

A sample was prepared by weighing 34.44 mg carnosine, 9.72 mg methylparaben and 3.26 mg propylparaben. The methylparaben and propylparaben were dissolved in 20 ml methanol in a volumetric flask, and the carnosine was dissolved in 40 ml water in the 200 ml volumetric flask. The paraben solution (5 ml) was transferred to the 200 ml volumetric flask containing the carnosine solution. The flask was then filled to volume with methanol (HPLC grade). The linearity of the ingredients was determined by plotting the mean peak areas against the concentration and performing linear regression analysis.

E.6.1.2 Results and discussion

E.6.1.2.1 Carnosine

Carnosine demonstrated outstanding linearity with a correlation coefficient (R^2) value of 0.999. This value indicates the HPLC system was stable over the concentration range of 34.33 – 206.64 µg/ml. The linearity results and the regression curve of linearity follow in Table E.2 and Figure E.2, respectively.

Table E.2: The linearity results of carnosine

Standard concentration (µg/ml)	Peak area 1	Peak area 2	Mean peak area
34.44	649.4	648.2	648.8
68.88	1320.5	1319.8	1320.2
103.32	1984.2	1985.6	1984.9
137.76	2653.3	2654.0	2653.7
172.20	3322.6	3319.0	3320.8
206.64	3986.1	3995.8	3991.0
		Slope	**19.397**
		y-intercept	**- 18.270**
		R^2	**0.999**

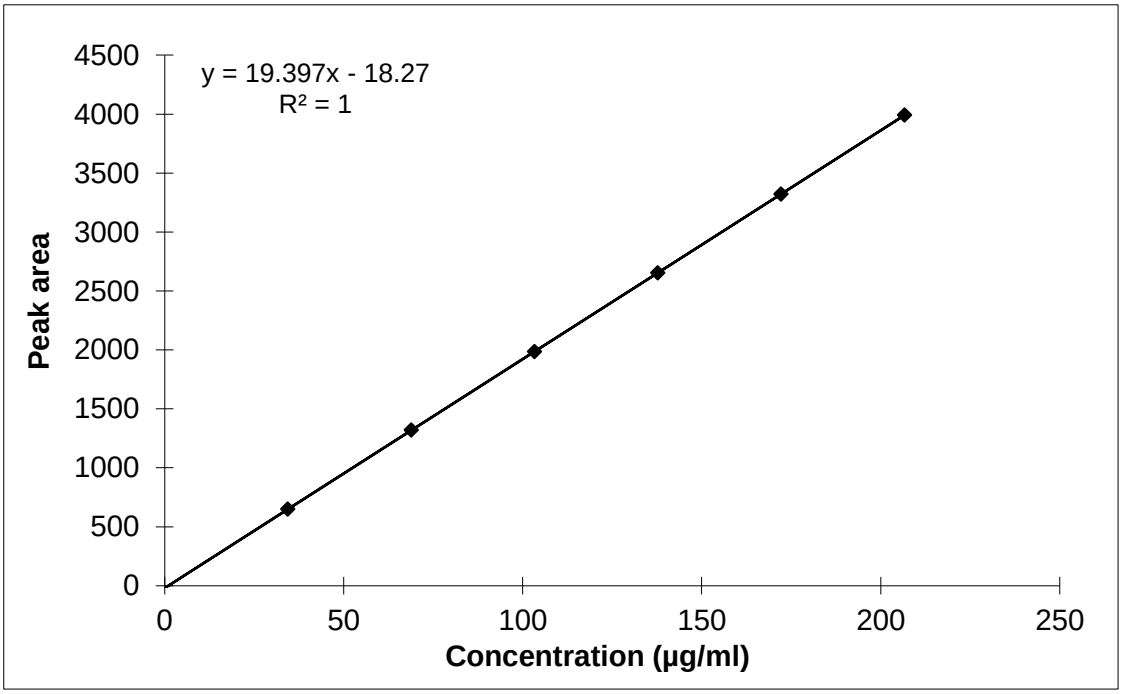


Figure E.2: The linear regression curve of carnosine standard solutions

E.6.1.2.2 Methylparaben

Methylparaben demonstrated outstanding linearity with a R^2 value of 0.999. This value indicates the HPLC system was stable over the concetration range of 2.43 – 14.58 µg/ml. The linearity results and the regression curve of linearity follow in Table E.3 and Figure E.3, respectively.

Table E.3: The linearity results of methylparaben

Standard concentration (µg/ml)	Peak area 1	Peak area 2	Mean peak area
2.43	153.9	162.9	158.4
4.86	301.0	338.4	319.7
7.29	448.7	448.9	448.8
9.72	598.2	597.9	598.1
12.15	746.1	746.3	746.2
14.58	893.2	894.8	894.0
		Slope	**60.044**
		y-intercept	**16.850**
		R^2	**0.999**

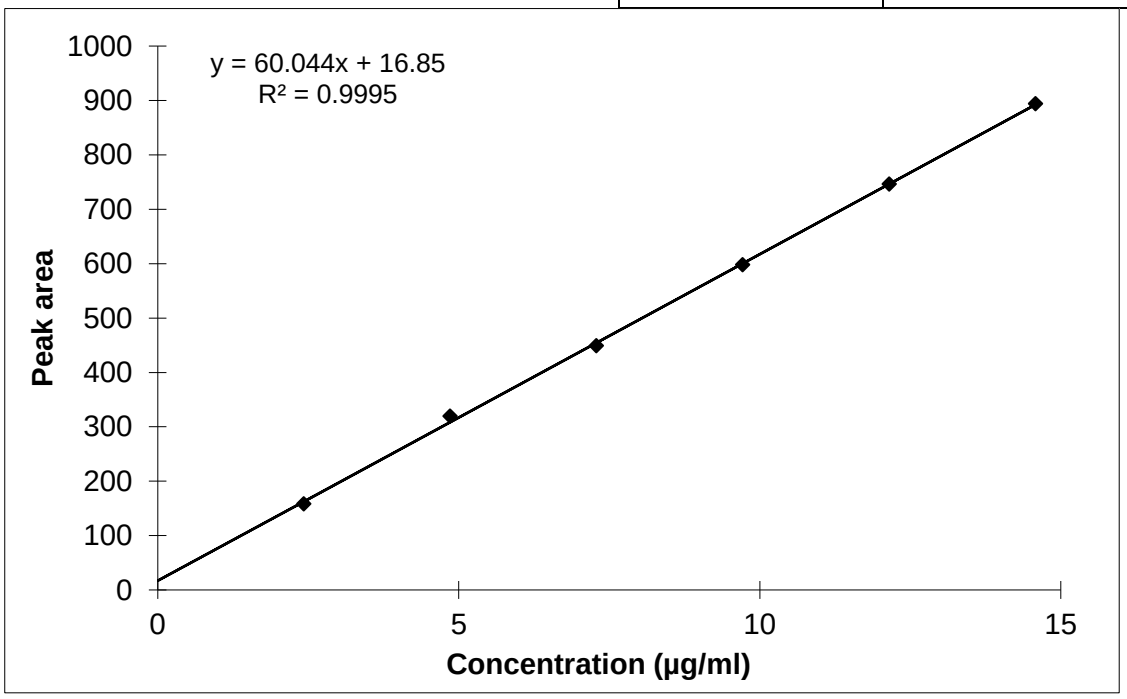


Figure E.3: The linear regression curve of methylparaben standard solutions

E.6.1.2.3 Propylparaben

Propylparaben demonstrated acceptable linearity with a R^2 value of 0.997. This value indicates the HPLC system was stable over the concentration range of 0.82 – 4.90 µg/ml. The linearity results and the regression curve of linearity follow in Table E.4 and Figure E.4, respectively.

Table E.4: The linearity results of propylparaben

Standard concentration (µg/ml)	Peak area 1	Peak area 2	Mean peak area
0.82	55.4	56.3	55.9
1.63	106.1	107.8	107.0
2.45	137.6	137.5	137.6
3.26	181.2	181.6	181.4
4.08	225.4	225.0	225.2
4.89	283.1	269.3	276.2
		Slope	**52.598**
		y-intercept	**13.823**
		R^2	**0.997**

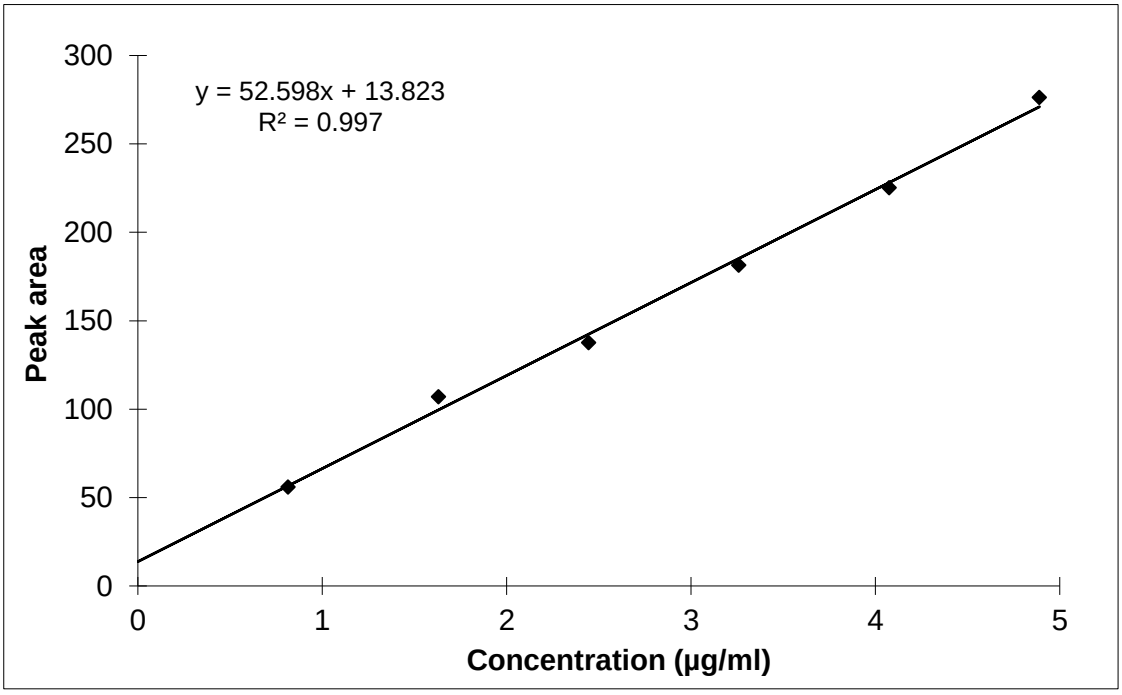


Figure E.4: The linear regression curve of propylparaben standard solutions

E.6.2 Accuracy

The accuracy is a validation parameter used to express the closeness between the experimental value and the known value (ICH, 2005:4). Accuracy is indicated by percentage recovery. The %RSD must be 2% or less (Shabir, 2005:9) and the acceptable range for the mean percentage recovery is 98 – 102% (Shabir, 2005:9). At least nine determinations of three concentrations are necessary for accuracy determinations (ICH, 2005:10).

E.6.2.1 Method

A standard solution was prepared as described in Section E.4. This solution was injected in duplicate in injection volumes of 2.5, 5.0, 10.0, 15.0 and 20.0 µl. The samples were prepared by dissolving carnosine (298.20 mg) in water and methylparaben (20.63 mg) and propylparaben (5.16 mg) in methanol, in 100 ml volumetric flasks, respectively. Next, 4, 5 and 6 ml of the solutions were diluted to 100 ml in volumetric flasks with water and methanol, respectively. All nine sample solutions were injected in duplicate, where after the recovery was calculated in concentration and percentage.

E.6.2.2 Results and discussion

E.6.2.2.1 Carnosine

The average recovery of carnosine was determined as 101.24%, with an RSD of 1.36%, which is within the acceptable ranges. The results follow in Table E.5.

Table E.5: The accuracy results of carnosine determination

Concentration (µg/ml)	Peak area 1	Peak area 2	Mean peak area	Recovery	
				(µg/ml)	%
119.28	2354.6	2352.2	2353	120.956	101.41
119.28	2341.9	2361.2	2352	120.863	101.33
119.28	2356.8	2362.0	2359	121.256	101.66
149.10	3042.0	3046.2	3044	155.535	104.32
149.10	2908.6	2892.4	2901	148.346	99.49
149.10	2912.2	2899.2	2906	148.606	99.67
178.92	3560.5	3579.2	3570	181.857	101.64
178.92	3566.1	3565.9	3566	181.664	101.53
178.92	3517.0	3514.2	3516	179.141	100.12
				Mean	**101.24**
				SD	**1.36**
				%RSD	**1.34**

E.6.2.2.2 Methylparaben

The average recovery of methylparaben was determined as 101.85%, with an RSD of 1.51%, which is within the acceptable ranges. The results follow in Table E.6.

Table E.6: The accuracy results of methylparaben determination

Concentration (µg/ml)	Peak area 1	Peak area 2	Mean peak area	Recovery	
				(µg/ml)	%
8.25	516.6	545.1	531	8.707	105.52
8.25	510.5	511.8	511	8.384	101.60
8.25	512.2	512.2	512	8.401	101.81
10.32	657.1	639.3	648	10.633	103.08
10.32	632.9	631.8	632	10.373	100.56
10.32	631.0	631.7	631	10.356	100.40
12.38	758.5	760.6	760	12.460	100.66
12.38	767.6	767.6	768	12.592	101.73
12.38	764.1	764.2	764	12.535	101.27
				Mean	**101.85**
				SD	**1.51**
				%RSD	**1.48**

E.6.2.2.3 Propylparaben

The average recovery of propylparaben was determined as 101.02%, with a RSD of 0.57%, which is within the acceptable ranges. The results follow in Table E.7.

Table E.7: The accuracy results of propylparaben determination

Concentration (µg/ml)	Peak area 1	Peak area 2	Mean peak area	Recovery	
				(µg/ml)	%
2.06	109.7	108.3	109	2.084	100.97
2.06	110.5	110.8	111	2.115	102.48
2.06	108.4	109.9	109	2.087	101.11
2.58	134.9	137.1	136	2.593	100.51
2.58	135.8	136.7	136	2.598	100.69
2.58	138.0	136.4	137	2.616	101.39
3.10	164.2	163.4	164	3.117	100.69
3.10	163.4	164.6	164	3.121	100.81
3.10	164.4	162.7	164	3.113	100.54
				Mean	**101.02**
				SD	**0.58**
				%RSD	**0.57**

E.6.3 Precision

The precision is a validation parameter to measure the closeness of agreement between a number of measurements done on the same sample (ICH, 2005:4). Intra-day and inter-day repeatability form part of precision. For the precision to be acceptable, the %RSD must ≤ 2% (Shabir, 2005:9).

E.6.3.1 Intra-day repeatability

E.6.3.1.1 Method

A standard solution was prepared, as described in Section E.4, and injected in duplicate in injection volumes of 2.5, 5.0, 10.0, 15.0 and 20.0 µl. The samples were prepared by dissolving approximately 0.4, 0.5 and 0.6 g semi-solid formulation in methanol in a 100 ml volumetric flask. The samples were prepared in triplicate. All nine samples were injected in duplicate, where after the recovery was calculated in concentration and percentage.

E.6.3.1.2 Results and discussion

E.6.3.1.2.1 Carnosine

The intra-day repeatability %RSD of carnosine was calculated as 1.97%, which is slightly high but still acceptable. The results follow in Table E.8.

Table E.8: The intra-day repeatability results of carnosine determination

Concentration (µg/ml)	Peak area 1	Peak area 2	Mean peak area	Recovery	
				(µg/ml)	%
126.06	2537.0	2544.3	2541	130.288	103.35
122.82	2445.4	2518.8	2482	127.355	103.69
120.81	2414.8	2462.8	2439	125.185	103.62
154.95	3120.9	3141.6	3131	159.880	103.18
150.39	2898.8	2937.3	2918	149.198	99.21
153.96	2942.6	3090.0	3016	154.121	100.10
181.47	3703.7	3638.7	3671	186.934	103.01
181.95	3495.8	3513.4	3505	178.586	98.15
192.30	3873.1	3756.2	3815	194.121	100.95
				Mean	**101.70**
				SD	**2.01**
				%RSD	**1.97**

E.6.3.1.2.2 Methylparaben

The intra-day repeatability %RSD of methylparaben was calculated as 1.75%, which is within the acceptable ranges. The results follow in Table E.9.

Table E.9: The intra-day repeatability results of methylparaben determination

Concentration (µg/ml)	Peak area 1	Peak area 2	Mean peak area	Recovery	
				(µg/ml)	%
8.40	512.8	519.8	516	8.468	100.77
8.19	488.9	495.7	492	8.075	98.64
8.05	506.7	510.5	509	8.342	103.58
10.85	667.5	665.8	667	10.935	100.82
11.03	674.5	674.0	674	11.060	100.29
10.26	611.8	624.1	618	10.136	98.76
12.10	724.9	718.2	722	11.836	97.84
12.13	750.5	755.6	753	12.353	101.84
12.82	769.5	771.0	770	12.635	98.56
				Mean	**100.12**
				SD	**1.75**
				%RSD	**1.75**

E.6.3.1.2.3 Propylparaben

The intra-day repeatability %RSD of propylparaben was calculated as 1.79%, which is acceptable. The results follow in Table E.10.

Table E.10: The intra-day repeatability results of propylparaben determination

Concentration (µg/ml)	Peak area 1	Peak area 2	Mean peak area	Recovery	
				(µg/ml)	%
2.49	132.0	130.6	131	2.505	100.51
2.43	127.1	133.6	130	2.487	102.45
2.39	125.5	127.3	126	2.412	101.01
3.06	157.5	155.8	157	2.983	97.38
2.97	154.2	152.0	153	2.916	98.08
3.04	158.2	157.3	158	3.003	98.68
3.59	186.2	182.8	185	3.508	97.79
3.60	182.4	185.8	184	3.500	97.32
3.80	192.5	197.5	195	3.706	97.49
				Mean	**98.97**
				SD	**1.78**
				%RSD	**1.79**

E.6.3.2 Interday repeatability

The inter-day repeatability was performed over three days.

E.6.3.2.1 Method

A standard solution was prepared, as described in Section E.4, and injected in duplicate in injection volumes of 2.5, 5.0, 10.0, 15.0 and 20.0 µl. The samples were prepared by dissolving approximately 0.5 g semi-solid formulation in methanol in a 100 ml volumetric flask. The sample was prepared in triplicate each day. All three samples were injected in duplicate, where after the recovery was calculated in concentration and percentage.

E.6.3.2.2 Results and discussion

E.6.3.2.2.1 Carnosine

The inter-day repeatability %RSD of carnosine was calculated as 1.51%. The variance between inter-day and intra-day repeatability did not differ significantly. The repeatability was in acceptable limits and, when executed in different laboratories by different personnel, the assay will therefore perform well. The results follow in Table E.11.

Table E.11: The inter-day repeatability results of carnosine determination

	Day 1	Day 2	Day 3	Between days
	103.2	101.1	101.4	
	99.2	101.8	98.8	
	100.1	98.2	99.5	
Mean	**100.83**	**100.37**	**99.89**	**100.36**
SD	**1.70**	**1.54**	**1.10**	**1.52**
%RSD	**1.69**	**1.53**	**1.10**	**1.51**

E.6.3.2.2.2 Methylparaben

The inter-day repeatability %RSD of methylparaben was calculated as 1.01%. The variance between intra-day and inter-day repeatability did not differ noticeably. The repeatability was within acceptable limits. The results follow in Table E.12.

Table E.12: The inter-day repeatability results of methylparaben determination

	Day 1	Day 2	Day 3	Between days
	100.8	98.1	98.8	
	100.3	98.5	98.4	
	98.8	97.9	97.8	
Mean	**99.95**	**98.16**	**98.31**	**98.81**
SD	**0.87**	**0.22**	**0.40**	**0.99**
%RSD	**0.87**	**0.23**	**0.41**	**1.01**

E.6.3.2.2.3 Propylparaben

The inter-day repeatability %RSD of propylparaben was calculated as 1.72%. The variance between inter-day and intra-day repeatability did not differ greatly. The repeatability was within acceptable limits. The results follow in Table E.13.

Table E.13: The inter-day repeatability results of propylparaben determination

	Day 1	Day 2	Day 3	Between days
	97.4	101.1	99.8	
	98.1	101.7	98.3	
	98.7	101.2	96.8	
Mean	**98.05**	**101.37**	**98.27**	**99.23**
SD	**0.53**	**0.26**	**1.22**	**1.71**
%RSD	**0.54**	**0.25**	**1.24**	**1.72**

E.6.4 Ruggedness

Sample stability and system repeatability form part of ruggedness. The ruggedness is a validation parameter measuring reproducibility of test results when the samples are analysed under different conditions, such as varying temperatures in different laboratories and at different time periods (ICH, 2005:5). The acceptance criteria are for the %RSD to be ≤ 2% (Shabir, 2005:9-10).

E.6.4.1 Sample stability

E.6.4.1.1 Method

A standard sample was prepared by dissolving 0.5 mg semi-solid formulation in methanol in a 100 ml volumetric flask. The sample was injected for analysis every hour for 24 h to measure the stability. The injection volume was 10 µl.

E.6.4.1.2 Results and discussion

E.6.4.1.2.1 Carnosine

The mean recovery of carnosine was 100.09% and the %RSD was 0.37%. Both values were within the acceptable limits and the sample was stable for 24 h. The results follow in Table E.14.

Table E.14: The sample stability of carnosine over 24 h

Time (h)	Peak area	%Recovery
0	3679.4	100.00
1	3648.7	99.17
2	3662.1	99.53
3	3676.8	99.93
4	3681.8	100.07
5	3698.6	100.52
6	3698.5	100.52
7	3676.2	99.91
8	3691.7	100.33
9	3695.3	100.43
10	3702.6	100.63
11	3677.7	99.95
12	3666.7	99.65
13	3674.6	99.87
14	3680.7	100.04
15	3679.0	99.99
16	3695.5	100.44
17	3699.6	100.55
18	3696.2	100.46
19	3690.4	100.30
20	3674.7	99.87
21	3667.2	99.67
22	3697.6	100.49
23	3686.2	100.18
24	3670.4	99.76
Mean	**3682.7**	**100.09**
SD	**13.49**	**0.37**
%RSD	**0.37**	**0.37**

E.6.4.1.2.2 Methylparaben

The mean recovery of carnosine was 99.60% and the %RSD was 0.31%. Both values were within the acceptable limits and the sample was stable for 24 h. The results follow in Table E.15.

Table E.15: The sample stability of methylparaben over 24 h

Time (h)	Peak area	%Recovery
0	740.8	100.00
1	736.6	99.43
2	737.4	99.54
3	736.6	99.43
4	737.3	99.53
5	737.9	99.61
6	737.8	99.60
7	735.2	99.24
8	735.9	99.34
9	731.0	98.68
10	736.8	99.46
11	740.2	99.92
12	736.4	99.41
13	736.5	99.42
14	737.7	99.58
15	740.2	99.92
16	740.0	99.89
17	739.3	99.80
18	736.3	99.39
19	737.0	99.49
20	739.8	99.87
21	742.0	100.16
22	737.0	99.49
23	741.4	100.08
24	738.2	99.65
Mean	**737.8**	**99.60**
SD	**2.28**	**0.31**
%RSD	**0.31**	**0.31**

E.6.4.1.2.3 Propylparaben

The mean recovery of carnosine was 98.22% and the %RSD was 1.12%. Both values were within the acceptable limits and the sample was stable for 24 h. The results follow in Table E.16.

Table E.16: The sample stability of propylparaben over 24 h

Time (h)	Peak area	%Recovery
0	154.9	100.00
1	153.5	99.10
2	154.4	99.68
3	153.3	98.97
4	153.7	99.23
5	154.5	99.74
6	154.2	99.55
7	152.5	98.45
8	153.4	99.03
9	150.1	96.90
10	153.2	98.90
11	150.2	96.97
12	150.3	97.03
13	148.8	96.06
14	151.7	97.93
15	150.2	96.97
16	153.8	99.29
17	153.2	98.90
18	150.9	97.42
19	151.5	97.81
20	152.3	98.32
21	151.3	97.68
22	151.1	97.55
23	150.1	96.90
24	150.3	97.03
25	152.1	98.19
Mean	152.1	98.22
SD	1.70	1.10
%RSD	1.12	1.12

E.6.4.2 System repeatability

E.6.4.2.1 Method

A standard sample was prepared by dissolving 0.5 mg semi-solid formulation in methanol in a 100 ml volumetric flask. The sample was injected seven times.

E.6.4.2.2 Results and discussion

E.6.4.2.2.1 Carnosine

The %RSD of the retention times was calculated as 0.815% and the %RSD for peak area was 0.34%. Both these values were less than two and within the acceptable ranges and the method was repeatable. The results follow in Table E.17.

Table E.17: The system repeatability results of carnosine

Injection number	Peak area	Retention time (min)
1	2881.6	5.065
2	2876.1	5.112
3	2874.3	5.112
4	2852.8	5.149
5	2881.3	5.108
6	2869.2	5.019
Mean	**2873**	**5.094**
SD	**9.79**	**0.042**
%RSD	**0.34**	**0.815**

E.6.4.2.2.2 Methylparaben

The %RSD of the retention times was calculated as 0.046% and the %RSD for peak area was 0.08%. Both these values were less than two and within the acceptable ranges and the method was repeatable. The results follow in Table E.18.

Table E.18: The system repeatability results of methylparaben

Injection number	Peak area	Retention time (min)
1	589.0	6.824
2	589.9	6.825
3	589.4	6.825
4	588.3	6.824
5	588.9	6.832
6	589	6.830
Mean	**589**	**6.827**
SD	**0.49**	**0.003**
%RSD	**0.08**	**0.046**

E.6.4.2.2.3 Propylparaben

The %RSD of the retention times was calculated as 0.042% and the %RSD for peak area was 0.86%. Both these values are less than two and within the acceptable ranges and the method was repeatable. The results follow in Table E.19.

Table E.19: The system repeatability results of propylparaben

Injection number	Peak area	Retention time (min)
1	132.6	7.505
2	131.0	7.503
3	129.9	7.503
4	131.0	7.505
5	132.7	7.503
6	129.9	7.512
Mean	**131**	**7.505**
SD	**1.13**	**0.003**
%RSD	**0.86**	**0.042**

E.6.5 Robustness

Robustness is a validation parameter to determine whether the analysis will remain unaffected when deliberate small changes in validation parameters are made (ICH, 2005:5). This will reveal the reliability of the method upon normal usage. The method must therefore tolerate the small changes.

E.6.5.1 Method

A standard sample was prepared by weighing 0.5 g semi-solid formulation and dissolving it in methanol in a 100 ml volumetric flask. The following changes were made to the chromatographic conditions:

Change 1:

Flow rate: 1.2 ml/min

Injection volume: 12 µl

Detection wavelength: 212 nm

Change 2:

Flow rate: 0.8 ml/min

Injection volume: 15 µl

Detection wavelength: 212 nm

Change 3:

Flow rate: 1.1 ml/min

Injection volume: 10 µl

Detection wavelength: 213 nm

E.6.5.2 Results and discussion

The HPLC method tolerated the small changes in chromatographic conditions, remaining unaffected and the peaks identifiable. The method will perform well when used. The results follow in Figure E.5.

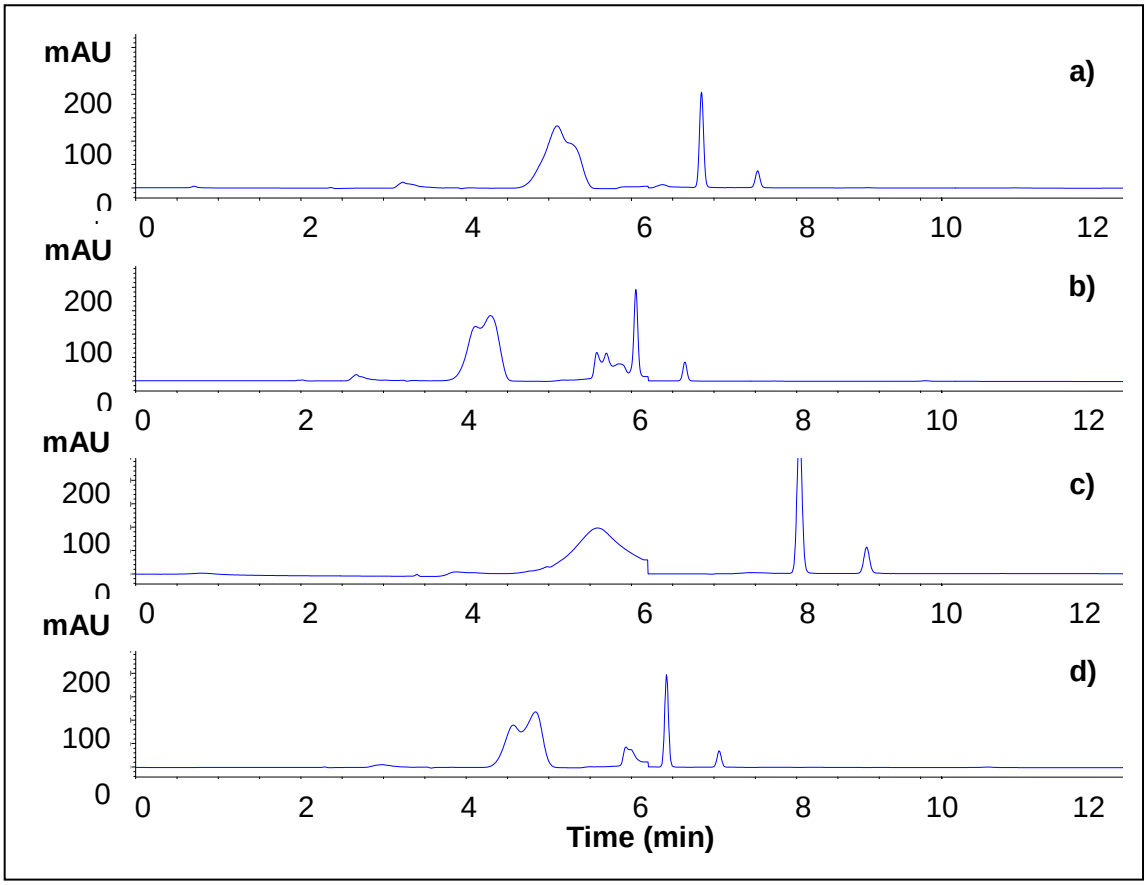


Figure E.5: The chromatogram obtained from the robustness analysis: a) standard sample, b) change 1, c) change 2 and d) change 3

E.6.6 Specificity

Specificity is a validation parameter to analyse ingredients in the presence of degradants and impurities (ICH, 2005:4). The degraded samples must not interfere with the active ingredients' determination.

E.6.6.1 Method

A standard sample was prepared by weighing 0.5 g semi-solid formulation and dissolving it in methanol in a 100 ml volumetric flask. The analytical samples were prepared by adding 1 ml of the standard sample to 1 ml HCl, NaOH, H_2O_2 and H_2O, respectively. These samples were vortexed for 5.00 min and injected in duplicate for HPLC analysis.

E.6.6.2 Results and discussion

The chromatograms obtained from the specificity test follow in Figures E.6 to E 10. The peaks of the active ingredient and excipients were identifiable in the sample mixed with HCl. The presence of NaOH and H_2O_2 slightly degraded both parabens in the samples, while water had no influence on the excipients.

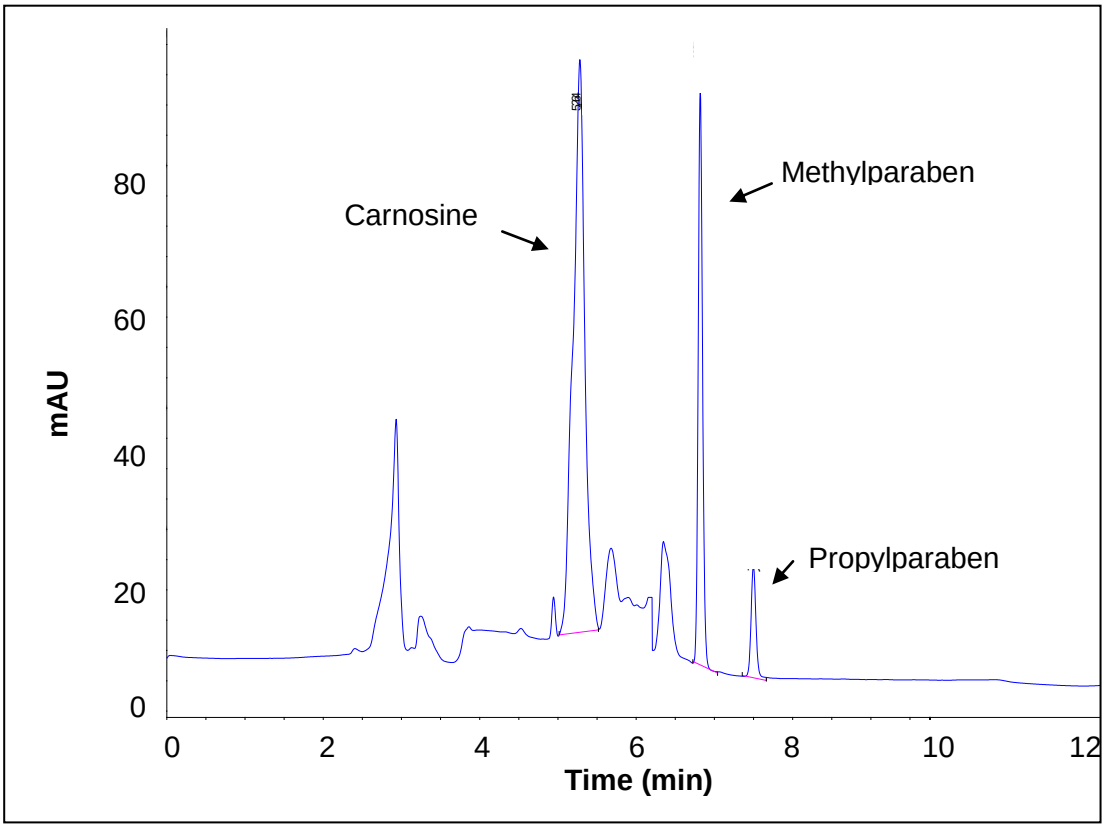


Figure E.6: The chromatogram obtained from the sample mixed with HCl

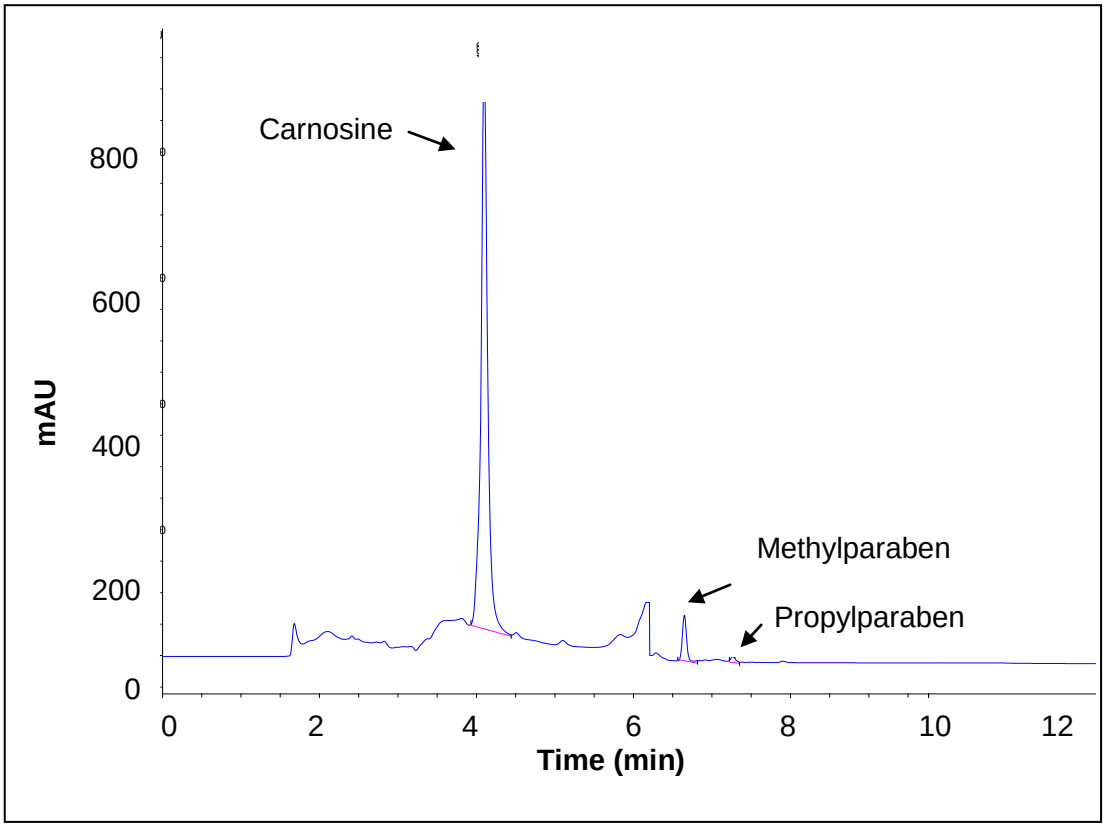


Figure E.7: The chromatogram obtained from the sample mixed with NaOH

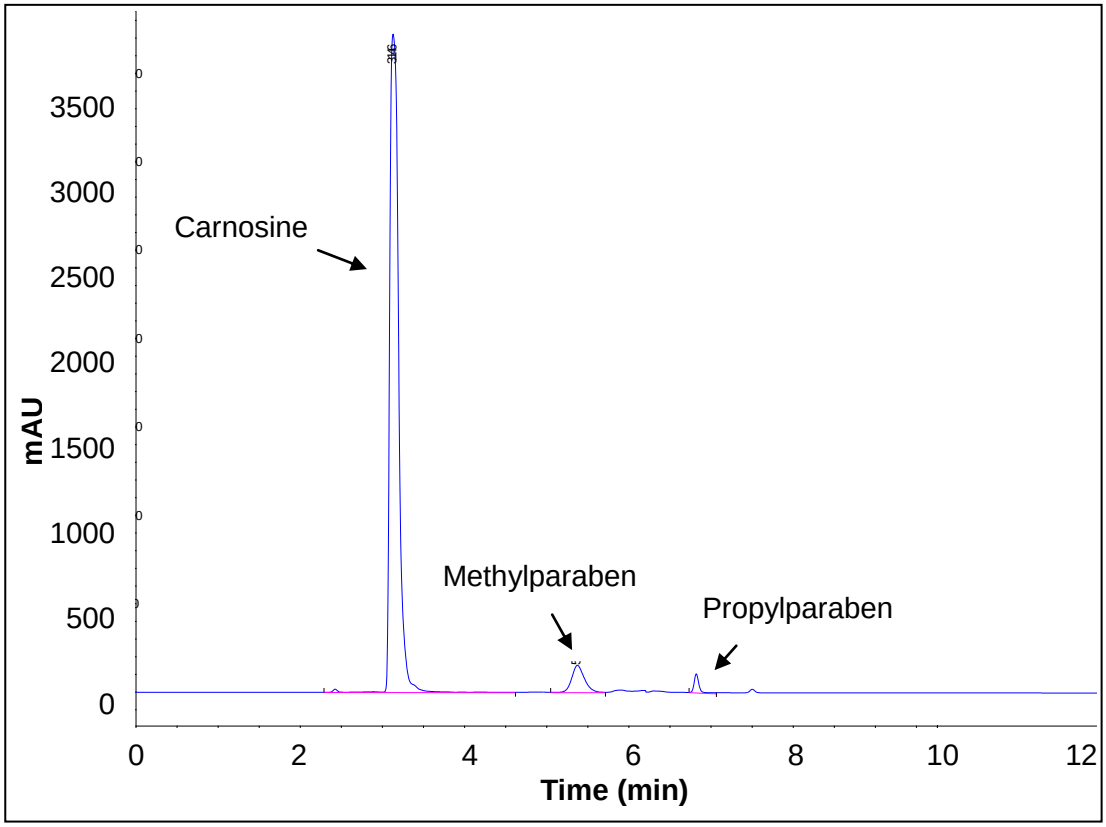


Figure E.8: The chromatogram obtained from the sample mixed with H_2O_2

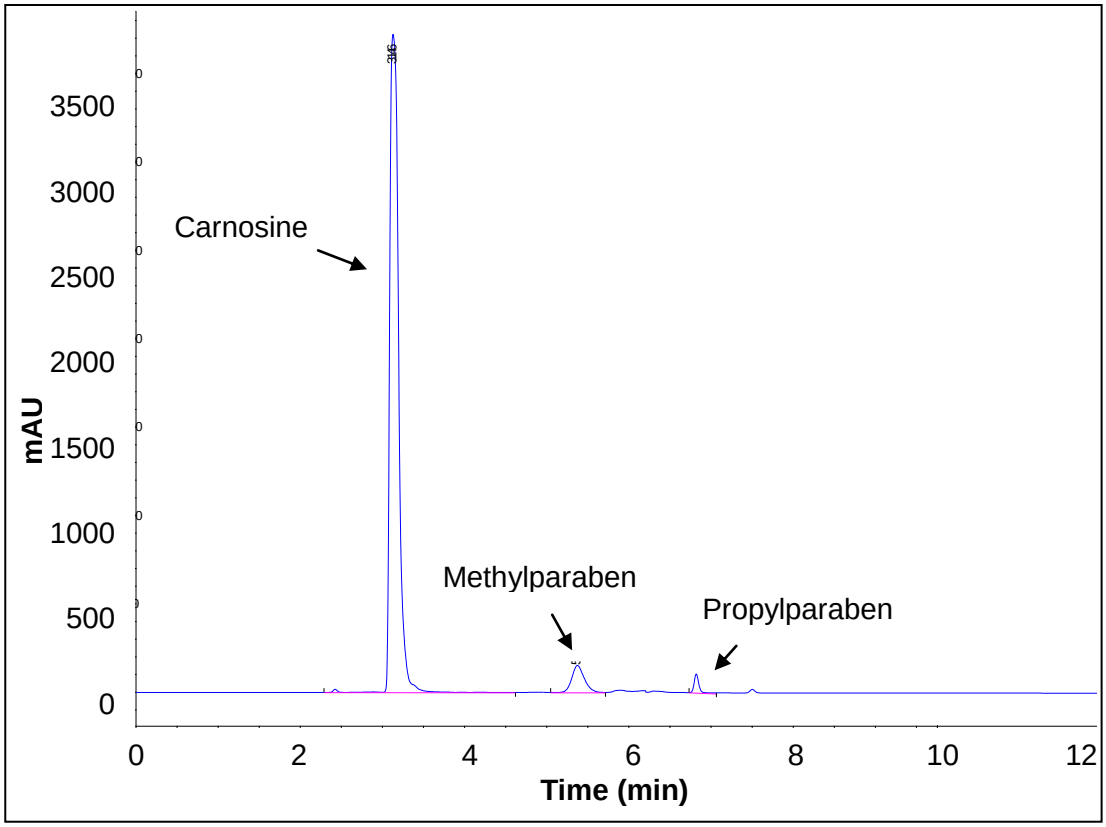


Figure E.9: The chromatogram obtained from the sample mixed with water

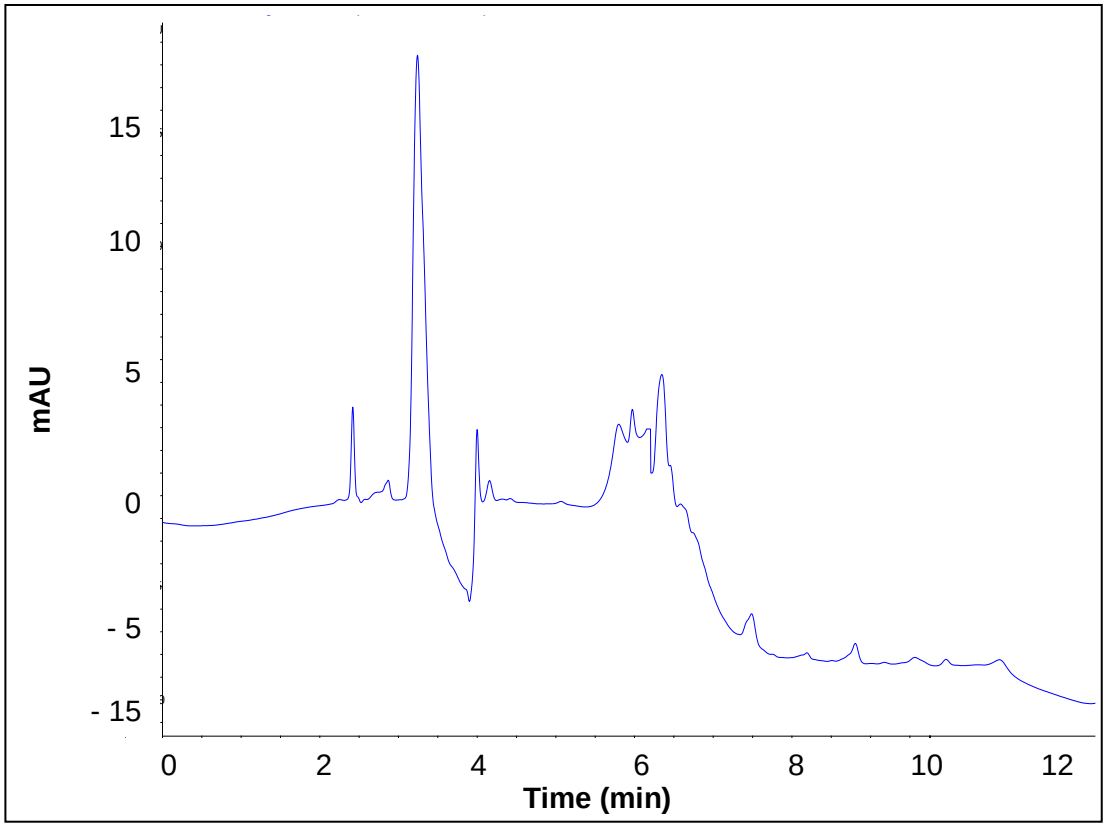


Figure E.10: The chromatogram obtained from the placebo sample

E.7 Conclusion

The HPLC method was successfully validated. It was adequately reliable and sensitive for the determination of the excipients during assay analysis.

References

ICH ***see*** ICH Expert Working Group.

ICH Expert Working Group. 2005. ICH harmonised tripartite guideline: validation of analytical procedures: text and methodology Q2 (R1). International conference on harmonisation of technical requirements for registration of pharmaceuticals for human use. http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q2_R1/Step4/Q2_R1__Guideline.pdf Date of access: 26 Jan. 2016.

Shabir, G.A. 2005. Institute of Validation Technology: step-by-step analytical methods validation and protocol in the quality system compliance industry. http://www.ivtnetwork.com/sites/default/files/Analytical%20Method%20Validation.pdf Date of access: 10 Jul. 2016.

APPENDIX F

STABILITY TESTING OF SEMI-SOLID FORMULATIONS

F.1 Introduction

Merriam-Webster's dictionary defines stability as "the quality or state of something that is not easily changed or likely to change" (Merriam-Webster Dictionary). According to Barnes (2007:650-661), pharmaceutical products deteriorate over time and have a predetermined shelf life and expiry date. The shelf life is described as the period during which a product is foreseen to retain acceptable stability on chemical, physical and microbiological levels. The expiry date, indicated on the product's packaging, represents the end of the shelf life. The purpose of stability testing is to determine the shelf life, storage conditions and expiry date of the pharmaceutical product.

Testing protocols include involving all relevant ingredients in the product, performing the tests on the final product stored in the exact packaging that is to be used for marketing, and using procedures that represent the storage and transport conditions to which the final product will be subjected to (Barnes, 2007:663; York, 2007:12-13). Most pharmaceutical products demand a shelf life of several years and stability testing would be too slow for the production process (Barnes, 2007:661). In order to shorten the test period, stress testing and applying the Arrhenius equation, for predictions on stability at room temperature, are generally performed (Barnes, 2007:661). Stress testing is valuable in pre-formulation studies to save time, but there is no substitute for long-term stability testing (Barnes, 2007:662). Stability testing will reveal the change in quality of the product over time under the influence of different environmental factors including light, temperature and humidity, while establishing a re-test period, or a shelf life, with recommended storage conditions (ICH, 2003:1).

Stability tests were performed according to the International Conference on Harmonisation (ICH) Guidelines and formulation changes were assessed during accelerated (40 ± 2 °C/75 ± 5% RH), intermediate (30 ± 2 °C/60 ± 5% RH) and long-term (25 ± 2 °C/60 ± 5% RH) storage conditions (ICH, 2003:3) over a three month testing period. Figure F.1 indicates the stability chamber used. According to the ICH (2003:9), the product should not change significantly. Significant changes include a 5% change from the baseline value, degradation of ingredients that exceed the acceptance criteria, failure to meet the acceptance criteria for appearance (colour, cracking and phase separation) and failure to meet the acceptance criteria for pH.

The following assessments were performed on two semi-solid formulations, a cosmeceutical cream and a cosmeceutical gel, initially and at month 1, month 2 and month 3:

- Concentration assay of active ingredients;
- pH;
- conductivity;
- viscosity;
- zeta-potential;
- mass loss determination;
- microscopic examination; and
- macroscopic/visual appearance examination.

Figure F.1: The stability chamber

F.2 Methods

F.3.1 Concentration assay

An HPLC method for the assay analysis of the active ingredient (carnosine) and the excipients (methylparaben and propylparaben) was previously developed and validated in cooperation with Professor Jan du Preez, at the Analytical Technology Laboratory, North-West University, Potchefstroom Campus, South Africa. The method is fully described in Appendix E. The three ingredients' concentrations were quantified and the mean percentages, relative to the initial assay results, were assessed.

Approximately 0.5 g of both the gel and the cream, representative of each storing condition, was accurately weighed and dissolved in methanol in a 100 ml volumetric flask. The samples were transferred to HPLC auto-sampler vials for analysis. The experiment was done in triplicate

initially and at all the time intervals. The standard sample was prepared as described in Section E.4.

F.3.2 pH

The pH of the samples, representative of each storage condition, was measured using a Mettler Toledo pH meter with a Mettler Toledo InLab 410 electrode (Mettler Toledo, Switzerland). The pH meter was calibrated at a pH of 4, 7 and 10 with buffer solutions before the experiment. The pH of each sample was measured in triplicate initially and at all the time intervals.

F.3.3 Conductivity

The conductivity of the formulation was determined using a Mettler Toledo SevenMulti™ pH/conductivity meter, equipped with a glass Mettler Toledo InLab 731 electrode (Mettler Toledo, Switzerland). The conductivity meter was calibrated with standard solutions before the experiment. The experiment was done in triplicate, initially and at all the time intervals, on the samples representing each storage condition. The conductivity was measured in micro Siemens per centimetre (µS/cm). The pH meter and electrode are indicated in Figure F.2.

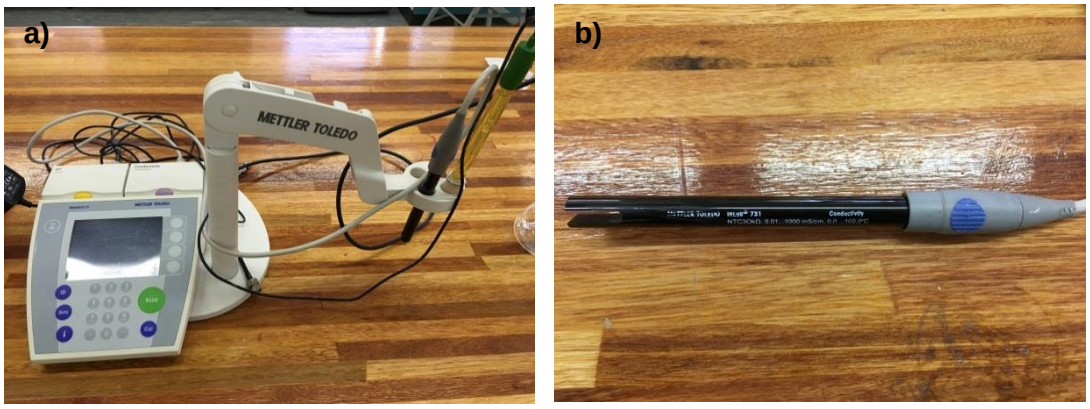


Figure F.2: a) The Mettler Toledo SevenMulti™ pH meter and b) Mettler Toledo InLab 731 electrode

F.3.4 Viscosity

The viscosity of the formulations was measured using Brookfield® Viscometer model DV-III Ultra (Brookfield Engineering Laboratories, United States of America). The viscosity was measured in centipoises (cP) at 25 °C. Prior to the experiment, the samples were placed in a water bath to reach 25 °C. Approximately 15 ml of each sample, representative of each storage condition, was transferred into a cylindrical sample chamber and placed in a flow jacket connected to the wsater bath. A SC25- spindle was used to measure the viscosity at a speed of 1 rpm and 2 rpm for the cream and gel, respectively. The average viscosity was determined from 32 data points,

collected at intervals of 10 sec for a total time of 5 min and 20 sec. The percentage torque was approximately 50%. Tests were performed intially and at all time intervals.

F.3.5 Zeta-potential

The zeta-potential of the formulations was determined using a Malvern Zetasizer Nano (Nano SZ) (Malvern Instruments, United Kingdom) at 25 °C. Each of the samples, representing the different storage conditions, were diluted (0.1 g/20 ml) with purified water to reduce particle interactions. This experiment was done in triplicate initially and at all the time intervals.

F.3.6 Mass loss determination

The mass loss of the formulations from the packaging was measured by weighing the exact same samples, representative of each storage condition, at all the time intervals. The empty containers with their lids were weighed initially, before adding the samples to them; the filled containers were weighed initially and at each time interval. The mass of the samples was determined by subtraction. The mean of three measurements was evaluated relative to the initial mass of each sample.

F.3.7 Microscopic analysis

A microscopic analysis of each sample, representative of each storage condition, was performed to inspect any changes in visual appearance. A Motic microscope (Motic, Hong Kong), equipped with a Moticam 3 camera (Motic, Hong Kong), using Motic Images Plus software was used during this experiment (Figure F.3). The samples were placed on a glass slide, spread out and covered with a glass slip before mounting it onto the microscope. Images were taken initially and at all the time intervals for comparison.

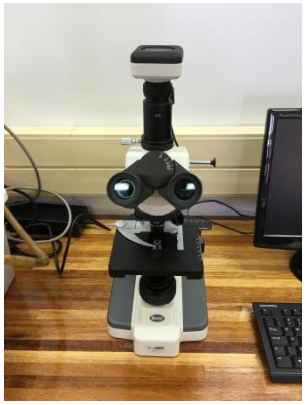


Figure F.3: The Motic microscope equipped with a Moticam 3 camera

F.3.8 Macroscopic analysis

A macroscopic analysis of each sample, representative of each storage condition, was performed to inspect any visible changes such as phase separation and colour changes. Images were taken initially and at all the time intervals for comparison with an iPhone 6s, 12-megapixel camera (Apple Inc., United States of America). Sensory assessments also formed part of the macroscopic analysis and included smelling and feeling the formulations. Tests were performed at the same time of the day at each time interval.

F.3.9 Statistical analysis

The mean percentage change, relative to the baseline values, of each stability parameter was calculated after completion of the stability tests. These values were used to determine whether any significant change had occurred over the three months. The following equation was used:

$$\%Change = (T0 - Tx)/T0 \times 100 \qquad \textbf{Equation E.1}$$

Where T0 is the initial value of the stability parameter and Tx is the stability parameter at the specific time interval.

F.4 Results and discussion

F.4.1 Concentration assay of active ingredients

The concentration assay results generated during the three months are summarised in Tables F.1 to F.6. The outcomes that did not comply with the acceptable 5% variation limit are indicated in red (ICH Guidelines Q1A (R2)).

As seen in the results, the initial concentrations are slightly higher than 100%. This is the result of an excess of the ingredients added to the formulation to provide for possible degradation. Approximately 20% excess was added to the formulations. According to Barnes (2007:650), this is a simple strategy to increase the shelf life of products. Two important aspects to consider when following this strategy are that the dose of the product must not be critical and that the degradation products must not be toxic. This usually applies to vitamins and other natural products. A USP monograph for vitamin E was used, because firstly, neither vitamin E nor carnosine are active pharmaceutical ingredients and the two substances are therefore comparable. The second reason is because there are no available monographs for a cream and gel containing carnosine as active ingredient. This vitamin E monograph indicated that the final product must not contain less than 95% and not more than 120% of the labelled amount (USP, 2016:6380).

The mean percentage concentration change of the active ingredient and excipients in the cream samples representing each different storage condition over the three months test period, relative to the initial values, were calculated. Of all the outcomes, only carnosine in the cream formulation, stored at 40 ± 2 °C/75 ± 5% RH, remained completely within the acceptable 5% variation limit (ICH Guidelines Q1A (R2)).

The variations of the carnosine concentration in the cream after three months were 2.9, 0.5 and 2.7% for the three different storage conditions (25 ± 2 °C/60 ± 5% RH, 30 ± 2 °C/60 ± 5% RH and 40 ± 2 °C/75 ± 5% RH), respectively. The variations of the methylparaben concentration in the cream after three months were 9.9, 11.2 and 15.5% for the three different storage conditions (25 ± 2 °C/60 ± 5% RH, 30 ± 2 °C/60 ± 5% RH and 40 ± 2 °C/75 ± 5% RH), respectively. Methylparaben had the highest variations of all the ingredients in the cream. The variations of the propylparaben concentration in the cream after three months were 1.3, 2.5 and 2.8% for the three different storage conditions (25 ± 2 °C/60 ± 5% RH, 30 ± 2 °C/60 ± 5% RH and 40 ± 2 °C/75 ± 5% RH), respectively.

The variations of the carnosine concentration in the gel after three months were 3.9, 1.3 and 5.3% for the three different storage conditions (25 ± 2 °C/60 ± 5% RH, 30 ± 2 °C/60 ± 5% RH and 40 ± 2 °C/75 ± 5% RH), respectively. The variations of the methylparaben concentration in the gel after three months were 5.4, 9.5 and 18.0% for the three different storage conditions (25 ± 2 °C/60 ± 5% RH, 30 ± 2 °C/60 ± 5% RH and 40 ± 2 °C/75 ± 5% RH), respectively. The variations of the propylparaben concentration after three months were 3.9, 2.2 and 6.8% for the three different storage conditions (25 ± 2 °C/60 ± 5% RH, 30 ± 2 °C/60 ± 5% RH and 40 ± 2 °C/75 ± 5% RH), respectively.

In summary, the active ingredient and excipients in the cream and gel revealed more or less the same results after the three months testing period; the concentrations in both the cream and the gel fluctuated during this period. The main reason for fluctuations could be due to poor mixing of the semi-solids during bulk formulation. Poor mixing also possibly contributed to some glass containers containing more of the active ingredient than what others did. The ingredients showed an overall decrease in concentration, but because of the excess at the start of the testing period, the final concentrations of all the ingredients remained between 95 and 120% of the labelled amount, as required by USP (USP, 2016:6380).

Table F.1: The concentration assay results of carnosine in the cosmeceutical cream

	Initial (T0)	Month 1 (T1)	Month 2 (T2)	Month 3 (T3)
25 ± 2 °C/60 ± 5% RH				
%Carnosine	113.1	107.9	119.9	109.9
30 ± 2 °C/60 ± 5% RH				
%Carnosine	113.1	106.6	121.9	112.5
40 ± 2 °C/75 ± 5% RH				
%Carnosine	113.1	109.2	117.6	110.0

Table F.2: The concentration assay results of methylparaben in the cosmeceutical cream

	Initial (T0)	Month 1 (T1)	Month 2 (T2)	Month 3 (T3)
25 ± 2 °C/60 ± 5% RH				
%Methylparaben	124.6	114.5	120.9	112.3
30 ± 2 °C/60 ± 5% RH				
%Methylparaben	124.6	114.9	119.3	110.6
40 ± 2 °C/75 ± 5% RH				
%Methylparaben	124.6	114.1	114.6	105.2

Table F.3: The concentration assay results of propylparaben in the cosmeceutical cream

	Initial (T0)	Month 1 (T1)	Month 2 (T2)	Month 3 (T3)
25 ± 2 °C/60 ± 5% RH				
%Propylparaben	110.9	113.2	127.2	112.4
30 ± 2 °C/60 ± 5% RH				
%Propylparaben	110.9	126.5	129.0	113.7
40 ± 2 °C/75 ± 5% RH				
%Propylparaben	110.9	114.5	128.7	113.9

Table F.4: The concentration assay results of carnosine in the cosmeceutical gel

	Initial (T0)	Month 1 (T1)	Month 2 (T2)	Month 3 (T3)
25 ± 2 °C/60 ± 5% RH				
%Carnosine	110.9	95.7	114.7	106.5
30 ± 2 °C/60 ± 5% RH				
%Carnosine	110.9	102.8	116.9	109.4
40 ± 2 °C/75 ± 5% RH				
%Carnosine	110.9	99.3	116.2	104.9

Table F.5: The concentration assay results of methylparaben in the cosmeceutical gel

	Initial (T0)	Month 1 (T1)	Month 2 (T2)	Month 3 (T3)
25 ± 2 °C/60 ± 5% RH				
%Methylparaben	119.6	112.5	120.5	113.1
30 ± 2 °C/60 ± 5% RH				
%Methylparaben	119.6	112.1	112.7	108.2
40 ± 2 °C/75 ± 5% RH				
%Methylparaben	119.6	108.2	107.9	98.0

Table F.6: The concentration assay results of propylparaben in the cosmeceutical gel

	Initial (T0)	Month 1 (T1)	Month 2 (T2)	Month 3 (T3)
25 ± 2 °C/60 ± 5% RH				
%Propylparaben	109.328	79.666	127.305	113.543
30 ± 2 °C/60 ± 5% RH				
%Propylparaben	109.328	111.082	117.693	106.886
40 ± 2 °C/75 ± 5% RH				
%Propylparaben	109.328	115.577	121.290	101.931

F.4.2 pH measurements of the samples

The pH values of the cream and gel samples, representing each storage condition, are indicated in Tables F.7 and F.8, respectively. The mean percentage pH changes over the three month test period, relative to the initial values, were calculated and the outcomes that did not comply with the acceptable 5% variation limit are indicated in red (ICH Guidelines Q1A (R2)).

According to Hach Company (2010:6), the pH value expresses the ratio between the hydrogen ions [H^+] and the hydroxide ions [OH^-] in a solution, and will remain constant in a stable solution. Consequently, a change in the pH value of a formulation suggests a disturbance of the [H^+] balance.

None of the cream samples complied with the acceptable 5% variation limit and the pH of the samples changed significantly during the test period (ICH Guidelines Q1A (R2)). The pH values of all the cream samples decreased over time, with the largest decrease calculated as 9.494% for the sample stored at 40 ± 2 °C/75 ± 5% RH after three months. The pH of the gel samples also revealed a small decrease, but remained within the acceptable 5% variation limit. The largest decrease observed for the gel was calculated as 2.122% for the sample stored at 30 ± 2 °C/60 ± 5% RH during the first month. The decreases in pH are indicative of the release of [H^+], possibly due to water release from the formulation resulting in both [H^+] and [OH^-] concentration variations (Hach Company 2010:4). The water release due to condensation, possibly supporting the pH variations, is described in Section F.4.7.

When considering the pH of the formulations, the gel was more stable over the three month testing period (Hach Company 2010:6).

Table F.7: The pH results of the cosmeceutical cream

	Initial (T0)	Month 1 (T1)	Month 2 (T2)	Month 3 (T3)
25 ± 2 °C/60 ± 5% RH				
pH	8.20	7.99	7.82	7.62
Mean %change relative to T0				
		T0 – T1	**T0 – T2**	**T0 – T3**
		2.625	4.682	7.068
30 ± 2 °C/60 ± 5% RH				
pH	8.20	7.91	7.84	7.53
Mean %change relative to T0				
		T0 – T1	**T0 – T2**	**T0 – T3**
		3.544	4.410	8.254
40 ± 2 °C/75 ± 5% RH				
pH	8.20	7.71	7.61	7.42
Mean %change relative to T0				
		T0 – T1	**T0 – T2**	**T0 – T3**
		5.962	7.173	9.494

Table F.8: The pH results of the cosmeceutical gel

	Initial (T0)	Month 1 (T1)	Month 2 (T2)	Month 3 (T3)
25 ± 2 °C/60 ± 5% RH				
pH	7.65	7.58	7.64	7.57
Mean %change relative to T0				
		T0 – T1	**T0 – T2**	**T0 – T3**
		1.006	0.226	1.084
30 ± 2 °C/60 ± 5% RH				
pH	7.65	7.49	7.55	7.56
Mean %change relative to T0				
		T0 – T1	**T0 – T2**	**T0 – T3**
		2.112	1.315	1.254
40 ± 2 °C/75 ± 5% RH				
pH	7.65	7.59	7.59	7.58
Mean %change relative to T0				
		T0 – T1	**T0 – T2**	**T0 – T3**
		0.797	0.862	0.980

F.4.3 Conductivity measurements of the samples

The conductivity results of the cream and gel samples, representing each storage condition are indicated in Tables F.9 and F.10, respectively. The mean percentage conductivity changes over the three months test period, relative to the initial values, were calculated. The outcomes that did not comply with the acceptable 5% variation limit are indicated in red (ICH Guidelines Q1A (R2)).

The character of a formulation can easily be evaluated by the electrical conductivity procedure (Ferreira *et al.,* 2010:1385). According to Lamba *et al.* (2015:719), electrical conductivity indicates to what extent a formulation will allow electrical current to pass through it. This property is generally associated with charged ions. Formulations with an aqueous nature will present high conductivity values (Ferreira *et al.,* 2010:1385). Any phase separation or release of encapsulated active ingredient can result in conductivity changes (Lamba *et al.,* 2015:719).

All the cream samples revealed significant conductivity changes during the test period (ICH Guidelines Q1A (R2)). The conductivity showed an overall decrease after three months. The condensation that took place (described in Section F.4.7), possibly lead to a smaller amount of free water in the formulation, and possibly contributed to the decrease in conductivity values (Korać *et al.*, 2014:272).

The gel samples revealed even higher conductivity changes than the cream during the test period. The conductivity showed an overall increase after three months. As mentioned above, these changes could possibly be the result of release of encapsulated carnosine or phase separation in the formulation, since visible phase separation had occurred in the gel samples (Section F.4.7) (Lamba *et al.,* 2015:719). Furthermore, the pH changes suggesting a change in ion concentrations could possibly have led to a higher concentration of free positively and negatively charged ions in the formulation, resulting in the increase in conductivity values of the gel (Hach Company, 2014-2015:1).

The variance between the conductivity values of the niosome cream (Table F9) and the niosome gel (Table F.10) is indicative of the aqueous nature of the gel (resulting in higher overall conductivity values) and the more oily nature of the cream (resulting in lower overall conductivity values) (Ferreira *et al.,* 2010:1385). The cream is an o/w emulsion and, despite the oily nature, still consists of an aqueous phase enabling electrical conductivity. Due to the significant fluctuations in conductivity values for both the cream and the gel, following no specific pattern, no valuable conclusions regarding the stability of the product based on conductivity could be drawn.

Table F.9: The conductivity results of the cosmeceutical cream

	Initial (T0)	Month 1 (T1)	Month 2 (T2)	Month 3 (T3)
25 ± 2 °C/60 ± 5% RH				
Conductivity (µs/cm)	214.00	153.27	173.60	113.20
Mean %change relative to T0				
		T0 – T1	**T0 – T2**	**T0 – T3**
		28.380	18.879	47.103
30 ± 2 °C/60 ± 5% RH				
Conductivity (µs/cm)	214.00	123.70	111.27	83.43
Mean %change relative to T0				
		T0 – T1	**T0 – T2**	**T0 – T3**
		42.196	48.006	61.012
40 ± 2 °C/75 ± 5% RH				
Conductivity (µs/cm)	214.00	86.03	77.33	67.77
Mean %change relative to T0				
		T0 – T1	**T0 – T2**	**T0 – T3**
		59.798	63.863	68.333

Table F.10: The conductivity results of the cosmeceutical gel

	Initial (T0)	Month 1 (T1)	Month 2 (T2)	Month 3 (T3)
25 ± 2 °C/60 ± 5% RH				
Conductivity (µs/cm)	153.13	495.33	541.33	566.33
Mean %change relative to T0				
		T0 – T1	**T0 – T2**	**T0 – T3**
		223.465	253.505	269.830
30 ± 2 °C/60 ± 5% RH				
Conductivity (µs/cm)	153.13	1259.00	755.67	595.33
Mean %change relative to T0				
		T0 – T1	**T0 – T2**	**T0 – T3**
		722.159	393.470	288.768
40 ± 2 °C/75 ± 5% RH				
Conductivity (µs/cm)	153.13	567.00	618.67	557.33
Mean %change relative to T0				
		T0 – T1	**T0 – T2**	**T0 – T3**
		270.266	304.005	263.953

F.4.4 Viscosity measurements of the samples

The viscosities of the cream and gel samples, representing each storage condition are indicated in Tables F.11 and F.12, respectively. The mean percentage viscosity changes over the three months test period, relative to the initial values, were calculated. Indicated in red, are the outcomes that did not comply with the acceptable 5% variation limit (ICH Guidelines Q1A (R2)).

The initial viscosity of the cream samples was 227146.9 cP. The mean viscosity of the cream samples after three months were, 334121.9, 264059.4 and 273428.1 cP for the three different storage conditions (25 ± 2 °C/60 ± 5% RH, 30 ± 2 °C/60 ± 5% RH and 40 ± 2 °C/75 ± 5% RH), respectively. The initial viscosity of the gel samples was 77301.7 cP. The mean viscosity of the gel samples after three months were, 130631.3, 107350.0 and 106406.5 cP for the three different storage conditions (25 ± 2 °C/60 ± 5% RH, 30 ± 2 °C/60 ± 5% RH and 40 ± 2 °C/75 ± 5% RH), respectively. All the samples underwent significant changes during the testing period (ICH Guidelines Q1A (R2)).

Despite the fact these changes are referred to as significant, other comparable viscosities must be brought into consideration. For instance, the viscosity of honey is 10 000 cP and that of golden syrup is 100 000 cP (Barnes *et al.*, 1989:11). There is a 90% difference between the two substances, but upon physical examination, the golden syrup is only slightly thicker than the honey. The same applies to ketchup, having a viscosity of 50 000 cP and mustard, 70 000 cP (Raw material suppliers, 2016). The difference is 28.571%, but upon physical examination, they are considered almost equal.

Therefore, after considering the previously mentioned comparisons and upon looks and feel of the samples at all the time intervals, the viscosity did not differ notably during the three months testing period.

Table F.11: The viscosity results of the cosmeceutical cream

	Initial (T0)	Month 1 (T1)	Month 2 (T2)	Month 3 (T3)
25 ± 2 °C/60 ± 5% RH				
Viscosity (cP)	227 146.9	98 863.8	128 383.8	334 121.9
Mean %change relative to T0				
		T0 – T1	**T0 – T2**	**T0 – T3**
		56.468	43.470	47.121
30 ± 2 °C/60 ± 5% RH				
Viscosity (cP)	227 146.9	180 300.0	316 340.6	264 059.4
Mean %change relative to T0				
		T0 – T1	**T0 – T2**	**T0 – T3**
		20.610	39.292	16.271
40 ± 2 °C/75 ± 5% RH				
Viscosity (cP)	227 146.9	295 581.3	247 393.8	273 428.1
Mean %change relative to T0				
		T0 – T1	**T0 – T2**	**T0 – T3**
		30.128	8.914	20.375

Table F.12: The viscosity results of the cosmeceutical gel

	Initial (T0)	Month 1 (T1)	Month 2 (T2)	Month 3 (T3)
25 ± 2 °C/60 ± 5% RH				
Viscosity (cP)	77 301.7	114 571.9	91 654.2	130 631.3
Mean %change relative to T0				
		T0 – T1	**T0 – T2**	**T0 – T3**
		48.214	18.567	68.989
30 ± 2°C/60 ± 5% RH				
Viscosity (cP)	77 301.7	104 266.9	87 786.3	107 350.0
Mean %change relative to T0				
		T0 – T1	**T0 – T2**	**T0 – T3**
		34.883	13.563	38.872
40 ± 2 °C/75 ± 5% RH				
Viscosity (cP)	77 301.7	104 266.9	87 786.3	106 406.5
Mean % change relative to T0				
		T0 – T1	**T0 – T2**	**T0 – T3**
		34.883	13.563	38.459

F.4.5 Zeta-potential of the samples

According to Lamba *et al.* (2015:719), the zeta-potential indicates the surface charge of each particle in a formulation. The surface charge will determine whether the particles will repulse each other with enough force to prevent aggregation. This is an easy way to evaluate stability of the formulations. Particles will repel each other in order to prevent flocculation if a formulation has a highly positive or negative zeta-potential (Malvern Instruments, 2015:3; Marianecci *et al.,* 2013:75). However, if the zeta-potential of the particles is low, the tendency of particles to aggregate and cluster together will increase (Malvern Instruments, 2015:3). A zeta-potential lower than - 30 mV or higher than + 30 mV is indicative of a stable formulation (Malvern Instruments, 2015:3).

The zeta-potential results of the cream and gel samples, representing each storage condition, are indicated in Tables F.13 and F.14, respectively. The mean percentage zeta-potential changes over the three months test period, relative to the initial values, were calculated. The outcomes that did not comply with the acceptable 5% variation limit are indicated in red (ICH Guidelines Q1A (R2)).

All the samples underwent significant changes (ICH Guidelines Q1A (R2)). The cream's changes varied from 2.096 - 21.438% and the gel's changes varied from 0.293 - 25.930% from the baseline values respectively. The changes observed were more or less the same for both of the formulations. Despite these significant changes, all the samples' results remained highly negative and the repulsive forces exceeded the attractive forces, suggesting the particles in the formulations remained dispersed and that the systems were deflocculated after the three months testing period (Roland *et al.*, 2003:87).

Table F.13: The zeta-potential results of the cosmeceutical cream

	Initial (T0)	Month 1 (T1)	Month 2 (T2)	Month 3 (T3)
25 ± 2 °C/60 ± 5% RH				
Zeta-potential (mv)	- 52.91	- 60.58	- 60.72	- 59.79
Mean %change relative to T0				
		T0 – T1	**T0 – T2**	**T0 – T3**
		14.483	14.760	12.994
30 ± 2 °C/60 ± 5% RH				
Zeta-potential (mv)	- 52.91	- 58.17	- 63.98	- 54.02
Mean %change relative to T0				
		T0 – T1	**T0 – T2**	**T0 – T3**
		9.928	20.908	2.096
40 ± 2 °C/75 ± 5% RH				
Zeta-potential (mv)	- 52.91	- 64.26	- 62.33	- 62.74
Mean %change relative to T0				
		T0 – T1	**T0 – T2**	**T0 – T3**
		21.438	17.803	18.580

Table F.14: The zeta-potential results of the cosmeceutical gel

	Initial (T0)	Month 1 (T1)	Month 2 (T2)	Month 3 (T3)
25 ± 2 °C/60 ± 5% RH				
Zeta-potential (mv)	- 64.53	- 62.08	- 59.03	- 64.34
Mean %change relative to T0				
		T0 – T1	**T0 – T2**	**T0 – T3**
		3.802	8.523	0.293
30 ± 2 °C/60 ± 5% RH				
Zeta-potential (mv)	- 64.53	- 69.89	- 56.53	- 60.36
Mean %change relative to T0				
		T0 – T1	**T0 – T2**	**T0 – T3**
		8.295	12.397	6.474
40 ± 2 °C/75 ± 5% RH				
Zeta-potential (mv)	- 64.53	- 69.47	- 53.58	- 81.27
Mean %change relative to T0				
		T0 – T1	**T0 – T2**	**T0 – T3**
		7.645	16.978	25.930

F.4.6 Mass loss of the samples

The mass of the cream and gel samples, representing each storage condition, are indicated in Tables F.15 and F.16, respectively. The mean percentage mass loss over the three months test period, relative to the initial values, were calculated. All the samples complied with the acceptable 5% variation limit (ICH Guidelines Q1A (R2)). The largest decrease in mass for the cream was the sample stored at 40 ± 2 °C/75 ± 5% RH over three months, calculated as 0.515%. The same applied to the gel, and the largest decrease in mass was again the sample stored at 40 ± 2 °C/75 ± 5% RH over three months, calculated as 0.237%. These small changes in mass loss of the samples indicate the containers used for storage of the products sealed well enough and therefore limited moisture absorption and evaporation during the three months testing period.

Table F.15: The mass loss results of the cosmeceutical cream

Initial (T0)	**Month 1 (T1)**	**Month 2 (T2)**	**Month 3 (T3)**
25 ± 2 °C/60 ± 5% RH			
34.799 g	34.799 g	34.587 g	34.526 g
30 ± 2 °C/60 ± 5% RH			
39.217 g	39.191 g	39.148 g	39.142 g
40 ± 2 °C/75 ± 5% RH			
38.517 g	38.325 g	38.142 g	37.998 g

Table F.16: The mass loss results of the cosmeceutical gel

Initial (T0)	**Month 1 (T1)**	**Month 2 (T2)**	**Month 3 (T3)**
25 ± 2 °C/60 ± 5% RH			
20.616 g	20.605 g	20.574 g	20.571 g
30 ± 2 °C/60 ± 5% RH			
19.031 g	18.995 g	18.949 g	18.937 g
40 ± 2 °C/75 ± 5% RH			
21.109 g	20.975 g	20.931 g	20.912 g

F.4.6 Microscopic analysis of the samples

The images obtained from the microscopic assessment of the cream are indicated in Table F.17 and Figure F.4. The images were taken at a 10 x magnification. Microscopic analysis did not reveal any substantial changes regarding the structure of the emulsion. In Table F.17, visible air bubbles in the formulation are revealed. Closer images (40 x magnification) indicated in Figure F.4 showed the emulsion droplets with no visible flocculation after three months. These images also revealed the slight formation of crystals. The crystals are the green shades visible in Figure F.4.b).

The images obtained from the microscopic assessment of the gel are indicated in Table F.18 and Figure F.5. The images were taken at a 10 x magnification. Microscopic analysis of the gel revealed significant changes. Visible air bubbles are revealed in the initial image in Table F.18. These air bubbles suggested that the degassing method used during the formulation of the gel was unsuccessful. During the three months testing period, the air bubbles completely disappeared. A closer image, indicated in Figure F.5, revealed a large amount of crystals and suggested that carnosine crystallised during the testing period.

In summary, when considering the microscopic assessment, the cream remained more stable during the three months testing period.

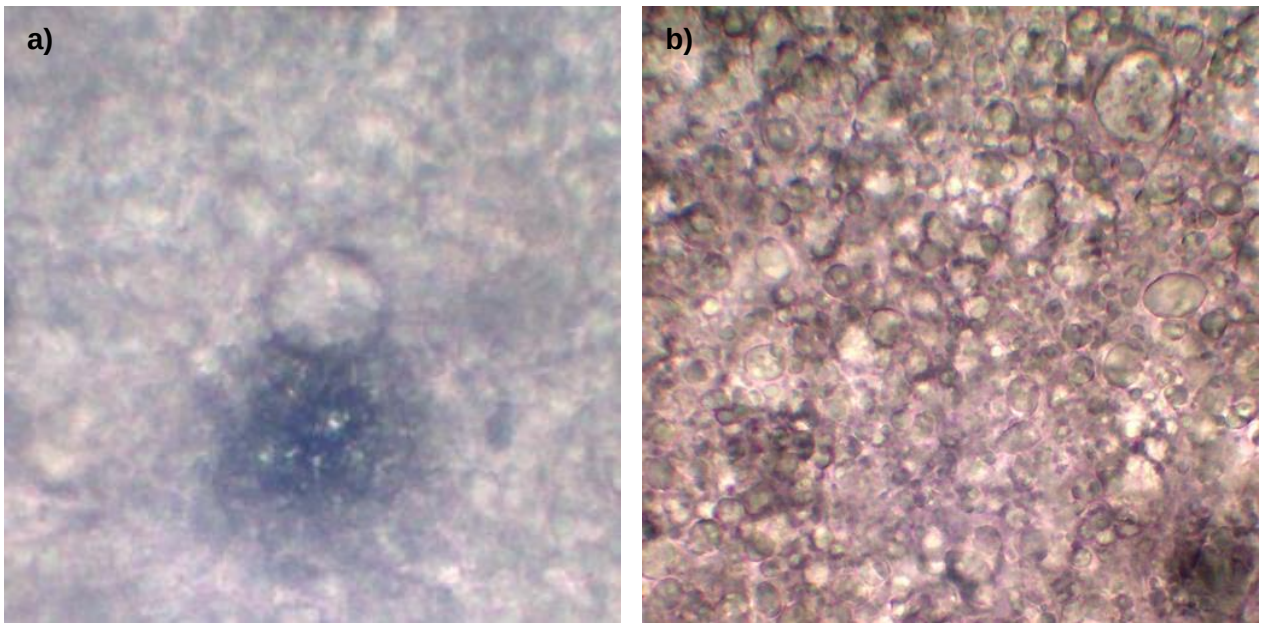


Figure F.4: The 40 x magnification of the cream sample; a) at T0 and b) at T3

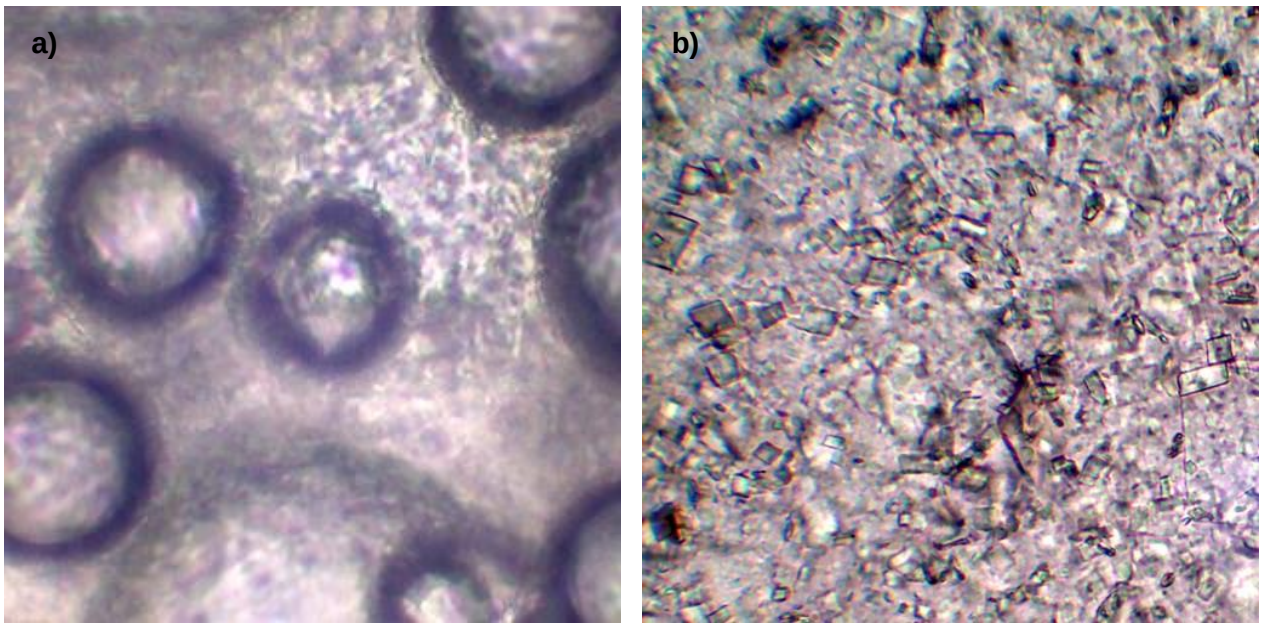


Figure F.5: The 40 x magnification of the gel sample: a) at T0 and b) at T3 (crystal formation is visible)

Table F.17: The microscopic images of the cosmeceutical cream

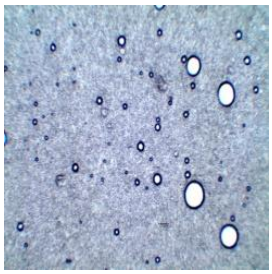
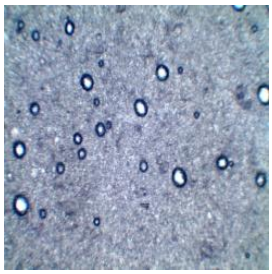
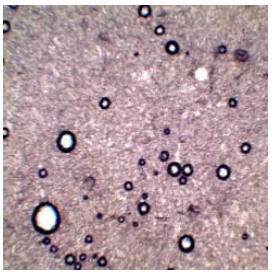
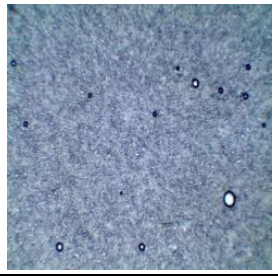
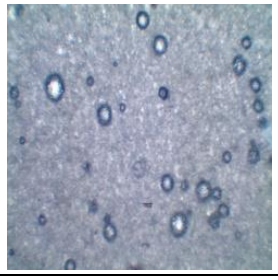
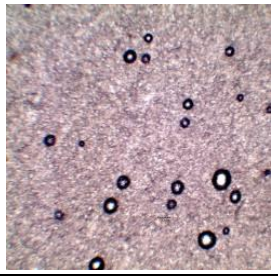
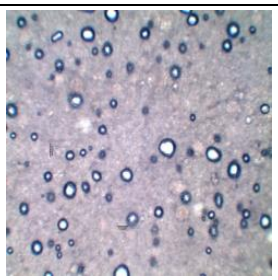
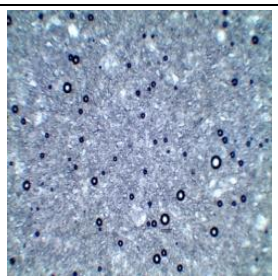
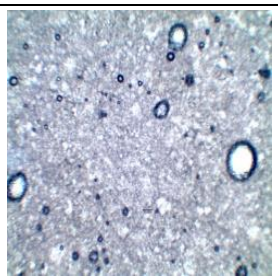
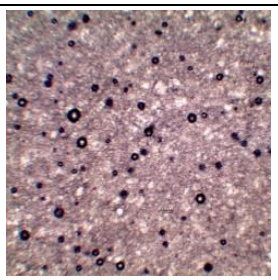
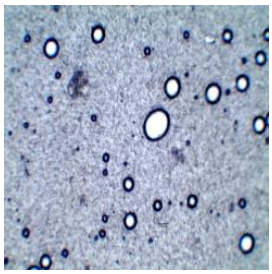
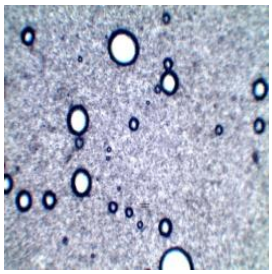
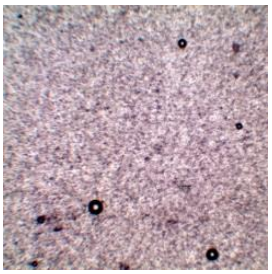
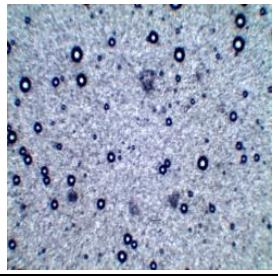
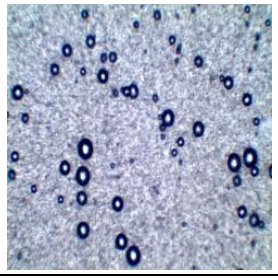
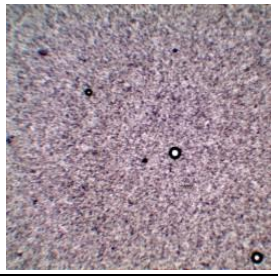
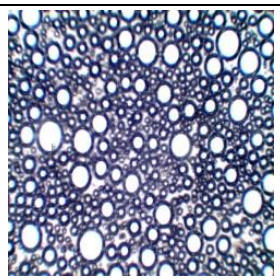
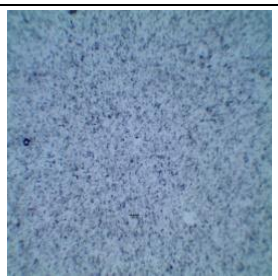
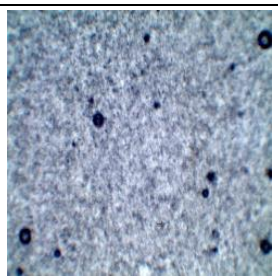
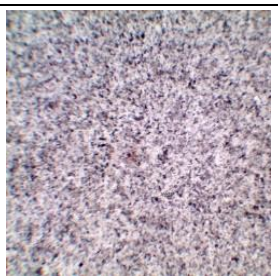
Initial (T0)	Month 1 (T1)	Month 2 (T2)	Month 3 (T3)
25 ± 2 °C/60 ± 5% RH			
			
30 ± 2 °C/60 ± 5% RH			
			
40 ± 2 °C/75 ± 5% RH			
			

Table F.18: The microscopic images of the cosmeceutical gel

Initial (T0)	Month 1 (T1)	Month 2 (T2)	Month 3 (T3)
25 ± 2 °C/60 ± 5% RH			
			
30 ± 2 °C/60 ± 5% RH			
			
40 ± 2 °C/75 ± 5% RH			
			

F.4.7 Visual appearance assessment of the samples

The images of the cream samples are indicated in Table F.19. The freshly prepared cream samples were smooth, off-white, semi-solid formulations with no particular smell. Visual examination of the samples revealed no texture change or phase separation during the three months testing period. The colour of the samples became slightly darker during the testing period, turning from off-white to light yellow. The samples stored at 40 ± 2 °C/75 ± 5% RH revealed the most visible colour change. The smell of the samples remained neutral during the three months testing period. Figure F.6 indicates the visible water droplets on the lids of the containers. This indicated that water evaporated from the o/w emulsion and that there was water-loss from the final product.

The images of the gel samples are indicated in Table F.20. The freshly prepared gel samples were light, foamy and bright white formulations with a characteristic smell. Visual examination of the samples revealed texture change and possible phase separation during the three months

testing period. The gel formulations went from being smooth and light to irregular, with visible transparent carbopol pieces in the formulation. Only after mixing, the formulations became smooth again. The colour of the samples became slightly darker during the testing period and went from bright white to off-white. The smell of the samples did not change during the three months testing period. Figure F.7 indicates the visible water droplets on the lids of the containers. This indicated that water evaporated from the gel formulations and that there was water-loss from the final product.

In summary, both of the formulations had a slight colour change. The cream samples had no visible phase separation whilst the gel formulation revealed an irregular texture, possibly suggesting phase separation. The smell of both of the formulations remained unchanged. Finally, both the formulations revealed signs of condensation and water loss from the final products. Considering the visual examination of the formulations, the cream performed better during the three months testing period.

Table F.19: The macroscopic images of the cosmeceutical cream

Initial (T0)	**Month 1 (T1)**	**Month 2 (T2)**	**Month 3 (T3)**
25 ± 2 °C/60 ± 5% RH			
			
30 ± 2 °C/60 ± 5% RH			
			
40 ± 2 °C/75 ± 5% RH			
			

Figure F.6: Signs of condensation on the inside of the lids of the cream containers

Table F.20: The macroscopic images of the cosmeceutical gel

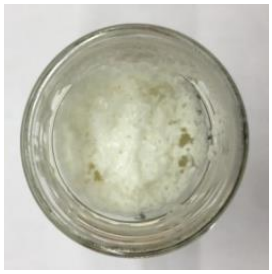
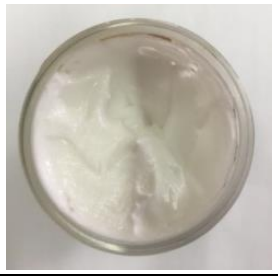
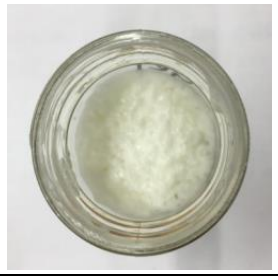
Initial (T0)	Month 1 (T1)	Month 2 (T2)	Month 3 (T3)
25 ± 2 °C/60 ± 5% RH			
			
30 ± 2 °C/60 ± 5% RH			
			
40 ± 2 °C/75 ± 5% RH			
			

Figure F.7: Signs of condensation on the inside of the lids of the gel containers

F.5 Conclusion

Two semi-solid formulations, a cream and a gel containing carnosine encapsulated in niosomes, were formulated in bulk and underwent stability tests at different storage conditions. ICH guidelines were followed to perform the stability tests and formulation changes were assessed during accelerated (40 ± 2 °C/75 ± 5% RH), intermediate (30 ± 2 °C/60 ± 5% RH) and long-term (25 ± 2 °C/60 ± 5% RH) storage conditions (ICH, 2003:3). Furthermore, the testing involved all relevant ingredients, as well as the exact packaging that will be used for marketing and storage (Barnes, 2007:663; York, 2007:12-13).

According to the ICH (2003:9), a significant change is defined as a 5% change in stability parameter from the initial value. Significant changes, for both the cream and the gel, had occurred regarding the concentration assays, conductivity, viscosity and zeta-potential.

Additionally, the degradation of ingredients, acceptance criteria for appearance (colour, cracking and phase separation) and acceptance criteria for pH should also be brought into consideration (ICH, 2003:3, 9). The cream revealed significant changes, whereas the gel showed good stability regarding the pH. The microscopic assessments of the formulations revealed crystal formation in both of the formulations and disappearance of air bubbles in the gel formulation. The macroscopic assessments revealed colour changes and condensation for both formulations, while the smell remained unchanged. Visible phase separation was also revealed during visual assessment of the gel formulation.

Both the cream and gel formulations revealed more or less the same concentration assay results after the three months testing period. Fluctuations in the concentrations of all three of the ingredients had occurred, possibly due to inconsistent mixing of the semi-solids during bulk formulation, resulting in variations in concentrations among the containers. All the ingredients in both formulations showed an overall decrease in concentrations.

All of the samples underwent a decrease in pH, which is indicative of the release of [H^+], possibly due to condensation (water release), resulting in both [H^+] and [OH^-] concentration variations in the formulation (Hach Company 2010:4).

Both formulations also revealed significant conductivity, viscosity and zeta-potential changes. The decrease in conductivity, together with the increase in viscosity of the cream, could be correlated with water release from the formulation. In contrast with this statement, the gel revealed an increase in both conductivity and viscosity after three months. The possibility of the release of encapsulated carnosine or phase separation in the formulation is therefore included here, since the gel formulation also revealed visible phase separation (Lamba *et al.,* 2015:719). The pH changes, suggesting changes in ion concentrations in the formulations, could possibly contribute to the conductivity changes (Hach Company, 2014-2015:1). The immense variations in conductivity revealed that no valuable conclusions, regarding the stability of the formulations, could be drawn. Despite the significant zeta-potential changes for both of the formulations, the zeta-potential remained highly negative and the formulations could still be considered stable with regards to the zeta-potential (Malvern Instruments, 2015:3, Roland *et al.*, 2003:87).

Both formulations revealed good stability regarding the mass loss of the products. The products were stored in the exact same containers used for storage and marketing. The glass containers had screw caps with polyvinylidene chloride (PVDC) liners. The mass loss results proved that the containers sealed good enough to limit moisture absorption and evaporation.

In summary, when considering all the stability parameters, the two products did not meet the ICH requirements for stability. As a result, the products could not be considered stable and therefore not suitable for manufacturing.

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APPENDIX G

FRANZ CELL DIFFUSION STUDIES

G.1 Introduction

Transdermal delivery is described as the controlled release of active ingredients through the skin (Muzzalupo *et al.*, 2011:28). It provides numerous advantages such as easy usage, quick withdrawal in the case of adverse effects, increased patient compliance and the avoidance of first-pass metabolism (Jepps *et al.,* 2013:153; Zhang *et al.,* 2009:227). Despite all these advantages, the poor permeation of hydrophilic active ingredients is still a major drawback for this delivery route (Marianecci *et al.,* 2013:63; Muzzalupo *et al.*, 2011:28).

According to Leveque *et al.* (2004:323), skin permeation studies are an essential part of optimising formulation design in transdermal and dermal delivery. Evaluation of the concentration of the active in the skin is complex and often associated with invasive procedures such as biopsies. A good alternative to overcome this challenge is the use of excised human skin positioned on appropriate *in vitro* test systems.

Franz cells are increasingly emerging as the test system of choice for skin permeation studies (Copley Scientific, 2016:66). According to Copley Scientific (2016:66), the cell comprises of a donor and receptor compartment; the donor compartment is filled with the semi-solid formulation or dispersion, and the receptor compartment is filled with a solution to mimic blood and body temperature (pH 7.4; 37 °C). These two compartments are separated either by a suitable membrane or by excised skin, depending on the study. The receptor compartment is extracted and replaced at predetermined intervals for HPLC analysis.

The aim of this study was to target topical delivery of carnosine, since it is a cosmetic substance. Targeted delivery is defined as a mode of delivering the active to the specific site of interest, to improve efficacy and reduce side effects by reducing the concentration of the active at other sites (Kamboj *et al.,* 2013:121). Vesicular systems have proven to deliver drugs successfully into the deeper layers of the skin and therefore targeting specific sites (Varun *et al.*, 2012:632).

Membrane release studies were conducted over a 6 h period to determine the release of carnosine from both the vesicular systems and the semi-solid formulations. Transdermal diffusion studies were conducted over a 12 h period to determine whether topical and/or transdermal delivery had been reached. The effects that the formulations had on the delivery from the pre-formulations were also investigated.

G.2 Ethics

This part of the study requires ethical approval, as biological material is used in the study, which was obtained from the North-West University Ethics Committee for the "application for the use of biological material obtained from human subjects in experiments" for the period of 25 August 2011 to 24 August 2016 (NWU-00114-11-A5). Written consent was given by patients for their skin to be used for research, and the patient information was handled confidentially. The laboratory (Biosafety level 2) is approved and equipped for transdermal studies on human skin. The biological waste was legally disposed of according to the prescribed methods after completion of the experiments.

G.3 Methods

G.3.1 HPLC analysis of carnosine

An HPLC method for the analysis of carnosine in study samples was previously developed and validated in cooperation with Prof Jan du Preez, at the Analytical Technology Laboratory, North-West University, Potchefstroom Campus, South Africa. The complete method is described in Appendix A. Analyses of the study samples collected form membrane release studies, transdermal diffusion studies and tape stripping (stratum corneum-epidermis (SCE) and epidermis-dermis (ED)) were performed using HPLC.

G.3.2 Aqueous solubility

Adequate aqueous solubility is important for molecules to cross the skin (Naik *et al.,* 2000:319). The aqueous solubility of carnosine (Sigma-Aldrich, Aston Manor, SA) was determined over a period of 24 h. A water bath was preheated to 25 °C before the procedure started. An excess amount of carnosine was added to a test tube containing a magnetic stirrer and 5 ml of the chosen buffer (phosphate buffer solution (PBS) pH 7.4). The solution was stirred continuously for 24 h, while the temperature remained at 25 °C, after which the samples were removed and filtered through 0.45 µm polytetrafluoroethylene (PTFE) filters. The solution was diluted 1 ml to 100 ml PBS (pH 7.4) before HPLC analysis. The experiment was done in triplicate.

G.3.3 Octanol buffer distribution coefficient (Log D)

The octanol-water partition coefficient (log P) describes the partitioning of the molecule between the octanol and water phase (Bolzinger *et al.,* 2012:157). Log D describes the distribution between octanol and a buffer at a certain pH, which therefore determines the partitioning between the stratum corneum and the underlying hydrophilic layers. Co-saturation of the *n*-octanol phase and the PBS (pH 7.4) phase occurred after equilibration of equal volumes of *n*-octanol and PBS (pH 7.4) for 24 h. A carnosine solution was prepared with the

pre-saturated PBS (pH 7.4) phase by adding approximately 60 mg to 3 ml PBS (pH 7.4). This solution was combined with 3 ml of the pre-saturated *n*-octanol phase, placed in a shaker bath overnight at 32 °C and centrifuged. The PBS and *n*-octanol solutions were diluted 1 ml to 20 ml with PBS (pH 7.4) and methanol, respectively, before HPLC analysis. The experiment was done in triplicate.

G.3.4 Preparation of phosphate buffer solution

The same method as described in Section A.4 was used to prepare the PBS (pH 7.4) for the Franz cell diffusion studies (BP, 2015b).

G.3.5 Membrane release studies

Membrane release experiments were conducted on the niosome and proniosome dispersions, as well as the niosome cream and the niosome gel. The objective of this experiment was to determine whether carnosine was released from the preparations. Figure G.1 represents the water bath used during diffusion studies.

Figure G.1: Grant JB series water bath

A Grant JB series water bath (Grant instruments) was preheated to 37 °C and a magnetic stirrer plate (Variomag) was placed inside the water bath. The experiments were performed using vertical Franz cells, which had a diffusion area of approximately 1.075 cm^3 and a receptor capacity of about 2 ml. Each cell consisted of a donor and receptor compartment. Before the study commenced, PBS (pH 7.4) was preheated in a water bath at 37 °C (normal body temperature). A magnetic stirrer was placed in the receptor compartment of each cell. Small circles of lipophilic polyvinylidene difluoride (PVDF) membrane filters with a 25 mm diameter and a pore size of 0.45 µm (Pall Corportion, United States of America) were placed on the receptor compartment and the donor compartment was positioned carefully on the top. Dow Corning® high vacuum grease was then used to seal the Franz cells to prevent leakage before attaching the clamps. The receptor compartments of the cells were carefully filled with 2.0 ml

PBS (pH 7.4) and the donor compartment was filled with 1.0 ml of the formulation. The donor compartment was covered with Parafilm® to prevent loss of any active. The cells were then finally placed in the water bath at a controlled temperature of 37 °C. The receptor phase was extracted every hour and refilled with PBS (pH 7.4 at 37 °C) for up to 6 h; this ensured sink conditions throughout the experiment. The extracted samples were analysed with HPLC to determine the concentration active that released from the vesicle system through the membrane into the receptor phase. Figures G.2 to G.6 indicate the apparatus used during diffusion studies.

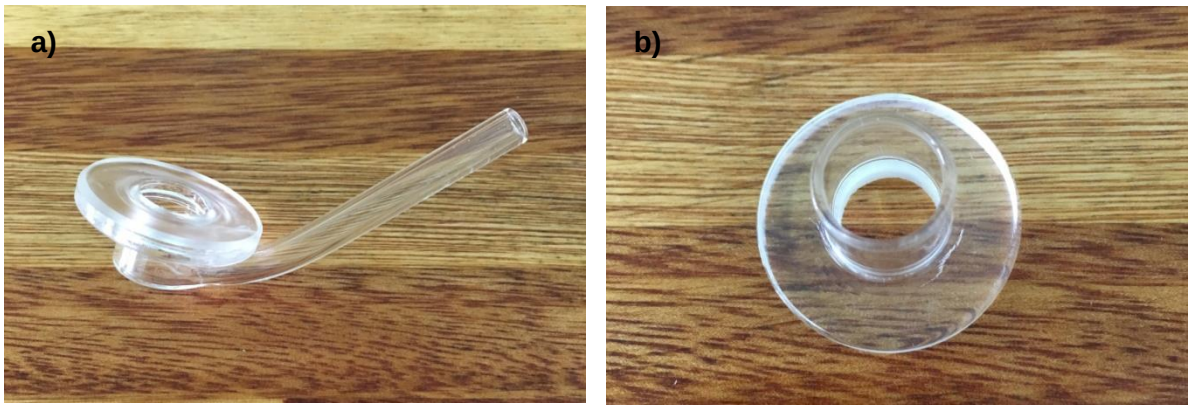

Figure G.2: Franz diffusion cell; a) is the receptor compartment and b) is the donor compartment

Figure G.3: Dow Corning® high vacuum grease used to seal the Franz cells

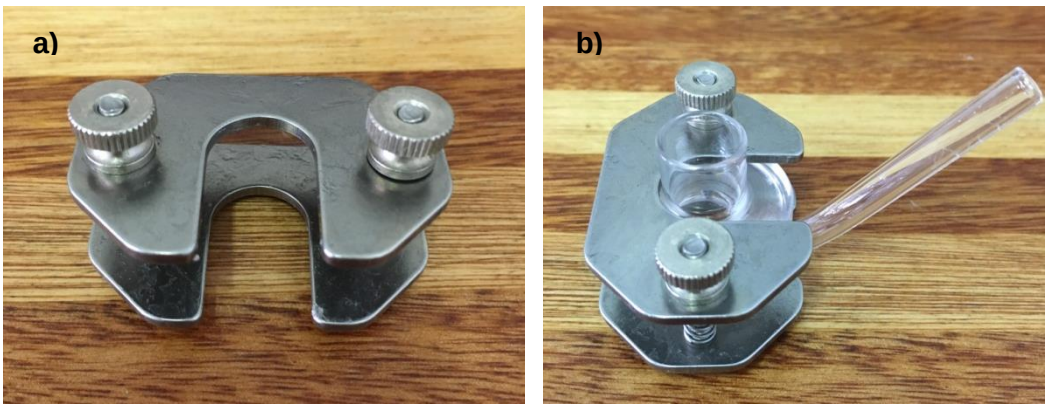

Figure G.4: a) Metal horseshoe clamp, and b) the Franz cell and clamp assembled together

Figure G.5: Syringe (5 ml) and 18 gauge needle with silicon tube used to extract and replace the receptor compartment

G.3.6 Skin preparation

Female, Caucasian skin (ethical approval: NWU-00114-11-A5) obtained from medical institutions performing abdominal plastic surgery was used to perform the transdermal diffusion studies. After collection, the skin was kept frozen at – 20 °C for a maximum of 24 h. The skin was defrosted and placed on paper towel with the stratum corneum facing upwards. A Zimmer™ electric dermatome model 8821 was used to obtain the preferred thickness of 400 µm. This part contains the stratum corneum, viable epidermis and upper dermis. The skin was cut by pressing the dermatome with constant pressure and force at an angle of 30 to 40 ° against the stratum corneum. The dermatomed skin was then placed onto Whatman® filter paper, with the stratum corneum facing upwards, and allowed to dry. Small circles were cut out and the prepared skin was wrapped in aluminium foil and stored at – 20 °C until needed. The skin was examined visually for any defects before performing the transdermal diffusion studies.

Figure G.6: The Zimmer™ electric dermatome model 8821

G.3.7 Transdermal diffusion studies

The same method as described in the membrane release studies (Section G.3.5) was used in skin diffusion studies, except that dermatomed skin was used instead of PVDF membrane filters. The prepared small circles of skin were placed between the two compartments of the Franz cells with the stratum corneum facing upwards. Samples were extracted from the receptor compartment every 20 min up to 2 h and then every 2 h up to 12 h. The samples were analysed using HPLC methods.

G.3.8 Tape stripping

The following method was obtained from Pellet *et al.* (1997:94). Before the tape stripping started, a piece of Parafilm® was stapled to a solid surface. After the diffusion studies, the skin was carefully removed, pinned to the Parafilm® and dabbed dry with paper towel. Pieces of tape, large enough to cover the whole diffusion area were cut out. The first tape strip was discarded for it may have been contaminated with the active from the formulation. The next 15 tape strips were placed in 5 ml extraction solution (PBS at pH 7.4) and kept overnight in the refrigerator at 4 °C. After the tape stripping, the areas of the skin where diffusion did occur place were separated from the diffusion areas and discarded. The diffusion areas were then cut into small pieces and placed in 5 ml extraction solution (PBS at pH 7.4) overnight at 4 °C. Both solutions were filtered and analysed using the HPLC.

G.3.9 Data analysis

The data obtained from the membrane release studies was used to determine if carnosine was released from the four different preparations. The data obtained from the skin diffusion studies was used to calculate the % carnosine, which had diffused into the SCE and ED. The effect of the semi-solid formulation on the topical delivery of carnosine from the pre-formulation was investigated. The data used for statistical analysis were the results obtained from the SCE and

the ED, since there were significant carnosine concentrations in both of these phases for all experiments.

Statistica (StatSoft Inc., 2016) was used to analyse the data. Non-parametric tests were performed, seeing that the p-p-plot obtained from this study did not indicate normality of data. The non-parametric tests involved a Kruskal-Wallis rank test to determine a statistical significant effect of the four preparations, followed by multiple comparisons between the different pairs of preparations. In the cases where ED was compared with SCE, the nonparametric Mann-Whitney rank sum test was performed. A significant statistical difference is indicated with a p-value of less than 0.05 ($p < 0.05$).

G.4 Results and discussion

G.4.1 Aqueous solubility

According to Naik *et al.* (2000:319), it is essential for a molecule to have an aqueous solubility of at least 1 mg/ml for passive skin diffusion. The aqueous solubility of carnosine in PBS (pH 7.4) at 25 °C was determined as 122.804 ± 0.716 mg/ml. The experiment was firstly performed at 32 °C, since it is the temperature of the skin during diffusion studies, but precipitation had occurred with the decrease in temperature as soon as the sample was removed from the water bath. Due to the excessive solubility, the experiment was finalised at 25 °C. The aqueous solubility of carnosine determined by Singh *et al.* (2009:734) is 205.869 mg/ml at 25 °C with pH 7.0 and 52.033 mg/ml at pH 8.0. The slight difference between the determined value and the value obtained from literature could be attributed to the higher pH of the PBS (pH 7.4). Carnosine therefore has adequate solubility to be a good candidate for topical delivery.

G.4.2 Octanol buffer distribution coefficient (log D)

According to Khalid *et al.* (2016:129), compounds with a Log P of 1 to 3 are expected to diffuse freely across the skin. According to Goebel *et al.* (2012:282), the Log P of carnosine is - 2.972 ± 0.463; in this study, the Log D of carnosine was determined as - 2.891 ± 0.013, which correlates well with literature. A Log D of - 2.891 will possibly result in poor transdermal permeation and external interventions might be needed to improve dermal delivery.

G.4.3 Membrane release studies

The aim of the membrane release studies was to determine if carnosine was released from the vesicular systems and semi-solid formulations. The average and median flux values (µg/cm^2.h) of the four different preparations that diffused through the membranes during the 6 hr membrane release studies are indicated in Table G.1 and Figure G.7.

Table G.1: The average and median flux values of the vesicular systems and semi-solid formulations

Preparation	Average flux (µg/cm^2.h)	Median flux (µg/cm^2.h)
Niosomes	1 122.11 ± 120.581	1 139.10
Proniosomes	1 127.07 ± 73.789	1 096.20
Cream	387.06 ± 78.019	378.64
Gel	813.94 ± 75.167	796.41

The proniosomes had the highest average flux of 1 127.07 ± 73.789 µg/cm^2.h, while the cream had the lowest average flux of 387.06 ± 78.019 µg/cm^2.h. The niosomes had the highest median flux value (1 139.10 µg/cm^2.h), while the cream had the lowest median flux value (378.64 µg/cm^2.h). The average values were determined by adding all the data points and dividing it by the number of data points of each preparation. The median values were the centre point of the data points in each preparation. The median flux value of the niosome dispersion was 1.51% higher than the average flux value. For the proniosomes, cream and gel, the median flux values were 2.74, 2.18 and 2.15% lower than the average flux values, respectively. The median values were used, as they are less affected by outliers in the data, and are therefore a more accurate representation of the true flux values (Dawson & Trapp, 2001:30; Gerber *et al*., 2008:190). With the highest median flux, it is evident that the niosomes revealed the best release during the membrane release studies. The cream had the lowest median flux and revealed poor release of carnosine.

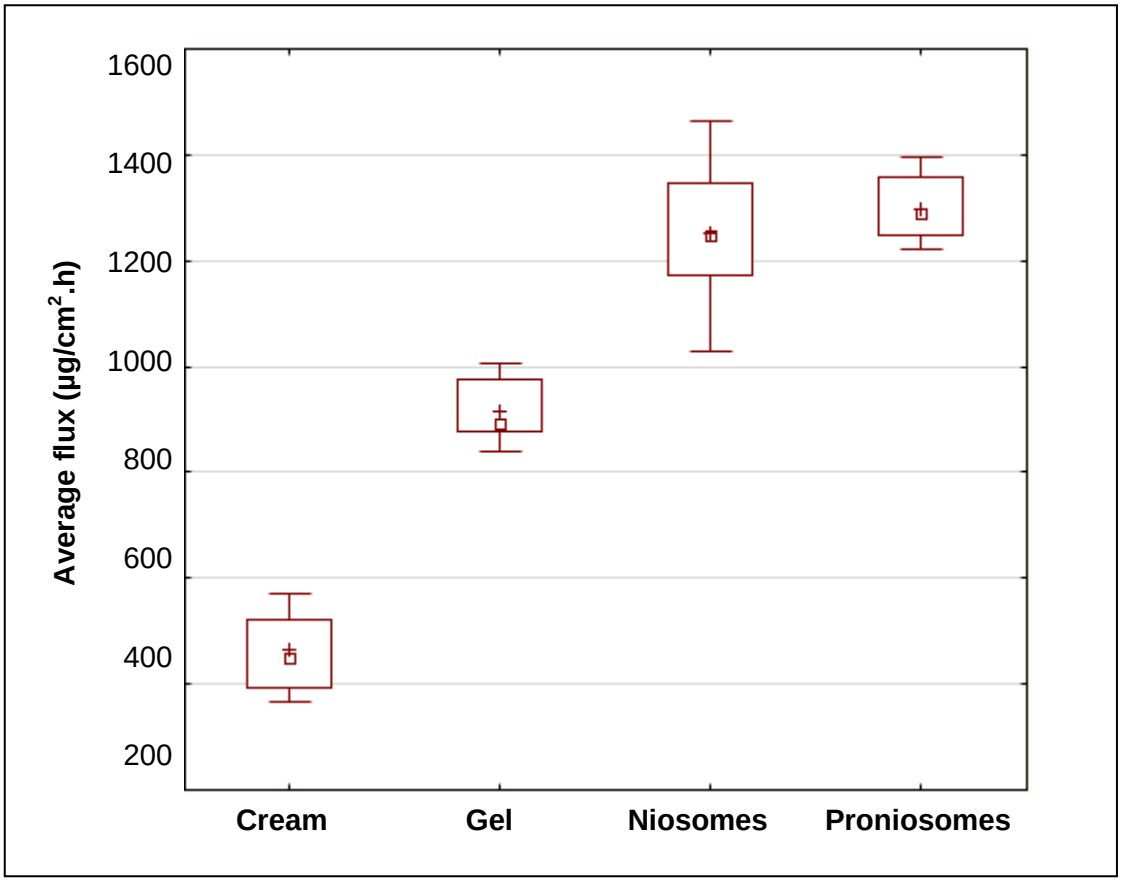

Figure G.7: Box-plots of the flux values of the four preparations illustrating the average (+) and median (□), as well as minimum and maximum values

The average %carnosine release results obtained from the membrane release studies of the four different preparations are indicated in Figure G.8. The preparation with the best %release was the proniosomes (14.145 ± 0.644%), then the niosomes (13.508 ± 1.361%), followed by the gel (9.963 ± 0.552%) and the cream (5.131 ± 0.798%). There is a much bigger difference between the vesicular systems and the semi-solid formulations compared to proniosomes and niosomes and the gel and the cream. These results therefore suggest that the formulations (cream and gel) had a negative influence on the release of carnosine from the pre-formulations (niosomes and proniosomes).

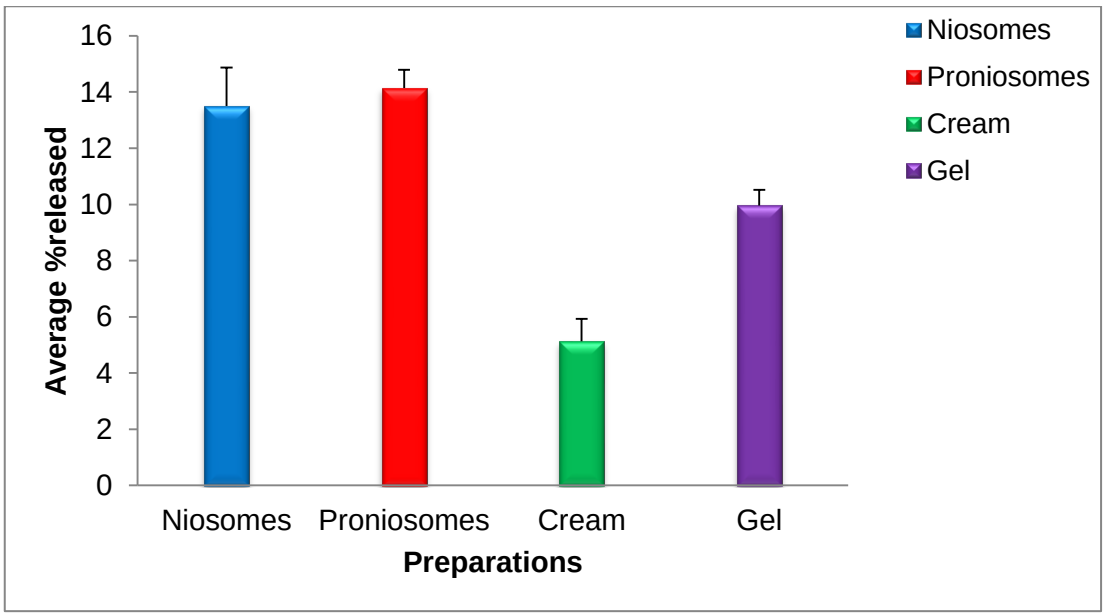

Figure G.8: The average %carnosine released for the four preparations after the 6 hr membrane release studies

Figures G.9 to G.16 indicate the cumulative concentrations of carnosine per area (µg/cm^2) for each individual sample and the average cumulative concentrations of carnosine per area (µg/cm^2) that had diffused during the 6 hr membrane release studies as a function of time for the niosomes, proniosomes, cream and gel.

It is important to keep in mind that the results obtained from membrane release studies and skin diffusion studies had no direct correlation between them. The results obtained from the membrane release studies indicated that, regardless of the amounts, all four preparations had successful carnosine release. Poor release from the preparation could therefore be ruled out if poor topical/transdermal delivery was revealed.

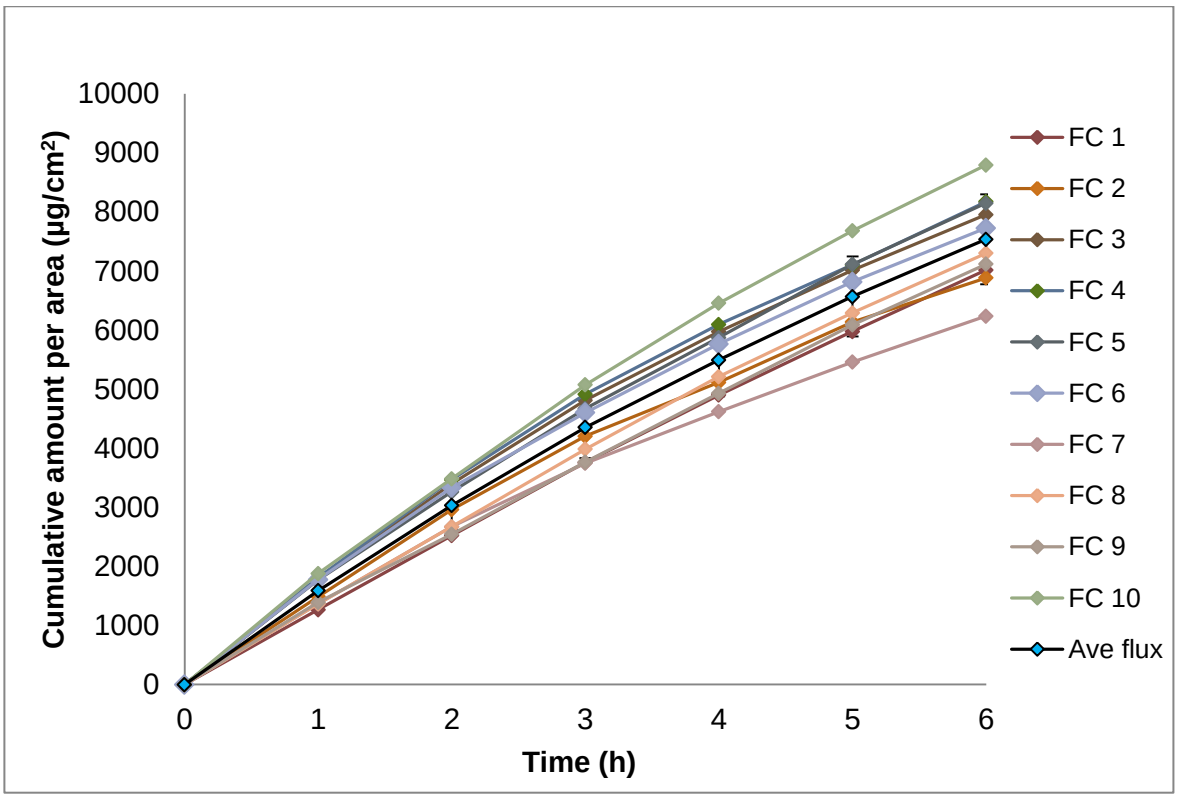

Figure G.9: Cumulative amount per area (µg/cm^2) of carnosine released from niosomes for each individual Franz cell as a function of time after 6 h (n = 10)

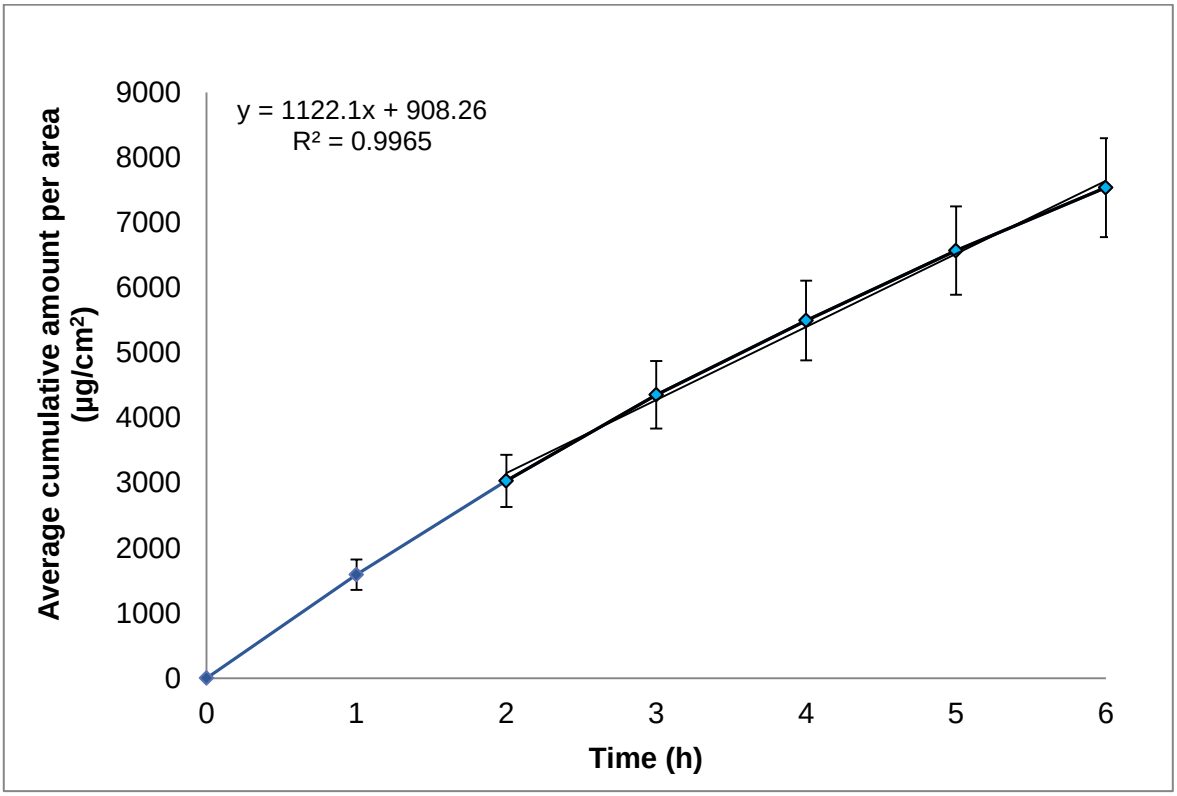

Figure G.10: Average cumulative amount per area (µg/cm^2) of carnosine released from niosomes as a function of time after 6 h

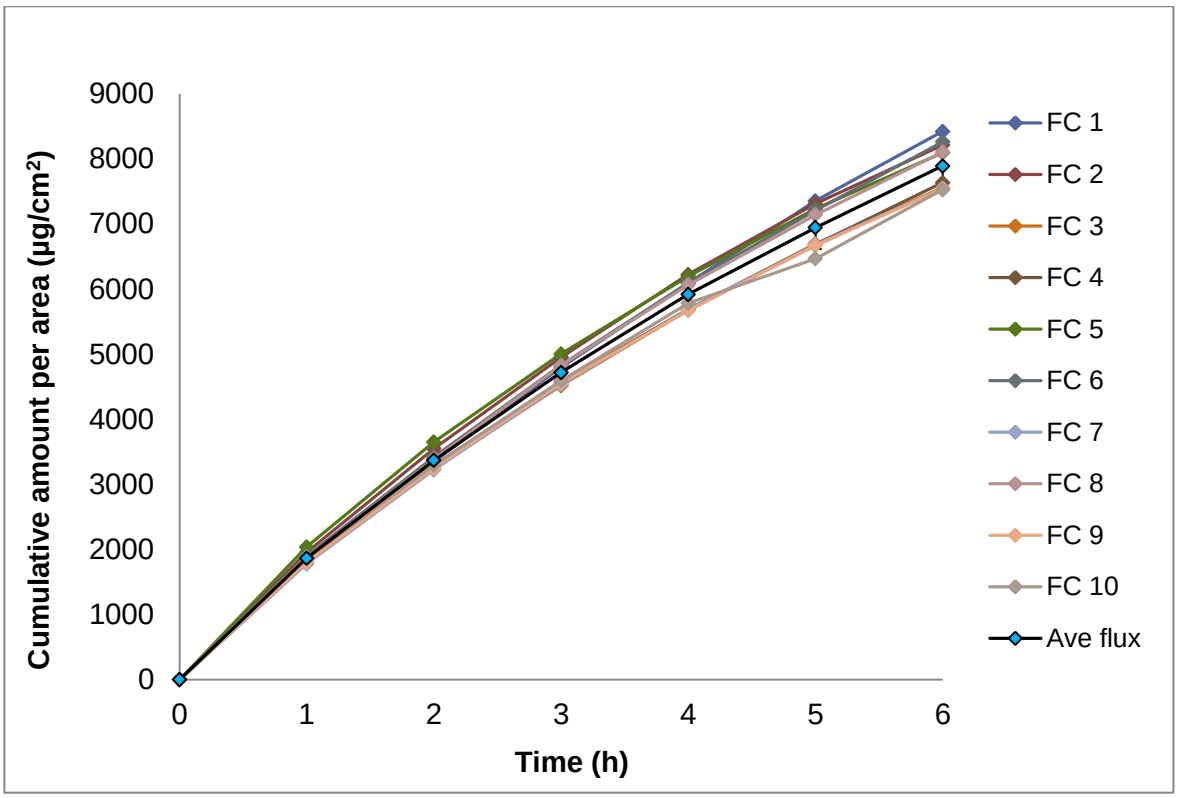

Figure G.11: Cumulative amount per area (µg/cm^2) of carnosine released from proniosomes for each individual Franz cell as a function of time after 6 h (n = 10)

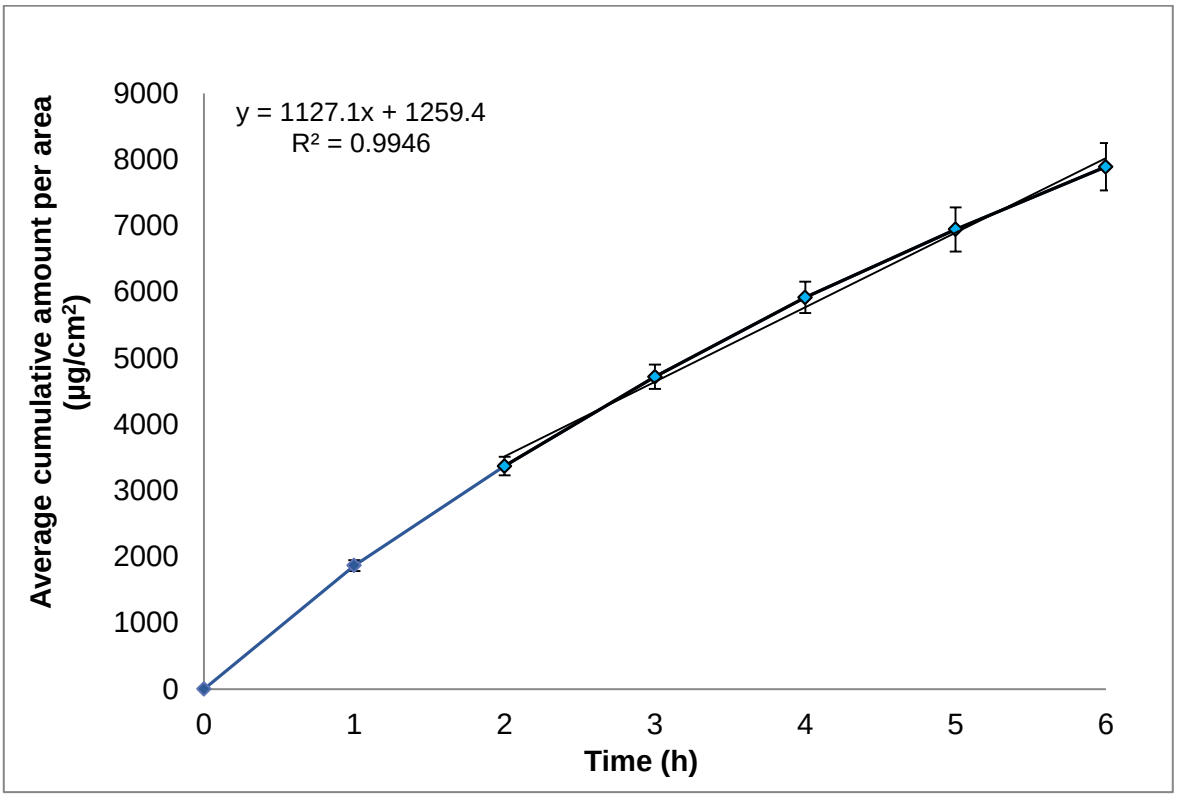

Figure G.12: Average cumulative amount per area (µg/cm^2) of carnosine released from proniosomes as a function of time after 6 h

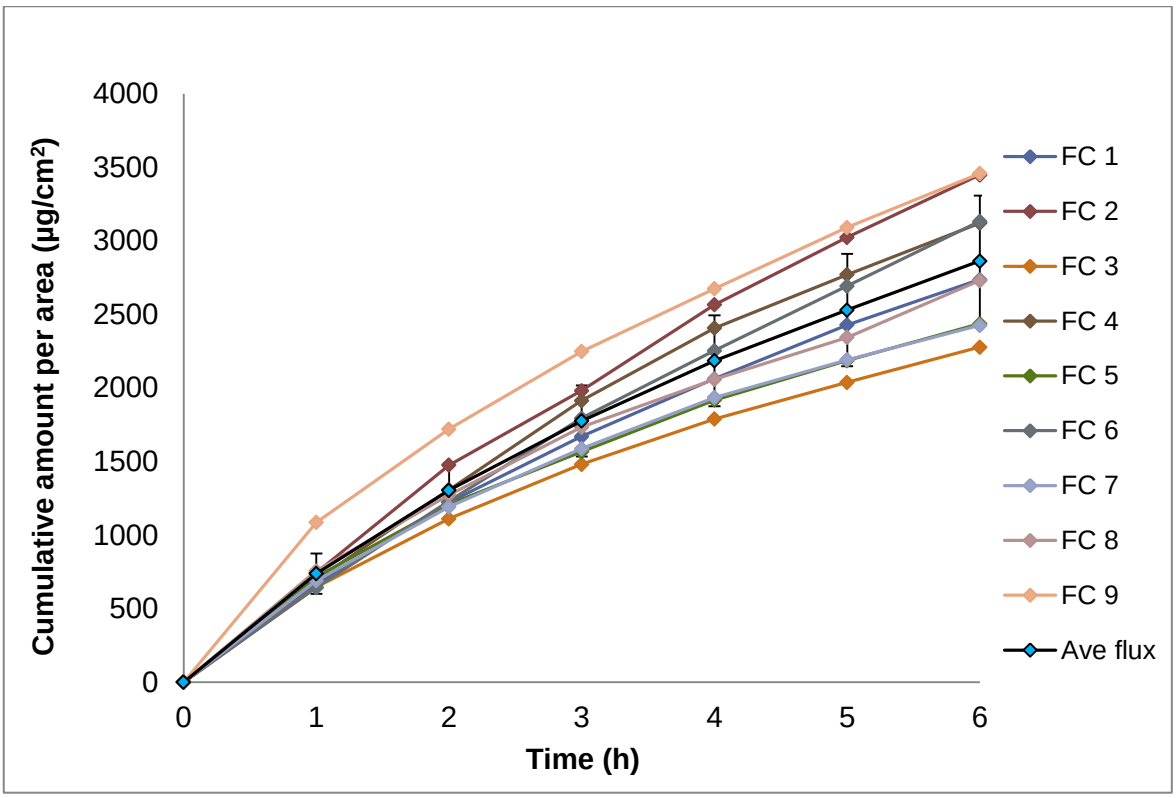

Figure G.13: Cumulative amount per area (µg/cm^2) of carnosine released from the cream for each individual Franz cell as a function of time after 6 h (n = 9)

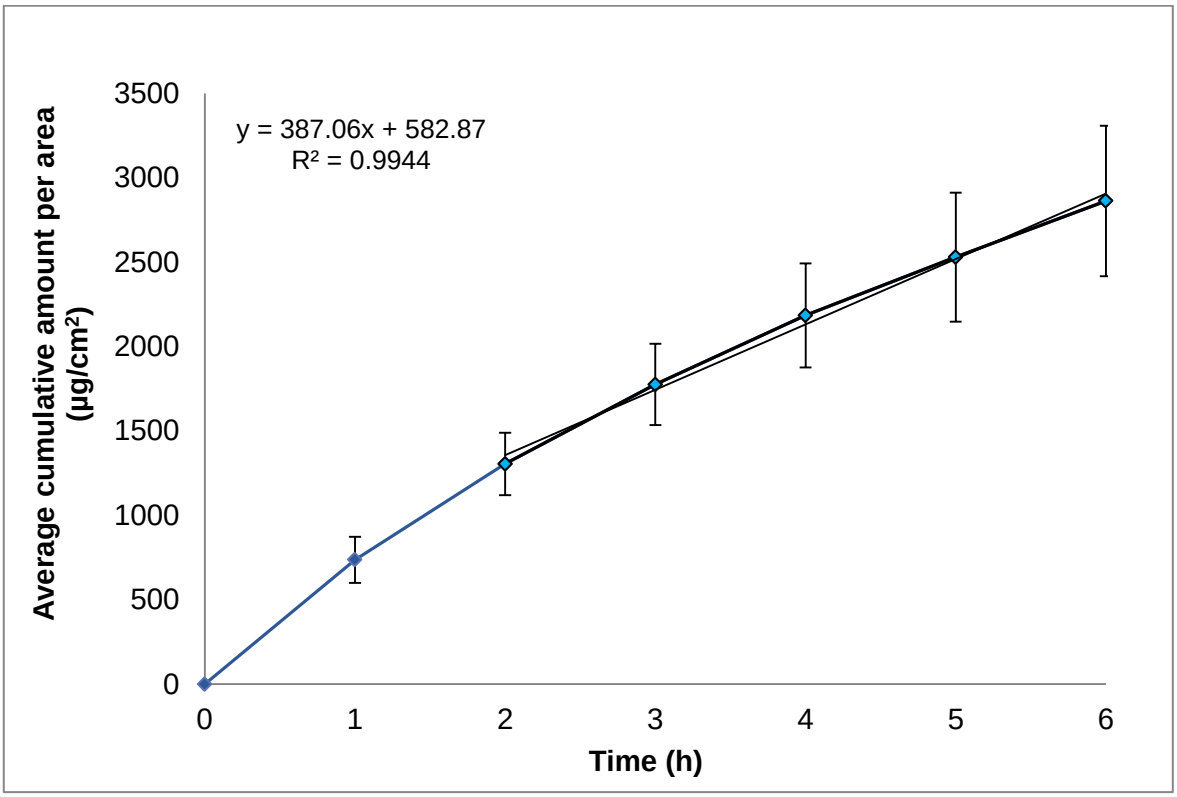

Figure G.14: Average cumulative amount per area (µg/cm^2) of carnosine released from the cream as a function of time after 6 h

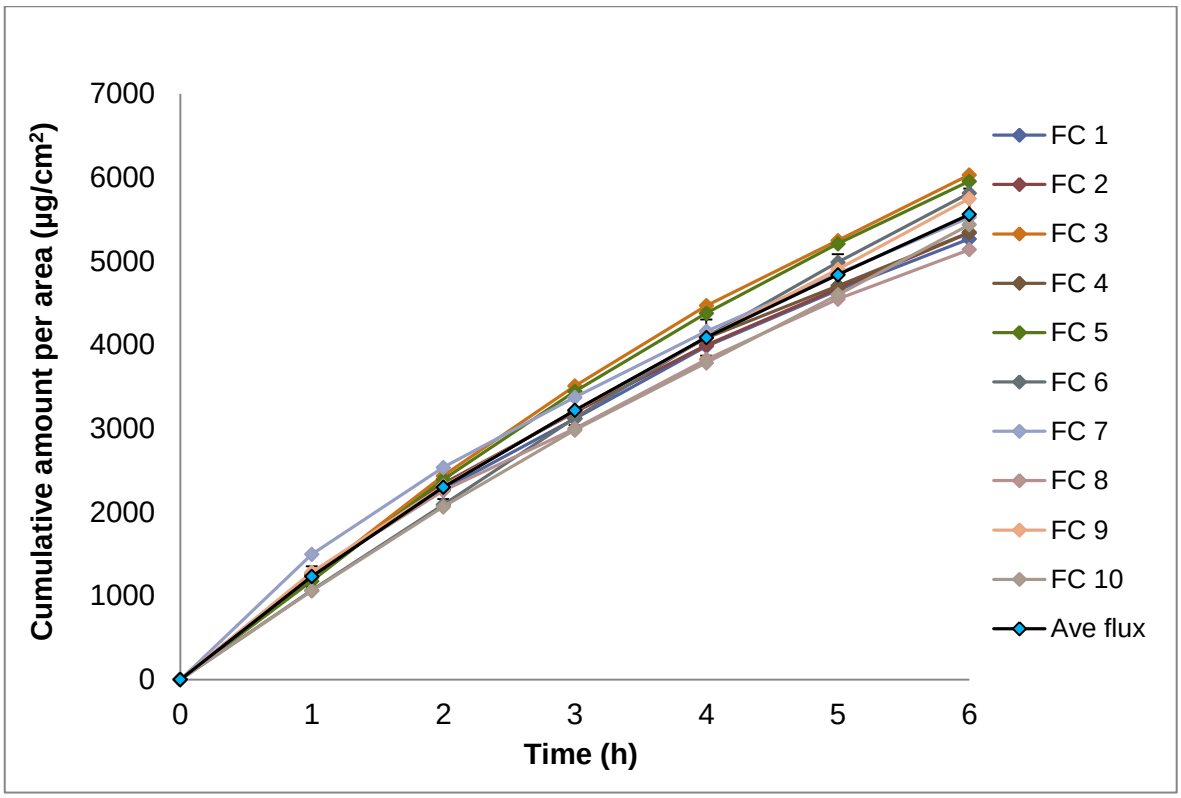

Figure G.15: Cumulative amount per area (µg/cm^2) of carnosine released from the gel for each individual Franz cell as a function of time after 6 h (n = 10)

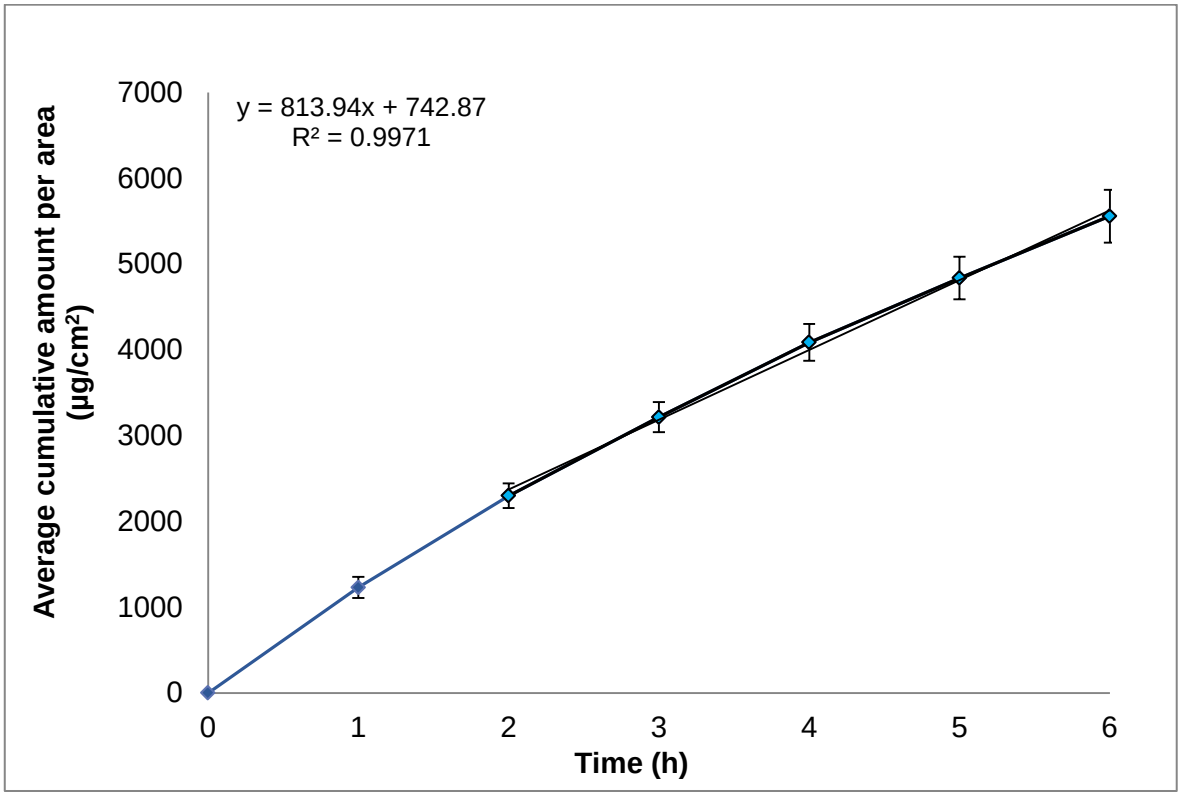

Figure G.16: Average cumulative amount per area (µg/cm^2) of carnosine released from the gel as a function of time after 6 h

G.4.4 Transdermal diffusion studies

The aim of the diffusion studies was to determine whether transdermal and/or topical delivery were reached and to investigate the effects the semi-solid formulations had on the transdermal and/or topical delivery from the pre-formulations. As carnosine is a cosmeceutical substance, the aim was to reach topical delivery.

The results obtained revealed that no transdermal diffusion was reached and none of the four preparations reached the receptor phase. Due to the focus of this study, being topical and not transdermal delivery of carnosine, the preferred outcome was reached and no carnosine was delivered into the blood stream (receptor phase).

G.4.5 Tape stripping

The results obtained from analysis of the tape strip samples are indicated in Figure G.17, and revealed that carnosine was targeted in the SCE by all four preparations. The gel delivered the highest average concentration carnosine (2.745 ± 1.367 µg/ml) to the SCE, followed by the niosomes (1.759 ± 0.595 µg/ml), proniosomes (1.333 ± 0.464 µg/ml) and lastly, the cream (1.149 ± 0.626 µg/ml). When considering the median concentrations, the same pattern was followed as the gel also delivered the highest concentration (2.458 µg/ml) to the SCE, followed by the niosomes with 1.662 µg/ml, the proniosomes with 1.161 µg/ml and finally, the cream with 1.051 µg/ml. All the median concentration values were lower than the average concentration values and since the median values are not affected by the outliers in the data, it is a more accurate representation of the data (Dawson & Trapp, 2001:30; Gerber *et al.*, 2008:190).

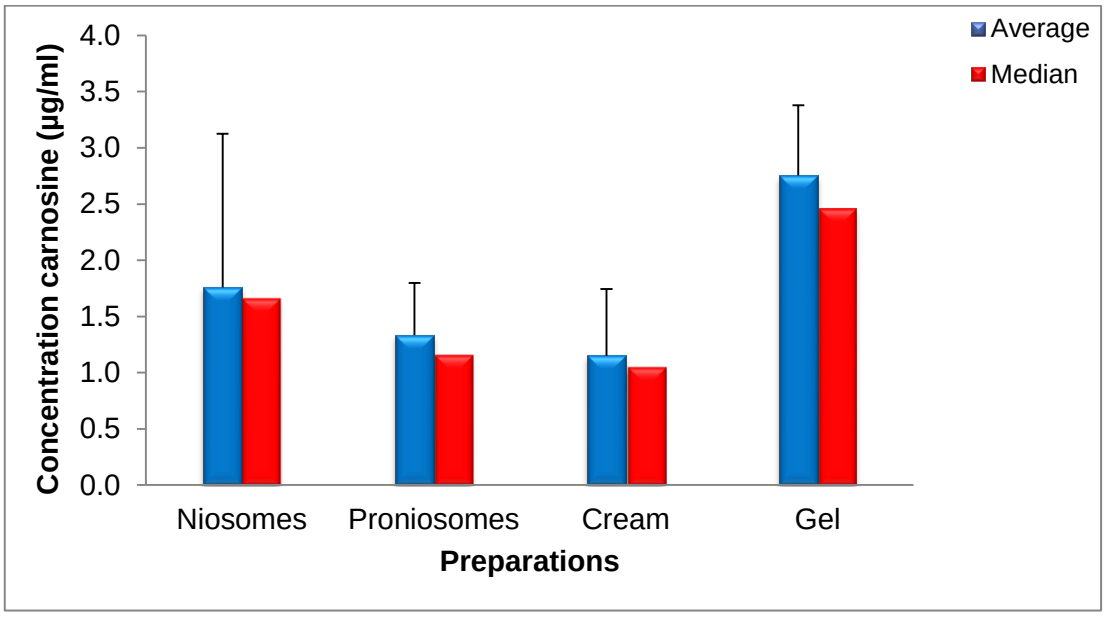

Figure G.17: The average and median concentrations of carnosine (µg/ml) in the SCE for each of the four preparations

A possible reason for the increased delivery to the SCE by the gel and the niosomes could be due to better release from the preparations. As revealed by the membrane release experiments, the niosomes pre-formulation and the gel semi-solid formulation showed the best release of the active. Better release provided for higher concentration gradients at the stratum corneum, and since diffusion is directly proportional to the concentration gradient, the skin permeation will increase (Abbott, 2012:219). Increased permeation of the stratum corneum by the gel compared to by the cream could also be attributed to the aqueous nature of the gel. According to Benson and Watkinson (2012), water is a natural hydrating substance and skin permeability will increase as the hydration of the stratum corneum increases. The water content in the gel hydrated the stratum corneum resulting in increased permeability to improve SCE delivery.

G.4.6 Epidermis dermis

The ED was the intended target site for the preparations. The results obtained from analysis of the ED are indicated in Figure G.18 and revealed that carnosine was delivered to the ED by all four preparations. The niosomes delivered the highest average concentration carnosine (3.855 ± 4.483 µg/ml) to the ED, followed by the gel (2.614 ± 2.518 µg/ml), the cream (1.423 ± 1.461 µg/ml) and lastly, the proniosomes (0.941 ± 0.277 µg/ml). When considering the median concentrations the niosomes delivered the highest concentration (2.465 µg/ml) to the ED, followed by the gel with 1.377 µg/ml, the proniosomes with 0.957 µg/ml and finally, the cream with 0.921 µg/ml. As mentioned, the median represented the skewed data more accurately and therefore, was a better representation of the true concentration values (Dawson & Trapp, 2001:30; Gerber *et al.*, 2008:190).

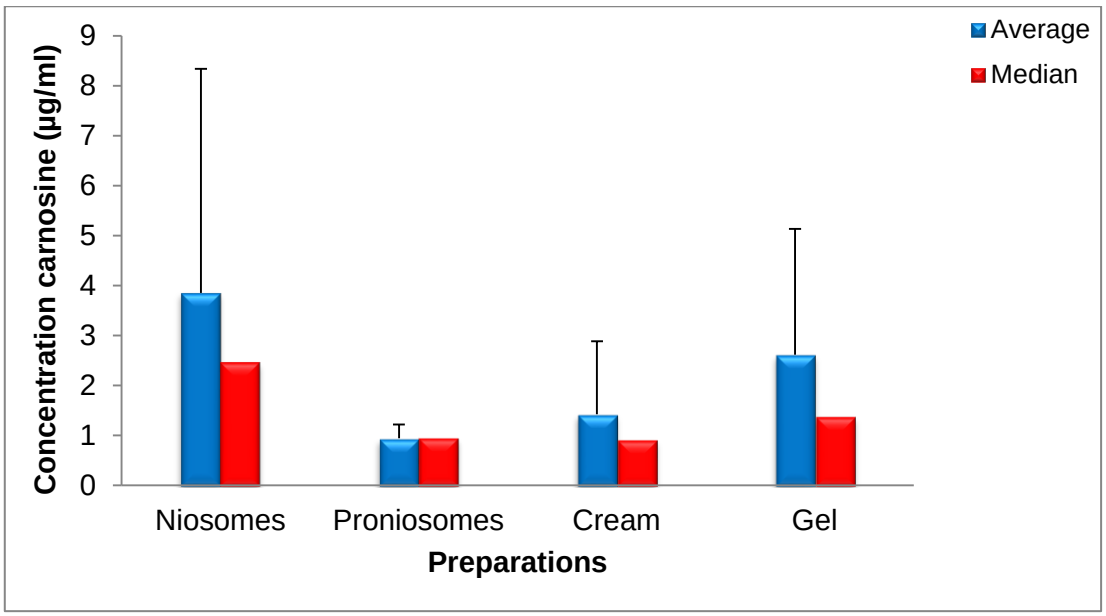

Figure G.18: The average and median concentrations of carnosine (µg/ml) in the ED for each of the four preparations

The niosomes and niosome gel have proven to be the best pre-formulation and semi-solid formulation for targeting topical delivery, respectively. A possible reason for the increased topical delivery of these two preparations could be attributed to better release of carnosine from them. Better release of carnosine from the formulation will result in a higher available concentration of carnosine for topical diffusion (Barry, 2007:571). When taking the release into consideration (Section G.4.3), the niosomes and proniosomes revealed almost the same results, but the niosome gel revealed much better release of carnosine than the niosome cream. Better release of carnosine resulted in a higher concentration at the stratum corneum. Since diffusion is proportional to the concentration gradient, a higher concentration at the delivery site will provide for a higher concentration gradient, and finally result in increased diffusion (Barry, 2007:571). Another possible contributor to better skin permeation from the gel than from the cream is the aqueous nature of the gel. According to Benson and Watkinson (2012), skin permeability increases as the hydration of the stratum corneum increases and water is considered as a natural skin hydrator. The aqueous nature of the gel hydrated the stratum corneum and consequently increased the permeability to improve topical delivery of carnosine.

Furthermore, the characteristics of the vesicular systems must be brought into consideration. Firstly, the niosomes had slightly better EE% results (Section C.2.5) compared to the proniosomes and increased transdermal diffusion was expected. Secondly, a larger amount of small niosome vesicle droplets formed (Section B.6.1.1.1), compared to the small amount of large proniosome vesicle droplets (Section B.6.2.1.1) that formed. According to Williams (2003:36), the size of molecules is inversely proportional to diffusion, therefore smaller droplets will be expected to penetrate the skin easier than larger droplets. The voluminous amount of

small niosome droplets also provided for a generous membrane surface of entrapped carnosine, enabling many of these small droplets to effortlessly reach the stratum corneum at once and release carnosine. The proniosomes had the lowest concentration in the ED, which is an indication of the contribution of the characteristics to skin permeation.

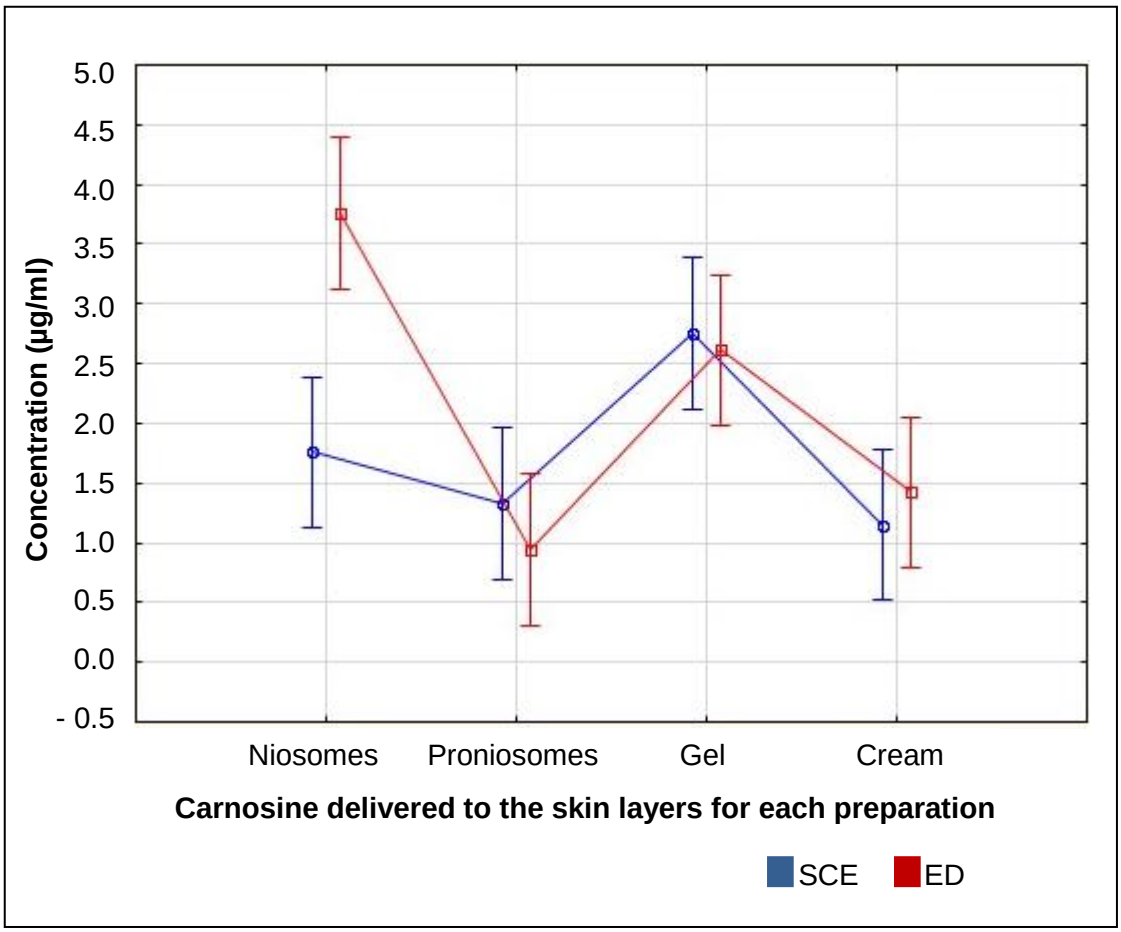

Figure G.19: The concentration carnosine (µg/ml) in the SCE and ED respectively for each of the four preparations

It is evident in Figure G.19, that when compared, the niosomes had a much higher concentration in the ED than in the SCE. The other three preparations delivered more or less the same amount carnosine to both the SCE and the ED. Figure G.20 indicates the concentrations of carnosine delivered to the SCE and ED by the four preparations and includes the average, median as well as minimum and maximum values. The niosomes had one value for the ED that was a lot higher than that of the others; this value was considered as an outlier.

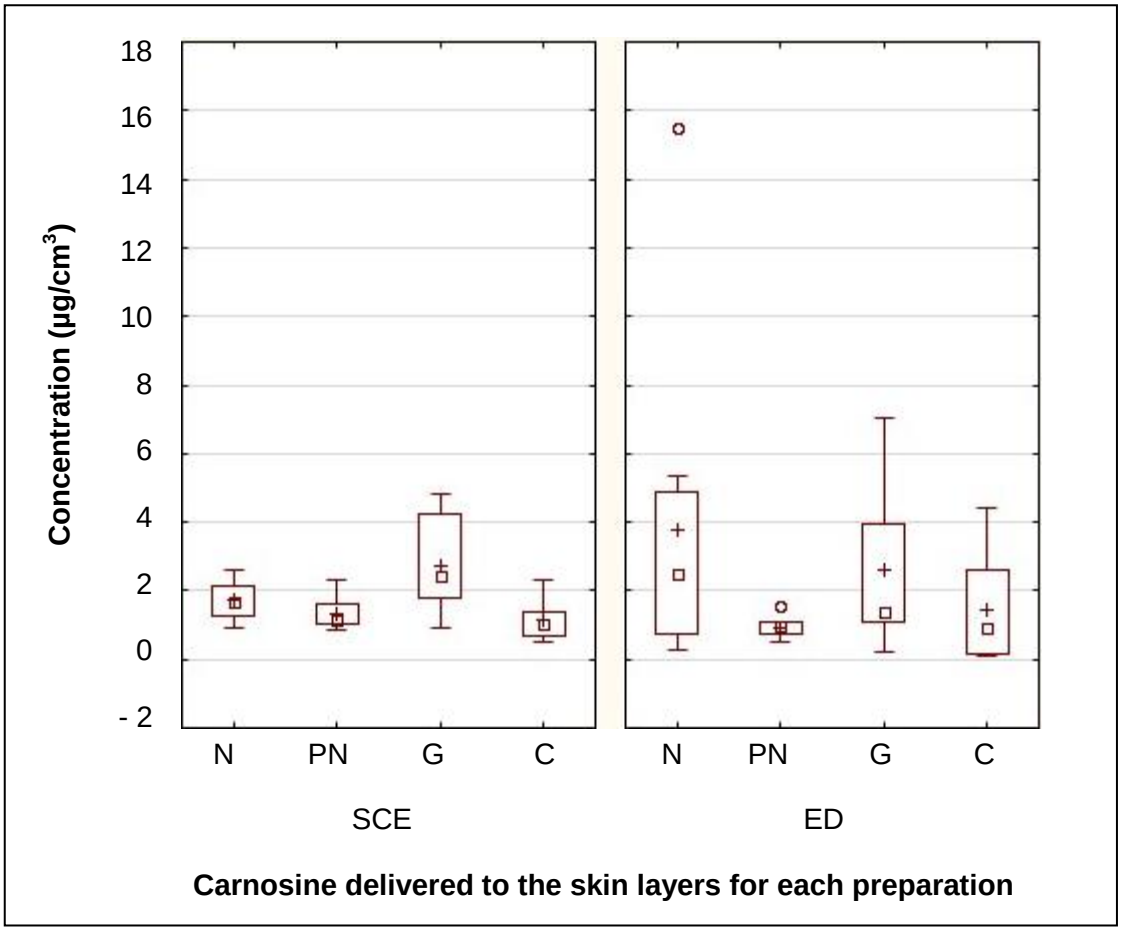

Figure G.20: Box-plots of the SCE and ED values of the four preparations illustrating the average (+) and median (□), the minimum and maximum values as well as the outliers (o). The following key can be used for the different preparations, N: niosomes, PN: proniosomes, G: gel and C: cream

G.4.7 Statistical data analysis of diffusion studies

Non-parametric tests were performed on the flux data obtained from the membrane release studies after 6 h. Significant differences were revealed with the Kruskal-Wallis test. The multiple comparisons test was performed after the Kruskal-Wallis test to determine where the significant differences lay. There was neither significant statistical difference between the niosomes and the proniosomes ($p = 1.000$) nor between the gel and the cream ($p = 0.419$), but there was significant statistical difference ($p < 0.05$) between a) the cream and the niosomes ($p = 0.000$), b) the cream and the proniosomes ($p = 0.000$), c) the gel and the niosomes ($p = 0.048$) as well as d) the gel and the proniosomes ($p = 0.007$).

Non-parametric tests were performed on the data obtained from the tape-stripping and epidermis-dermis after 12 h diffusion studies. The Kruskal-Wallis test revealed significant statistical differences in the tape stripping (SCE) ($p = 0.042$), but no significant statistical differences in the cut pieces of skin (ED) ($p = 0.156$). Furthermore, the Mann-Whitney test

indicated there were no statistical differences between the SCE and ED for the niosomes ($p = 0.676$), cream ($p = 0.791$) and gel ($p = 0.345$), but the proniosomes had a significant statistical difference between the SCE and ED ($p = 0.023$).

G.5 Conclusion

As mentioned before, the aim of this study was to target topical delivery of carnosine. Carnosine is a cosmetic substance with anti-oxidation and anti-glycation properties (Babizhayev, 2006:2344; Kyriazis, 2010:46). By targeting topical delivery, carnosine could possibly act as an anti-ageing substance to reverse cell senescence and prevent ageing in the skin.

An aqueous solubility of at least 1 mg/ml is essential for skin diffusion (Naik *et al.*, 2000:319). Furthermore, compounds with a log P of 1 to 3 are expected to cross the skin more easily (Khalid *et al.*, 2016:129). Carnosine proved to have adequate aqueous solubility to be a good candidate for topical delivery. Despite the aqueous solubility, the log D of carnosine suggested that external interventions might be needed to improve dermal delivery. These interventions involved incorporating carnosine into vesicular systems. According to Kumar and Rajeshwarrao (2011:209) and Varun *et al.* (2012:633), the use of vesicles will improve skin penetration and allow targeting of the active in the epidermis-dermis.

The 6 h membrane release studies proved that carnosine was released from both the pre-formulations, as well as both semi-solid formulations. The niosomes showed the best release, followed by the proniosomes, the gel and finally the cream. Targeted topical delivery was successful and there was carnosine in the SCE and ED for all four experiments. None of the experiments revealed carnosine in the receptor phase. The stratum corneum is generally seen as the physical barrier between the exterior environment and the inside of the organism (Baroni *et al.,* 2012:258). Having carnosine in both the SCE and the ED proved it successfully penetrated the external barrier after successful release from the preparations.

The gel delivered the highest concentration to the SCE, followed by the niosomes, proniosomes and finally the gel. Furthermore, the niosomes delivered the highest concentration to the ED, followed by the gel, the cream and finally the proniosomes. The difference in carnosine concentration in the SCE and ED between the gel and the cream could be attributed to the difference in release from the two formulations and the aqueous nature of the gel. The gel had much higher release of carnosine from the formulation than the cream, resulting in a higher concentration gradient and better diffusion (Barry, 2007:571). The gel also hydrated the stratum corneum to increase skin permeability (Benson & Watkinson, 2012). The poor targeting of the proniosomes in the ED could be attributed to the poor characteristics of the formulation. The size and amount of the droplets that formed are believed to be the largest contributor to the

poor topical delivery. The proniosome dispersion revealed a small amount of large droplets, in contrast with the large amount of small droplets that formed in the niosome dispersion.

In conclusion, the aim of this study was met as carnosine was successfully targeted to the SCE and ED. The niosomes and niosome gel were the best pre-formulation and semi-solid formulation when considering topical delivery of carnosine.

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APPENDIX H

AUTHOR GUIDELINES: DIE PHARMAZIE

H.1 Introduction

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

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

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

Original articles: Publications from all fields mentioned above.

Short communications: Brief publications about the fields mentioned above.

H.2 Conditions

1. For submitted manuscripts, it is the responsibility of the author(s) to demonstrate novelty or a new approach taken in his research. The references should reflect the most recent relevant articles, and the discussion should compare the author's findings with the results of former investigations. For an experimental work, the data have to be determined and classified in a suitable way, problems must be formulated in view of the data, hypotheses should be suggested and/or the author should give possible explanations for any inconsistencies. If possible, the author(s) should perform mathematical or statistical calculations, fit the curves appropriate, and carry out the experiments under controlled conditions. Studies involving animals or human volunteers must include details of ethical approval.
2. Authors are requested to submit all manuscripts online. Paper copy submissions are no longer acceptable. Articles are considered for publication depending on their value and

pharmaceutical relevance and with the understanding that they have not been published previously and are submitted exclusively to the journal Die Pharmazie.

3. All manuscripts are subject to experts review. Additional corrections may be done by the editors.

4. A PDF-file of the article is delivered free of charge after the paper has been published in the journal. Please note that, by copyright reasons, this is for personal use of the authors only and *must not* be made available, e.g. by posting on a freely accessible website.

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7. The quotation of registered names, trade names, trademarks, etc. in this journal does not imply, even in the absence of a specific statement, that such names are exempt from the relevant laws and regulations and therefore free for general use.

H.3 Preparation of manuscripts

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

1. Below the title, the surname(s) of the author(s) with initials should be given without academic and professional degrees. The full address of the author for correspondence should appear below author names. Details on the institution where the work was done are requested and should be given above the title.

2. Each manuscript should start with an abstract, containing the most essential results of the study. Extensive review papers and articles for continuous education should be preceded by an outline of topics. Papers should be subdivided into chapters and subchapters according to the decimal system (e.g. 2.1.3.).

3. To achieve clarity and brevity of the presentation, original contributions should be subdivided after the abstract (see 2.) as follows:

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

   3.2. Investigations and results or synthesis of compounds: Methods should only be described generally (see "Experimental"), referring to previous or analogous studies. The

presentation of results should be precise, with necessary formulas (numbered in sequence with Arabic numerals), diagrams, tables and figures added separately (together with the legend) to the manuscripts. Numerical values of results should generally be presented either in tables or curves (please mark statistical limits).

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

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

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

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

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Journal articles:

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